University of Alberta

MESENCHYMAL STEM CELLS IN ISLET TRANSPLANTATION

by

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This thesis is dedicated to my parents who have supported me in all of my academic pursuits.

ABSTRACT

Type 1 diabetes mellitus (T1DM) is a chronic disorder of glucose metabolism due to autoimmune destruction of insulin producing β-cells. Although insulin therapy is the standard treatment for T1DM, islet transplantation, which has emerged as an alternative to insulin injection, offers a more physiologic means of glycemic control. Unfortunately, the sustainability of islet function is poor. Most islet recipients experience loss of graft function and need to resume insulin therapy. Post-transplant inflammation, allograft rejection and anti-rejection drug toxicity are several factors that contribute to the loss of graft function. The primary cause of islet graft impairment immediately after transplantation is inflammation. Our aim is to prevent or minimize islet dysfunction after transplantation.

The growing tempo of discoveries in stem cell therapies has opened avenues to explore improvements in islet graft survival. Mesenchymal stem cells are currently being examined for clinical therapies of various inflammatory disorders, such as sepsis and graft versus host disease. The objective of the first study is to examine the cytoprotective effects of MSCs on islets in the presence of pro-inflammatory cytokines. Human islets were co-cultured with bone marrow derived MSCs followed by exposure to pro-inflammatory cytokines *in vitro*. Glucose stimulated insulin secretion was preserved and β-cell apoptosis was prevented in the islets cultured with MSCs. However, the mechanism of protection is unclear.

In the second study, we speculated the protection conveyed by MSCs was dependent on the physical interaction between islets and MSCs. Direct contact in islet and MSC co-cultures showed favorable results. When islets and MSCs were separated by a barrier, the MSCs were able to preserve islet function, but insulin content was decreased. We concluded that direct contact with MSCs is more beneficial than indirect contact for human islets.

In the third study, the protective effect of MSCs on islets was examined in a preclinical mouse model of islet transplantation. The kidney is not an optimal site to assess the beneficial effect of co-transplanting islets and MSCs. On the other hand, intravenous MSC injection after islet transplantation improved islet function, but the effect was short-lived. These results suggest that MSCs are a promising solution to prolong islet graft function.

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APC	Antigen presenting cell
BSA	Bovine serum albumin
BM	Bone marrow
BMT	Bone marrow transplantation
bMSCs	Bone marrow derived mesenchymal stem cells
CD	Cluster of differentiation
CK	Cytokeratin
DNA	Deoxyribonucleic acid
EC	Endothelial cell
EDTA	Ethylenediaminetetraacetic acid
ECM	Extracellular matrix
ESC	Embryonic stem cell
EGF	Epidermal growth factor
FACS	Flow activated cell sorting
FBS	Fetal bovine serum
FGF	Fibroblast growth factor
bFGF	Basic fibroblast growth factor (FGF-2)
FITC	Fluorescein Isothiocyanate
GFP	Green fluorescent protein
GSIS	Glucose stimulated insulin secretion
GVHD	Graft versus host disease

HBMC	Human bone marrow cell
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HGF	Hepatocyte growth factor
IBMIR	Instant blood-mediated inflammatory reaction
IEQ	Islet equivalent
iPS cell	Induced pluripotent stem cell
IgG	Immunoglobulin G
IL-6	Interleukin-6
IFN-γ	Interferon-γ
ILC	Islet-like clusters
MHC	Major histocompatibility complex
MSC	Mesenchymal stem cell
MMP	Matrix metalloproteinase
Ngn3	Neurogenin 3
NPI	Neonatal porcine islet
PCNA	Proliferating cell nuclear antigen
pMSCs	Pancreatic derived mesenchymal stem cells
PBS	Phosphate buffered saline
PD-1	Programmed cell death 1
PDL	Pancreatic ductal ligation
PDX-1	Pancreatic duodenal homeobox gene-1
SEM	Standard error of the mean
SI	Stimulation index

T1DM	Type 1 diabetes mellitus
TGF-β	Transforming growth factor beta
TNFα	Tumor necrosis factor alpha
TUNEL	TdT-mediated dUPT Nick-End Labeling
VEGF	Vascular endothelial growth factor

CHAPTER 1

GENERAL INTRODUCTION

1.1 TYPE 1 DIABETES MELLITUS

Diabetes or diabetes mellitus is a group of metabolic disorders characterized by high levels of blood glucose as a result of a defect in insulin production or insulin use [1-3]. When the pancreas is unable to produce insulin, this syndrome is known as type 1 diabetes mellitus (T1DM). On the other hand, when insulin production from the pancreas does not meet the body's need or the body becomes less responsive to insulin, this defect in insulin use is called type 2 diabetes mellitus [1-3]. Diabetes mellitus has become a serious health concern around the world. Currently, diabetes affects almost 250 million individuals and has become a leading cause of morbidity and mortality worldwide [4]. In addition to costly health expenditures, diabetes has a considerable impact on the economies of both first world countries and the developing nations [4]. The American Diabetes Association (ADA) estimates that the annual burden of medical expenses, disability and loss of work productivity is approximated at $174 billion US dollars [4]. Between the two main types of diabetes, type 1 diabetes mellitus constitutes approximately 10% of all diabetes cases, but the cost for T1DM is disproportionally higher [5]. Based on health expenses and absentees at work, T1DM is reported to account for 25% of the costs for all cases of diabetes [5]. Besides, the growing incidence of T1DM at a rate of 3% per year

is a concern because of escalating costs to the healthcare system [1]. T1DM can develop at any age, but tends to occur in individuals younger than 20 years old [1,2]. T1DM occurs when the patients' insulin producing cells are attacked and destroyed by the body's own immune system, which is known as autoimmunity. The exact mechanism that activates this autoimmune response is still unknown [1,2]. Many believe environmental factors trigger abnormal antibody responses which cause damage to the pancreatic cells [1-3]. Population-based studies have identified rising incidences of T1DM in many ethnic groups suggesting the influence of environment and the interaction between several etiologies like genetics and toxins (viruses and vaccinations) [1]. With the loss of insulin production, T1DM is a debilitating lifelong disorder. Primary prevention is desirable. Yet, clinical trials initiated by the National Institutes of Diabetes and Digestive & Kidney Diseases found no evidence that T1DM could be prevented by the induction of immune tolerance [1,6]. Prediction of diabetes onset is also difficult because the disease usually exhibits no symptoms until the body has lost most of its endogenous capacity to produce insulin [1,2]. These symptoms include polyuria (increased urination), polydypsia (increased thirst) and polyphagia (increased appetite), which are not specific to diabetes [1-3]. The standard diagnosis of diabetes requires an assessment of symptom history, confirmation with blood glucose measurements as well as detection of glycosuria [1]. Detection of diabetes can often be too late. By the time the signs and symptoms of diabetes have occurred, over 80% of the insulin producing cells in the endocrine pancreas have already been destroyed [1,2]. Currently, there is no

cure for diabetes but the disease can be managed. For treatment, patients with T1DM must rely on exogenous administration of insulin formulations.

1.1.1 Insulin Therapy

The main goal of diabetes management is to maintain optimal blood glucose levels that minimize acute complications, to prevent or delay the onset of chronic complications as well as to balance protein and nitrogen ratio [1]. An adequate protein and nitrogen ratio is required for normal body function, growth, and development [1]. To achieve treatment goals for T1DM, the main therapeutic options are insulin therapy and β-cell replacement. Currently, exogenous administration of insulin injection is considered as mainstay therapy for TIDM [1,2]. β-cell replacement better satisfies these therapeutic goals, but is only available to selective patients because the surgical procedure for whole pancreas transplantation is invasive and the long term outcome for islet transplanatation is currently unfavorable [1-3]. At present, β-cell replacement is prescribed only to diabetes patients for those whose insulin therapy is no longer effective or whose kidney disease has progressed to a seriously critical stage that requires renal replacement [1-3].

Insulin therapy is entirely self-managed by frequent measuring and monitoring of blood glucose levels together with multiple daily injections of insulin [1-3]. Meals and physical activities need to be well-planned in conjunction with daily glucose measurements and injections [1-3]. Managing diabetes with insulin therapy is a demanding task. Although intensive insulin

therapy improves glycemic control, the risk of serious hypoglycemic episodes and comas also increases substantially [1-3,6]. Nevertheless, since the discovery of insulin, the life expectancy of patients with T1DM has been prolonged. With the increase in life span, the incidences of renal failure, blindness and heart disease also have been on the rise [1-3]. Poor glycemic control was believed to increase the risk of long term complications including atherosclerosis, retinopathy, neuropathy, nephropathy and heart disease [1-3,6]. Clinical trials, conducted by the Diabetes Control and Complications Trial (DCCT) along with the Epidemiology of Diabetes Interventions and Complication (EDIC), proved strict control of blood glucose with intensive insulin therapy could prevent or delay the onset of complications in T1DM [1,7,8]. Therefore, stringent control of blood glucose levels is imperative to prevent or to delay the onset of late complications. As such, insulin therapy follows the 2008 Canadian Diabetes Association clinical practice guidelines, in which a defined range of acceptable blood glucose levels must be maintained [9]. Today, newer insulin formulations are available for helping to maintain a more balanced glycemic control throughout the day [1,2].

1.1.2 β-cell Replacement

The introduction of insulin therapy marked a significant turning point in diabetes management, but severe long term complications can continue to manifest even with good control of blood sugar levels [1-3]. β-cell replacement is another option for treatment that enables precise physiologic glycemic control and reverses diabetes by replacing endogenous cell function [1,10-13]. The two

prominent choices for β-cell replacement are whole pancreas transplantation and islet transplantation [10]. Pancreas transplantation was first introduced in the late 1960s [1,14]. Since then, over 20,000 whole pancreas transplants had been performed either alone or with kidney transplants [10,14]. Of that, approximately 50% of patients remained insulin independent for five years after transplantation [10,14]. When the procedure was introduced in the 1960s, whole pancreas transplant was invasive and unsafe with a high chance of mortality [10,14]. Over the years, advancements in surgical techniques and immunosuppressive drugs have made the transplant safer with better outcomes [14]. At present, patients with severe hypoglycemic unawareness, poorly controlled diabetes together with end stage renal disease are eligible for whole pancreas transplant [14]. For patients, the concern is whether the benefits of glycemic control can outweigh the risks of invasive surgery and complications from immunosuppression [8,10].

On the other hand, islet transplantation has recently achieved success as a treatment for T1DM [11,12]. The Edmonton Protocol is a less invasive procedure than whole pancreas transplantation [11,12]. This procedure utilizes a catheter to infuse isolated islets into the hepatic portal vein [11,12]. Using a combination of three anti-rejection drugs, daclizumab, tacrolimus, and sirolimus, the Edmonton Protocol procedure was initially able to achieve total insulin independence in seven out of seven recipients [11]. In an international multi-centered trial, sixteen out of thirty-six patients also achieved the same result at one year post-transplantation [12]. While short term success has been achieved, long term outcomes are less promising [13]. A five year post-transplant follow up study

demonstrated that only 10% of the sixty-five islet-transplanted patients remained insulin independent [13]. However, approximately 80% remained C-peptide positive suggesting islet grafts had survived for at least five years after transplantation. Thus, the loss of islet graft function was identified as the main reason that recipients would lose insulin independence [13]. Maintenance of islet function is critical for sustainability of islet graft. Islet transplantation must also be proven safe before being accepted and approved as standard treatment. The current requirement for strong immunosuppression after transplantation is a disadvantage. Oral ulcers, diarrhea and ovarian cysts have been commonly reported with the use of sirolimus after islet transplantation [13]. In addition, the use of long term immunosuppression can cause more serious side effects like hepatoxicity, renal toxicity and acquiring opportunistic infections [1,13]. Nevertheless, islet transplantation procedure is less invasive than whole pancreas transplantation and the risks of surgical related morbidity and mortality are much lower [10,11].

Although islet transplantation is a step forward treatment for diabetes care, several barriers have prevented its widespread clinical application. The problems are the insufficient supply of insulin producing tissue due to lack of human organs donors, the progressive loss of islet graft function, and the detrimental side effects caused by chronic immunosuppression [10-14]. In order to overcome these barriers, a readily available source of islets or insulin producing tissue together with safer anti-rejection strategies are necessary. With the remarkable progress in the fields of stem cell biology and regenerative medicine for cell repair and

renewal, the goal is to find a solution in which β-cells can be generated from the patient's own tissues. This can overcome the obstacles of limited β-cell supply and the requirement for immunosuppression [14-16]. The scope of this study is to review the alternative sources of insulin producing β-cells, to understand the factors that limit islet graft survival, to discuss strategies that can improve engraftment and protect islets from destruction using mesenchymal stem cells.

1.2 STEM CELLS AS A POSSIBLE SOURCE TO GENERATE β-CELLS

As the current means of β-cell replacement utilizes whole pancreas or isolated islets, the β-cell source for transplant continues to rely on harvesting cadaveric human pancreases. Consequently, organ donations are still necessary. But, only 4000 pancreases are donated each year in the United States [16]. This number of available organs does not meet the demands of transplantation. Thus, widespread β-cell replacement therapy is currently not possible. An option is to build human pancreases and islet tissues from synthetic materials or mechanical parts as organ substitutes. Another option is to assemble, integrate and network the pieces that are available in nature or in the human body. In the context of β-cell replacement, stem cell technologies for β-cell neogenesis may be used to produce human pancreatic tissues in order to resolve problem in the lack of sufficient donor tissues to meet the high demands for future β-cell replacement therapies [14-16]. Three strategies are proposed for investigation: the creation of β-cells by differentiation of embryonic stem cells, differentiation of pancreatic progenitor cells, and transdifferentiation of adult somatic stem cells (the non-pancreatic stem cells) [14-16].

1.2.1 Embryonic Stem Cells

The remarkable and exciting discovery of embryonic stem cells (ESCs) has stimulated great interest in using those cells to develop treatments to repair and replace tissues that are damaged by disease. Type 1 diabetes has been envisioned as one of those diseases that may benefit from stem cell therapy [14-16]. ESCs are derived from the inner cell mass of a developing blastocyst [14-16]. They exhibit unlimited self renewal and they have the ability to differentiate into any tissue type in the body [14-16]. When ESCs are cultured in suspension, these cells spontaneously form clusters of cells known as embryoid bodies that can differentiate into the three embryonic lineages (germ layers): endoderm, mesoderm and ectoderm [14,15]. Within the embryoid body, cells from both the outer layer that corresponds to endoderm and the inner cell mass can express insulin [14,15]. However, due to their relatively undifferentiated state, these insulin positive cells are likely much different than differentiated pancreatic β-cells. [14,15]. In addition to the spontaneous formation of insulin positive cells, the spontaneous secretion of insulin has also been reported [14,15]. When ESCs with a highly active insulin promoter were selected from a pool of undifferentiated ESCs and were cultivated in a low glucose medium with nicotinamide, insulin secretion from these cells was similar to mature β-cells [15]. Moreover, these undifferentiated ESCs were able to restore normoglycemia in diabetic mice [15].

Although scientists have observed the spontaneous formation of insulin secreting tissue from ESCs, finding a method to differentiate ESCs into pancreatic β-cells has been much more challenging. Most approaches to generate β-cells from ESCs attempt to follow the events of pancreatic development [14,15], but many aspects of development are still not clearly understood [14]. For instance, the spatial orientation of cells with respect to environmental cues, the temporally regulated release of soluble factors, and the interactions with other developing tissues are all unknown [14]. However, initial reports show that islet-like cells can be generated without recapitulating the steps of pancreatic development. Lumelsky *et al*. were the first to report the formation of islet-like clusters from ESCs [17]. By selecting and expanding cells positive for the neural stem cell marker nestin, Lumelsky discovered that ESCs were able to form clusters of insulin producing cells surrounded by other islet hormone expressing cells – glucagon and somatostatin positive cells [17]. But, insulin secretion from these cells was noticeably lower than mature β-cells [15,17]. These differentiated cells were unable to restore euglycemia in diabetic mice [15,17]. In addition, the detection of insulin expression was likely due to insulin uptake from the surrounding culture medium rather than endogenous insulin synthesis [15,17]. This observation led some to believe that the islet-like clusters described by Lumelsky *et al*. represented neuroectodermal derivatives instead of classical islet cells [15,17].

Following the developmental steps of endocrine pancreas formation, many researchers have recognized that formation of the endoderm is the limiting factor

for the generation of β-cells [14,18-21]. As such, several research groups have focused on deriving definitive endoderm from ESCs [14,20,21]. Activin A and nodal are mesenchymal factors responsible for endoderm formation [14,21]. Potent small molecules have now been identified that induce endoderm formation at much higher efficiency than these proteins [21]. Other soluble factors, including transforming growth factor beta (TGF-β) proteins and fibroblast growth factor (FGF) are also important to direct normal endocrine cell development [15,18,19,21].

Using these concepts, Jiang *et al.* developed a multi-step serum free approach with a variety of these growth factors to produce islet-like clusters (ILCs) [22]. C-peptide was detected in cell culture [22]. In transplanted diabetic mice, C-peptide positive cells accounted for only 2 - 8% of all cells in the cluster and insulin secretion from these clusters was similar to fetal islets but not mature adult islets [23]. Yet, the ILCs were not able to normalize blood glucose levels [23]. Novocell also reported the formation of insulin producing cells using a different protocol for differentiation [24,25]. After transplanting ILCs into diabetic mice, the level of human insulin and C-peptide was identical to 3,000 human islets [24,25]. Unfortunately, these results could not be replicated in a rat model [26]. Basford *et al.* described the first comprehensive approach to characterize ESC derived insulin production cells at a molecular and functional level [27]. By examining the maturation of ESCs, they elucidated targets to improve differentiation [28]. Thus, future attempts at differentiation may be better guided by correlating functional and molecular data [28].

1.2.2 Pancreatic Progenitor Cells

As the ethical controversy surrounding embryonic stem cell research has not been resolved, another potential source of insulin producing tissue may be from adult stem cells. In tissues such as blood and intestinal epithelium, adult stem cells play a role in tissue renewal [14,15]. In the pancreas, turnover of the β-cell is predicted to occur [15], which has led some to hypothesize that an adult stem cell for pancreatic islets does exist [14,15]. In experimental models of pancreatic injury such as physical or chemical ablation of the pancreas, β-cell regeneration has been detected [15,19]. This cell regeneration led many to believe that pancreatic derived precursors do exist and can differentiate into islet cells [15,20]. However, an alternative hypothesis stated that islet regeneration occurs due to mitotic division of existing β-cells rather than expansion from a progenitor population [20,29]. Lineage tracing studies, using a Cre-LoxP method to label cells, showed that pancreatic regeneration in most models of pancreatic injury occurred primarily through the expansion of existing cells rather than precursor cells [20,29]. But, the pancreatic ductal ligation (PDL) model of injury provided evidence of stem cells residing in the pancreas [20,30,31]. Within the ducts of the ligated pancreas, histological evidence demonstrated that neogenesis had occurred [30,31]. Detection of the gene transcript for neurogenin 3 (Ngn3), a marker that specifies for the formation of islet cells confirmed the presence of neogenesis in the pancreatic ducts [20,31]. Ngn3 is not normally expressed in the adult pancreas even during regeneration after partial pancreatectomy [20,31]. The ductal cells, in which the Ngn3 signal persisted, eventually formed new islet β-

cells [20,31]. After knockdown of Ngn3 expression, the doubling of β-cells was eliminated, which indicated the specific involvement of this marker in β-cell development [20,31].

Furthermore, PDL induced the doubling of β-cell mass within one week in the ligated portion of the pancreas only [20,31]. The β-cell mass in the unligated fraction remained unchanged [20,31]. Based on studies of cell proliferation, this increase in β-cell mass occurred at a faster rate than mitotic division of existing β-cells [20,31]. Thus, the increase likely occurred as a result of progenitor cell proliferation [20,31]. For patients with T1DM, the uncertainty is whether endogenous progenitor cells could be present and/or be activated after autoimmune destruction. Injury from PDL is different from the autoimmune destruction of pancreatic islets in T1DM because it does not involve the loss of pre-existing β-cells [20]. The ability to regenerate pancreatic cells in patients with T1DM is unclear [20]. Still, pancreatic acinar tissue, islets and mesenchyme have also been cited as possible sources of islet precursor cells [14,20,21]. Needless to say, the evidence remains compelling enough to suggest that pancreatic stem cells do exist. An inflammatory signal similar to ductal ligation may be necessary to activate differentiation and expansion of these progenitor cells into β-cells.

In the PDL model, pancreatic ductal cells have emerged as a likely source of islet progenitors [20,32,33]. But contamination of ductal tissue by β-cells is possible [20,33]. Removal of β-cells from ductal preparations eliminated neogenesis [33]. Therefore, these new β-cells may not arise from progenitor cells

but from pre-existing β-cells [34]. When mesenchymal and β-cells were removed from culture of human pancreatic preparations, β-cell formation was not detected [34]. When the pancreatic epithelial cells were transplanted with human fetal islet clusters into a mouse kidney capsule [34], the co-transplanted pancreatic epithelial cells and human fetal islets yielded 10 - 20% more insulin positive β-cells [34]. Another group purified pancreatic ductal cells and aggregated them with pancreatic stromal fibroblasts prior to transplanting into the mouse renal capsule [35]. They reported that only 1% of the ductal cells underwent differentiation into β-cells [35]. Both techniques utilized helper cells, either human fetal islets or pancreatic stromal cells, to induce differentiation of ductal cells. In addition, the mouse kidney capsule, which is a favourable site for differentiation of fetal islet tissue was utilized in both studies [20,34,35]. The limitation of the *in vivo* differentiation model is that the specific conditions and signals regulating differentiation are difficult to reproduce [20,34,35]. Thus, the ability to optimize and to replicate these results in cell culture is uncertain. Further investigation and testing are required.

Developing a defined differentiation protocol in which all conditions can be controlled and modified is necessary to produce β-cells consistently. Toward this end, Zhou *et al*. described the formation of β-cells by viral transduction [36]. His team utilized three pancreatic genes (Ngn3, Pdx-1 and Mafa) to reprogram mouse pancreatic exocrine cells [36]. The final products resembled islet β-cells which secreted insulin to reverse hyperglycemia in a diabetic mouse [36]. This technique provides an efficient and potentially reproducible approach to derive

tissue that can restore normoglycemia in diabetic animals. Certainly, the next step is to demonstrate if these results can be replicated using human pancreatic tissues.

Overall, the adult pancreatic stem cell remains elusive. However, both ductal and exocrine tissues have demonstrated some success in generating β-cells. Intercellular signals governing the creation of β-cells from these tissues are still under investigation [20]. As adult pancreatic tissue has the ability to form β-cells from non-islet tissue in the laboratory environment, the expansion of insulin producing tissue for transplantation may be possible to minimize the need of multiple islet donors in the future. Moreover, activating and stimulating β-cell regeneration in islet graft tissue may prolong survival and function after transplantation. But deriving β-cells from adult pancreatic stem cells still requires tissue from human pancreases. Thus, the acquisition of donor organs continues to be a limiting factor.

1.2.3 Adult Somatic Stem Cells

As many questions surrounding the properties of pancreatic stem cells are unanswered, adult stem cells from other organ tissues that have significant plasticity could be a potential source of insulin producing tissue. Bone marrow stem cells, for instance, can be differentiated into cells of all three embryonic lineages suggesting the capability to form almost any type of cells in the body [14]. However, this claim of pluripotency has been challenged, as fusion of donor stem cells with recipient cells may better explain the perception of plasticity [14]. Nevertheless, other adult stem cells have been reprogrammed by de-

differentiation followed by re-differentiation into other cell lineages [14]. The plasticity of some adult stem cells suggests that patient derived tissues can be used to develop autologous graft tissue and to circumvent the requirement for immunosuppression [14]. Another advantage is that fewer ethical and legal barriers regulate the use of adult derived stem cells versus embryonic stem cells.

1.2.3.1 Bone Marrow Cells

Reprogramming or transdifferentiation of adult stem cells to cross different lineages is another approach to generate pancreatic cells from non-pancreatic tissue. The challenge is to identify the conditions and cell types that are conducive to reprogramming. Bone marrow cells are thought to be an ideal source of adult stem cells for transdifferentiation because of their plasticity [14,15]. Bone marrow derived mesenchymal stem cells respond to tissue damage by migrating to the site of injury and contributing to both structural and functional repair and regeneration [14]. Clinically, bone marrow (BM) cells can be easily isolated for treatment. Currently, bone marrow cells are used for bone marrow replacement. In a bone marrow transplant patient, after one month, 3% of islet β-cells were of donor origin [37]. However, no evidence was reported to confirm that these BM cells had differentiated into β-cells [14,37].

Reprogramming bone marrow derived mesenchymal stem cells into pancreatic endocrine lineages has not yet been consistently reported [14,37-39]. One possible explanation for the presence of islet cell derived from donor tissues is the transdifferentiation from liver tissues because bone marrow cells can form

hepatocytes [14]. During embryogenesis, development of the liver and pancreas are closely associated [14,15]. The foregut endoderm that forms the ventral pancreas can also be differentiated into liver [14,21]. Due to their developmental similarity, liver cells have also been investigated as a potential source of tissue to generate pancreatic β-cells [40,41]. Using viral transfection, the induction of Pancreatic Duodenal Homeobox Gene-1 (PDX-1), a master regulator of pancreas organogenesis can induce hepatocytes to differentiate into insulin producing cells [40]. Yet, the complete differentiation into β-cells was not observed [40]. Detection of hepatocyte gene expression suggests that these cells were not pancreatic β-cells [40]. Human fetal liver cells were also virally transfected with a PDX-1 lentiviral vector, which resulted in pancreatic islet-like β-cells [41]. The transfected cells responded to glucose and reversed diabetes in mice after transplantation [41].

1.2.3.2 Induced Pluripotent Stem Cells (iPS Cells)

Although bone marrow and liver are possible sources of β-cells, acquiring these tissues require invasive procedures. Recently, Takahashi *et al*. discovered that differentiated adult tissues could be reverted to a pluripotent state using four transcription factors [21,42]. These reprogrammed cells are known as induced pluripotent stem (iPS) cells [42]. In cell culture, iPS cells and ESCs demonstrate similar capacities to self renew and differentiate [21,42,43]. Because iPS cells can be generated from any somatic tissue in the body, the procedure to generate iPS cells from a patient's own tissue is non-invasive as samples from the body can

be taken by a skin biopsy. After acquiring donor tissues, cells are reprogrammed by viral transduction methods with retroviruses that introduce four transcription factors, Oct-4, Sox-2, C-myc, and Klf-4 into the cell nucleus [21,42]. These are transcription factors that maintain an undifferentiated ESC-state [42,43]. After successful viral-transduction, cells are selected and grown in ESC specific cell culture conditions [21,42]. Therefore, the iPS cell is a promising alternative to human embryonic stem cells [21,42].

This iPS cell technology has been applied to generate insulin producing tissues. Tateishi *et al*. demonstrated that iPS cells can be differentiated and generated into β-cells [44]. Extracting foreskin fibroblasts to generate iPS cells, they used a multi-step protocol for differentiation, using a serum free medium with growth factors activin A and basic fibroblast growth factor [44]. The iPS cells formed islet-like clusters that expressed C-peptide, a marker of insulin secretion and glucagon [44]. These clusters secreted insulin in a glucose responsive manner [44]. In another study, skin samples from patients with T1DM were tested [45]. Using the iPS method, cells were generated that expressed C-peptide, insulin, glucagon, and somatostatin [45]. These cells were functional because release of C-peptide increased five-fold in responding to glucose [45]. The results represent an exciting advance in generating autologous islet grafts. Furthermore, the derivation of iPS cells from patients with T1DM indicates that patient specific therapies can be possible in the future. However, like many other directed differentiation protocols, the generation of β-cell for replacement therapy is limited by the efficiency of differentiation. For clinical application, a large

volume of islet tissue is required [13,14]. A highly efficient differentiation protocol is necessary. Generating iPS cells involves gene manipulation by viral transduction. The danger of this technique is mutagenesis due to aberrant insertion of viral DNA in undesirable locations. Mutagenesis can lead to the formation of malignant tissue. The reprogramming genes, c-myc and KLF-4, are also oncogenes, which also increase the risk of tumor formation [21]. Safety concerns for iPS technologies will need to be better addressed before clinical translation.

1.2.4 Stem Cells for β-cell Generation

What type of stem cells should be used to generate β-cells? Embryonic or adult stem cells continue to be a promising solution to expand the limited pool of readily available donor islet tissues. Thorough pre-clinical studies of each strategy are worthwhile to explore. Protocols to differentiate ESCs have shown success in generating β-cells but the efficiency of these techniques is poor [14,21]. ESCs are considered immunologically undefined, so the requirement for immunosuppression after transplantation may be less stringent [14]. But, the risk of developing tumors from ESCs such as teratomas is possible and is a major concern [14,15]. A better understanding of pancreas development is definitely necessary to improve the yield from these methods. On the other hand, protocols to generate β-cells from adult stem cells and iPS cells have also been reported, although there are reservations regarding the safety of transplanting virus-manipulated tissues [14]. The advantage of using iPS cells is the lack of immune

rejection because of the use of patient derived autologous tissue for transplantation. For T1DM, however, immuno-suppression may still be needed due to the concern for recurrent autoimmunity [14]. Considering the risks and benefits between ESCs and adult stem cells, safety concerns and reproducibility must be addressed prior to clinical applications.

1.3 FACTORS THAT ARE IMPORTANT IN ISLET FUNCTION AND SURVIVAL

Approximately one million islets are present in the normal human pancreas [2]. Of that, only 60% of this islet mass is required to maintain normal glucose metabolism [46]. In clinical islet transplantation, however, multiple islet donors are often required to achieve insulin independence [11-14]. While loss of β-cells after islet isolation does occur, Ryan *et al*. suggested that a larger than expected mass of transplanted islets is required because of impaired islet graft function [13]. At five-year post transplant, only 10% of islet recipients remained insulin independent [13]. Sustaining islet function is critical and is necessary for islet transplantation. Even though the initially promising results of the Edmonton Protocol did not translate into long term success, some evidence showed that long term graft survival did exist [13]. About 80% of islet transplant recipients remained positive for C-peptide, a byproduct of insulin processing and secretion. The existence of C-peptide indicates the presence of islet graft tissue [13]. But the prevalence of C-peptide positive individuals did not coincide with the proportion of islet recipients who are insulin independent. No therapeutic levels of insulin were present even though C-peptide did exist. Lack of improvement in

graft function with thiazolidinediones demonstrated that insulin resistance is not likely the cause for islet graft failure [13]. In islet transplanted recipients, during their insulin independent period, C-peptide levels in response to a meal challenge were normal [13]. When they resumed insulin therapy, C-peptide levels in response to a meal challenge were decreased [13]. This decline in C-peptide levels reflects a reduction in islet graft function [13]. Impaired islet graft function may be due to the decrease in β-cell renewal, toxicity of immunosuppression, loss of β-cell mass after islet isolation and culture, or decrease in islet engraftment post-transplantation [13]. Among these factors, Korsgren *et al*. predicted that the failure of islets to engraft accounts for the greatest amount of β-cell loss after transplantation [47]. Experimental islet transplant models confirmed events that occurred during islet engraftment caused 60% of islet loss [46-50].

In autologous and syngeneic islet transplantation, an inflammatory reaction occurs, which is characterized by inflammatory cytokines, macrophages and islet cell injury [46,50]. Another detrimental mechanism is a coagulatory pathway known as instant blood mediated inflammatory reaction (IBMIR) [47,49]. This innate immune response is activated immediately after transplantation [47,49]. Inadequate oxygenation is also a stress that occurs shortly after transplantation and contributes to islet cell loss by inducing apoptosis. Recent evidence has elucidated other stresses that do not occur immediately after transplantation but are important mechanisms of graft failure. Two independent autopsy reports of islet recipients revealed that transplanted islets undergo pathological changes [51,52]. In one T1DM islet recipient, auto-

antibodies and insulitis were absent but insulin staining in the remaining islets was intact [51]. The authors concluded that islet loss was not related to an immunologic cause [51]. This non-immunologic mechanism may be the formation of amyloid in islets. Amyloid is toxic to β-cells and is detrimental to β-cell function [47,52]. Westermark *et al*. reported that in one islet recipient, amyloid plaque deposits were present in 40% of the transplanted islets within the β-cells and in the extracellular space [52]. Although the mechanism of this plaque formation is largely unknown, similar amyloid deposits are observed in islets from patients with type 2 diabetes [52]. The pathogenesis of these plaques may be related to poor glucose control [52]. Improvements in graft performance as well as graft survival are important for the long term success of islet transplant protocols. Understanding and identifying the factors that lead to islet dysfunction and loss will help in the development of therapeutic treatments that prolong and sustain islet graft function.

1.3.1 Protection of Islets from Inflammation in the Immediate Post Transplant Period

Immediately following islet transplantation, inflammation causes significant loss of islets [46-48]. After infusion of islets in the hepatic portal vein, human islets are exposed to fresh human blood, which initiates a coagulatory process known as the instant blood-mediated inflammatory reaction (IBMIR) [47-49]. IBMIR is activated because isolated islets naturally express tissue factor as well as cytokines [47-49]. These signals activate thrombin + anti-thrombin complex formation, blood coagulation and platelet consumption [47-49]. In

addition to coagulation, leukocyte infiltration and activation of complement cascade characterizes this inflammatory reaction that is detrimental to islets [47-49]. Positron emission tomography (PET) and computed tomography (CT) demonstrates that nearly 25% of islet loss occurs during this period in patients who have been transplanted with islets. Coagulation inhibitors, such as the thrombin inhibitor megalatran, can prevent IBMIR [54]. However, administering a pharmacological inhibitor causes systemic dysregulation of coagulation pathways.

Localized control of IBMIR is a safer approach. Coating islets with heparin is effective for controlling IBMIR [55], but chemical modification of islets may alter islet function. Another solution is to modify the microenvironment by utilizing cells that do not stimulate IBMIR such as endothelial cells [56]. If these cells are delivered together with islets, this cell-mediated approach could enable localized control of inflammation [56]. Endothelial cells also have the ability to form new vessels by upregulating angiogenic factors such as vascular endothelial growth factor (VEGF), which could improve engraftment of islets after transplantation. Johansson *et al.* tested a method in which islets were coated with endothelial cells (EC) and exposed to human blood [56]. The coated islets demonstrated a decrease in infiltration of $CD11b^{+}$ leukocytes (monocytes, macrophages, granulocytes, and natural killer cells) as well as reduction in platelet consumption during activation of coagulation pathways [56]. Vessel like structures were detected in tissue grafts of EC coated islets, but were not found in untreated islets [56]. These results

provided evidence that EC therapy is a viable approach to prevent IBMIR and improve revascularization. Assessment of graft function of EC-coated islets in diabetic animals is necessary to determine the therapeutic value in islet transplantation.

1.3.2 Improving Islet Graft Oxygenation and Revascularization

After islet transplantation, donor tissues need to re-establish vascular networks with the recipient tissue to ensure long term function and survival. The disruption of the islet microvasculature upon isolation causes a hypoxic state and induces the loss of insulin producing cells that limits the ability of islets to engraft [48]. In addition, islets transplanted into the liver have markedly lower oxygen tension (~7.5–10 mmHg) compared to the native pancreas (~30 mmHg) [48]. Vascularization is required for tissue engraftment and graft oxygenation. Thus, strategies to improve revascularization after transplantation are necessary. Gene and cell therapy are among those that can increase islet revascularization. Targeting pathways to increase vascularization via gene therapy is effective. Over-expression of vascular endothelial growth factor (VEGF) in islets increases vascularization but does not improve survival of transplanted islets [57]. But, some concerns regarding gene therapy includes the danger of cancer cell formation after viral transfection and low efficiency of gene vector delivery into cells to achieve a therapeutic dose [14].

For cell-based therapy, the bone marrow (BM) cell is an attractive cell-based therapy. BM contains many progenitor cells including a population of

vasculogenic cells to initiate the formation of new blood vessels [58]. In animal studies, BM cells from wild-type mice have been used to test the ability of initiating new blood vessel growth. Wild-type BM cells were transplanted into mice that lacked genes for angiogenesis [58]. The wild type BM cells restored angiogenesis and participated in islet neo-vascularization in those mice [58]. In addition to participating in new blood vessel formation, the BM cells improved islet graft function [58]. Bone marrow vasculogenic cells were also co-transplanted with islets [58]. Fewer islets were required to normalize blood glucose levels in diabetic mice after co-transplantation than islets transplanted alone [58]. This confirmed that bone marrow cells were activated to increase vascularization in transplanted islets [58]. Interestingly, these BM vasculogenic cells were activated only with the absence of endogenous angiogenic pathways [58]. Disrupting angiogenesis systemically would be fatal. Localized gene knockout strategies like RNA interference at the transplant site may be an option to induce BM cell to undergo repair and revascularization of islet grafts [58].

1.3.3 Reconstituting a Microenvironment for Islet Engraftment

The intraportal site is relatively poor for islet survival and function [13,14]. Inflammation, coagulation and hypoxia occur shortly after transplantation following by amyloid deposition within the islet graft at a later time [13,14]. An alternative site may avoid these complications, but reconstituting a microenvironment conducive to long term islet survival is necessary for this approach to be successful. Processing of pancreatic tissue is

disruptive structurally and functionally to islets, which results in the loss of β-cell mass and glucose sensitivity [14]. Consequently, this causes delayed in islet engraftment after transplantation [14,48].

Within the native pancreas, cell to cell contact and cell to matrix interactions are crucial to the function and islet survival [14,48,59-63]. Reconstituting components of extracellular matrix (ECM) such as fibronectin and collagen on islet cultures has been a method to restore physiologic function [59-63]. Embedding pancreatic islets in a three dimensional collagen matrix preserves islets morphology, viability and glucose sensitivity [59-63]. Although restoring the natural compounds of the pancreas is desirable, artificial components can be superior substitutes [59]. For instance, the use of a synthetic matrix of self assembling nano-fibres proved to be more successful in maintaining islet morphology, viability and glucose response in culture than the natural ECM components [59]. After transplanting these islet-embedded matrices into diabetic mice, these grafts revealed that insulin content standardized to the amount of DNA per islet was the highest in this group [59]. DNA content also was greatest in the synthetic matrix after fourteen days [59]. These results indicate that islets, during the crucial period of islet engraftment, can be maintained in a synthetic matrix. However, the biocompatibility and the safety profiles of these biomaterials after transplantation need to be determined in a clinical setting. Further, suspension of islets in solid matrices could likely lead to necrosis of graft tissue upon transplantation due to the difficulty in vascularizing macrostructures.

An alternative method to recreate the islet microenvironment is to culture islets with pancreatic tissue [64]. Components of the exocrine pancreas, such as ductal epithelium, are believed to secrete trophic factors including the expression of ECM for islet development and differentiation into endocrine tissue [18,19]. Pancreatic ductal cells are also known to improve islet survival [65]. The long term metabolic success of islet transplantation was positively correlated with an increased proportion of transplanted pancreatic ductal cells [66]. The co-culture of islets with ductal epithelial cells preserved islet morphology and maintained the glucose responsive function of β-cells for up to ten days in a rotational suspension culture than the islets alone [64]. Alternative cell types including neural crest [67], fibroblasts [68] and bone marrow stromal cells [69] have also prolonged islet survival in cell culture.

This cell based strategy is a promising method to preserve islet function and to condition a microenvironment for implantation of islets at another site. The purpose and importance of engineering islets is to enable better engraftment of islets and to limit the destruction of islets upon transplant. Several approaches show favourable results in preserving islet function in long term culture [64,69]. Engineered islet tissue is an important model to improve its long term graft function and to investigate alternative islet transplant sites.

1.4 MESENCHYMAL STEM CELLS

Although the Edmonton Protocol first demonstrated that islet transplantation is a promising treatment for type 1 diabetes, unsustainable graft

function and anti-rejection drug toxicity have limited its clinical application. Most islet recipients resume daily insulin injections within 5 years post-transplantation. Therefore, only specific individuals are eligible for islet transplantation. For those whose insulin therapy is ineffective or whose T1DM is too severe and difficult to manage, islet transplantation is a possible treatment. To overcome these barriers, a regenerative medicine approach could be explored using stem cells to initiate graft cell renewal and repair. Multipotent mesenchymal stem cells (MSCs) are unique among most type of stem cells as they possess regenerative and immunoregulatory properties [69,70]. MSCs can stimulate new blood vessel formation, decrease inflammation and migrate to damaged tissues to initiate repair and renewal [69-70]. Moreover, the safety of MSC therapy has been widely reported [78]. Therefore, MSCs are currently being examined for clinical applications in various disorders including sepsis, graft versus host disease and Crohn's disease [78]. For islet transplantation, MSCs are being investigated as therapy to enhance islet engraftment, improve graft survival and reduce graft rejection.

1.4.1 Characterizing Mesenchymal Stem Cells

Mesenchymal stem cells (MSCs) were first reported as a population of plastic adherent fibroblast-like cells that are capable of undergoing differentiation into osteoblasts, adipocytes, chondrocytes and myoblasts [69-71]. MSCs were initially isolated from bone marrow. Recently, their existence in many other

tissues has been described. They now have been isolated from cord blood, peripheral blood, adipose, skeletal muscle, amniotic fluid and fetal tissues [69,70]. However, the differentiation potential of MSCs is not equal between these various tissue compartments [69,70]. Even in a single bone marrow sample, there are different sub-populations of MSCs that have varying capacities to form connective tissue cells [69,70]. Based on these observations, Phinney *et al*. suggested that MSCs are functionally heterogenous stem cells that may contain lineage restricted progenitors as well as undifferentiated stem cells [69]. Despite this heterogeneity, the International Society for Cell Therapy [72] proposed a consensus definition based on two criteria for MSCs: 1) A population of plastic adherent cells with the capability to differentiate into various connective tissue lineages: osteocytes, adipocytes and chondrocytes. 2) Cells with surface phenotype consisting of high expression of CD29, CD73, CD90, and CD105 as well as low expression of hematopoietic markers CD11b, CD14, CD19, CD34, CD45, CD79a and HLA-DR. In addition to their connective tissue plasticity, MSCs also possess immunomodulatory and regenerative properties [69,70,73]. In clinical cases studies, successful treatments of steroid refractory graft versus host disease, improvement of dense bone formation in osteogenesis imperfecta, and the hematopoietic engraftment for acute myeloid leukemia have been reported using human MSCs [69,70,73].

1.4.2 Properties of Mesenchymal Stem Cells for Tissue Repair and Regeneration

1.4.2.1 Differentiation of Mesenchymal Stem Cells into Pancreatic Islets

In the human body, MSCs are believed to naturally participate in tissue turnover by acting as replacement cells because MSCs could differentiate into bone, fat and cartilage [74]. In addition to replacing connective tissue, researchers have reported that MSCs can transdifferentiate into epithelial and neuroectodermal lineages [69,70]. After transplantation of MSCs into the central nervous system, the formation of astrocytes and neuron-like cells have been detected [69,70]. These results have prompted researchers to focus on harnessing the plasticity of MSCs to regenerate other tissues including pancreatic islets [76,77].

Several studies reported that MSCs derived from bone marrow have been differentiated into insulin producing cells, although insulin secretion was significantly lower than pancreatic β-cells [38,39]. Mesenchymal cells can also be isolated from adult pancreatic tissue. Seeberger *et al*. showed that pancreatic MSCs can express various β-cell development genes, but not their corresponding protein product [75]. Thus, adult pancreatic MSCs are considered as candidates for progenitors of β-cells [75-77]. During embryogenesis, these mesenchymal cells form a network of mesodermal derived cells, which secrete various soluble factors to regulate growth and development of the pancreas [18,19,65]. The pancreatic epithelium receives signals when in contact with mesenchyme [18,19,65]. Isolation of pancreatic epithelium from its surrounding mesenchyme leads to impairment of growth [65]; but mesenchymal cell extracts can restore

normal development of the pancreas [65]. Thus, soluble signals from mesenchymal cells are believed to induce proliferation and differentiation of pancreatic epithelium into endocrine and exocrine tissues [65]. Specifically, the notochord, a mesodermal lineage structure, secretes the TGF-β super-family protein, activin, that directs the formation of pancreatic endoderm by inhibiting the developmental factor Sonic Hedgehog [18,19]. Pancreatic endoderm is the precursor to islet and duct formation [18,19]. Fibroblast growth factors (FGF) 7 and 10 produced by the mesenchyme are also important in proliferation of the endocrine and exocrine pancreas [18,19,65]. Inhibition of FGF 7 and 10 receptors (cognate FGF receptor 2) causes the loss of islets during development [18,19,65].

While mesenchyme clearly directs pancreas development through intercellular signaling and interactions with the pancreatic epithelium, the role of mesenchymal stem cells in the adult pancreas has not been defined. One possibility is that MSCs may regulate β-cell mass in the body. β-cell mass is dynamic in the adult pancreas [15]. Expansion of β-cells can occur in response to various physiologic and pathologic states, such as pregnancy and obesity [15,65]. Because mesenchyme intimately interacts with epithelium in development, MSCs in the adult pancreas may retain a similar role to mediate proliferation and differentiation of endocrine tissue. Based on *in vitro* and *in vivo* evidence [75-79], transdifferentiation into insulin producing cells and secretion of soluble factors regulating pancreatic tissue repair are possible approaches for islet regeneration.

1.4.2.2 Secreted Factors from Mesenchymal Stem Cells

More recently, results from various *in vivo* repair models have highlighted an alternative mechanism of regeneration in which MSCs decrease inflammation, apoptosis and fibrosis by secretion of soluble factors rather than differentiate into replacement cells [78]. After transplantation, MSCs can initiate tissue repair in various animal models of injury [69,70,78]. For example, infusion of human MSCs into an immunodeficient mouse with acute myocardial infarction resulted in improved cardiac function [70]. However, engraftment of MSCs is uncommon [69,70]. In clinical trials of osteogenesis imperfecta, an inherited musculoskeletal disorder, only 1% of human MSCs engrafted into bone, skin and other tissues [69,70]. However, improved bone formation did occur [69,70]. The capacity of MSCs to produce a variety of soluble factors implicated in angiogenesis, inflammation, wound repair and immune function may be the primary means for tissue regeneration.

1.4.2.3 Mesenchymal Stem Cell Therapies

Given their ability to promote tissue repair, a number of investigators have explored the effect of MSC infusions in a variety of experimental models of diabetes [80-82]. Systemic administration by cardiac or intravenous infusion of human bone marrow mesenchymal stem cells into diabetic mice have resulted in decreased blood glucose and increased mouse insulin levels compared to untreated control mice [80,81]. In addition, the bMSC-treated mice showed increased numbers of pancreatic islets and improved glomerular morphology in

the kidneys [80]. Although human DNA was present in the pancreas and kidney, human insulin was not detected [80]. This suggests that MSCs migrate to injured tissue but did not differentiate into insulin producing cells. Many of these approaches used multiple or large infusions of human bone marrow cells to reverse diabetes in animal models [80,81]. A single injection of MSCs is likely safer; the effectiveness of a single cell infusion in reversing diabetes was tested with a combination of bone marrow cells (BMCs) with MSCs [82]. Restoration of normoglycemia was achieved only after co-transplantation of BMCs with syngeneic or allogeneic MSCs [82]. Transplantation of BMCs or MSCs alone was not able to reverse hyperglycemia [82]. The absence of infused donor cells in the pancreatic compartment confirmed that the BMCs and MSCs did not differentiate into pancreatic islets but mediated an alternate mechanism to regenerate islets, such as stimulating endogenous repair [82]. Recently, several clinical trials were initiated to investigate the role of MSCs in prevention, management and treatment of T1DM [http://www.clinicaltrials.gov/]. No results have been reported so far. However, these clinical trials are a clear indication of the translational possibilities of MSCs for diabetes therapies.

1.4.2.4 The Effect of Mesenchymal Stem Cells on Islets

As MSCs can facilitate repair of the pancreas, MSCs may also have potent effects on pancreatic islets after transplantation. Numerous studies have highlighted the beneficial effects of MSCs on islet function and survival in cell culture [69,83-85] . Chao *et al*. reported that human umbilical cord MSCs could

maintain long term survival and function of islet-like cell clusters (ICCs) in rat model [83]. Insulin secretion from the MSC treated islet-like clusters lasted for up to three months; whereas ICCs alone could not survive beyond twelve days [83]. A growing amount of evidence suggests that secretion of factors by MSC is the mode of action mediating islet survival [84,85]. Interleukin-6 (IL-6), transforming growth factor-β (TGF-β), vascular endothelial growth factor (VEGF) and hepatocyte growth factor (HGF) are commonly reported for their trophic and beneficial effects on islets in culture [83-85] or in islet transplantation [86-92]. These factors play roles in immune function, inflammation and tissue repair [84,89-92]. HGF, for instance, is important in pancreatic development and may participate in insulin secretion [85]. On the other hand, MSCs can also respond to signals from islets. Bone marrow mesenchymal stem cells (bMSCs) express chemokine receptors that are capable of promoting migration to pancreatic islets and are responsive to islet secretions in a dose dependent manner [86]. This migration of MSCs to pancreatic islets implies that the interactions between MSCs and islets require further investigation.

Although secreted factors are sufficient to improve islet function, direct contact between islets and MSCs may play a role in islet engraftment. For instance, when MSCs were cultured with endothelial cell coated islets, MSCs were able to augment vessel infiltration into the islets [87]. This experiment showed that MSCs expressed angiogenic factors like VEGF [87]. MSCs initiate vessel formation by secreting proteases that break down the vascular wall and enable EC migration and differentiation [87]. MSCs also have the capacity to

form small vessel coating pericytes [87]. Culturing MSCs with endothelial cell coated islets increased vessel sprout formation into the islets and the surrounding matrix compared to EC coated islets without MSC [87]. These vessel-like structures may form microvasculature for engraftment of donor tissues [87]. This co-culture approach with MSCs is a promising method to improve islet engraftment and prolong transplanted islet function. In animal studies, successful co-transplantation of islets and MSCs has been reported in the kidney capsule [88,91], omental pouch [99] and hepatic portal vein [89,92]. In each site, MSCs facilitated islet engraftment by promoting new blood vessel formation [88-92]. After transplantation, the non-hematopoietic bone marrow cells with fibroblast-like morphology localized around and inside pancreatic islets [90]. As fewer islets were required to reverse diabetes compared to the islets alone, islet survival and function was improved in the presence of MSCs. Berman *et al*. recently demonstrated similar beneficial effects of MSCs in islet transplantation in non human primates [92]. The objective for future studies is to investigate this co-transplant model with human mesenchymal stem cells and human islets.

1.4.3 Immunomodulation and Mesenchymal Stem Cells

MSCs have anti-inflammatory and immunoregulatory properties [79,93]. MSCs produce anti-inflammatory cytokines when stimulated by inflammation in cell culture or after injection into animal models of renal, neural and lung injury [79,93]. MSCs also lack the phenotype to stimulate the adaptive immune response. MSCs do not express major histocompatibility complex (MHC) class II

or the co-stimulatory molecules CD80, CD86, CD40 and CD40L [69,70,93]. Interferon-γ (IFN-γ) stimulates upregulation of MHC class II expression but does not induce co-stimulatory molecule expression from MSCs [69,70,93]. This lack of co-stimulation is a mechanism for peripheral tolerance; T cells that interact with MSCs will remain non-responsive [69,93]. In cell culture, lymphocyte alloreactivity is decreased in mixed lymphocyte reactions in the presence of MSCs [69,93]. MSCs interact with other immune cells including B cells, NK cells and dendritic cells to modulate their function [69,93]. Evidence also suggests that MSCs could evade allogeneic rejection in transplant models [69,93]. The immunosuppressive attributes of MSCs are due to several mechanisms like immunogenicity, disrupting normal function of autologous antigen presenting cells (APCs), and inhibiting allogeneic-activated T cell proliferation [69,93]. In T1DM, the autoimmune pathology is characterized by inflammation and infiltration of leukocytes into pancreatic islets [1,2]. To prevent or limit islet destruction, auto-reactive T cell responses could be suppressed with anti-rejection drugs; but the potential toxicity caused by these drugs is a concern. As inflammation and immune mediated destruction are characteristic of autoimmune diabetes as well as allogeneic islet transplantation, the immunomodulatory properties of MSCs may be well suited to improve islet transplant protocols for autoimmune diabetes [93,94].

1.4.3.1 Systemic Immunosuppression

Systemic delivery of MSCs has been investigated as a strategy to treat autoimmune diabetes. In a mouse model of autoimmune diabetes, administration of allogeneic MSCs delayed the onset of diabetes [95]. In another report, human MSCs were detected in the pancreases of mice with low grade inflammation [79]. These findings demonstrate that MSCs may have migrated to the sites of injury. In the context of islet transplantation, infusion of MSCs might protect β-cells and lead to better long term islet graft function after systemic injection. In one study, bone marrow transplantation (BMT) along with MSC infusion into myeloablated diabetic mice was curative for experimental diabetes [81]. T cells isolated from diabetic mice were responsive to autologous antigen presenting cells (APCs). The specificity of autoimmunity was confirmed by autologous APC activation of T cells only [81]. In the MSCs alone or MSCs with bone marrow cells treated mice, T cell reactivity was diminished [81].

In another similar study, Itakura *et al*. used MSCs to help induce immune tolerance of islet allograft tissue and to minimize the use of chronic immunosuppressive drugs [97]. In his model, BMT was performed to achieve specific immune tolerance of donor tissue. For engraftment of donor marrow, the recipient's bone marrow must be ablated or the immune system must be suppressed by administration of corticosteroids [97]. Treatment with corticosteroids causes a severe complication known as graft versus host disease (GVHD) after BMT [97]. The use of MSCs circumvented the requirement for immunosuppressive drugs and minimized GVHD after BMT [97]. Co-infusion of

allogeneic islets, bone marrow cells and MSCs into diabetic rats achieved immune tolerance of allograft tissue without the development of GVHD [97]. In addition, long term reversal of diabetes in the rat model was also observed [97]. Interestingly, administration of the immunosuppressive drug cyclosporine A did not lead to normalization of blood glucose, which suggests that systemic immunosuppression did not facilitate recovery from diabetes [97]. One possible explanation is that MSCs modulated the activity of adaptive immune cells like T lymphocytes and dendritic cells.

1.4.3.2 Graft Specific Immunosuppression

Other than systemic infusion of MSCs, local administration of MSCs is desirable for a graft specific immunoprotection [98-101]. Proliferation of a tumor in a mouse model after injection with MSCs demonstrated local immuno-suppression can be achieved [98]. For islet transplantation, combined delivery of autologous MSCs along with allogeneic islets in an omental pouch of diabetic rats improved insulin production and restored normoglycemia [100]. Administration of MSCs altered the adaptive immune response. Levels of the pro-inflammatory cytokines IFN-γ and TNF-α secreted from T cells after activation remained depressed in the allogeneic transplant group. In the same mice, the number of anti-inflammatory interleukin 10 (IL-10) secreting $CD4^+$ T cells was also increased within the mesenteric lymph nodes [100]. Ding *et al.* recently elucidated a molecular pathway that may account for these immunosuppressive effects [101]. MSCs expressed matrix metalloproteinase 2 and 9 to disrupt

interleukin 2 (IL-2) mediated activation of T cells by cleaving the IL-2 receptor, CD25 [101]. All in all, both systemically and locally administered MSCs exhibited immuno-modulation favorable to islet graft survival and function in several diabetic animal models. These findings may lead to the development of chronic immunosuppression free islet transplant protocols.

1.4.4 Mesenchymal Stem Cells for Islet Transplantation

To summarize, MSCs are known to reside in the developing pancreas to direct proliferation and differentiation of both the exocrine and endocrine compartments. But the role of the MSCs in the matured adult pancreas (pMSC) may be very different from the developing pancreas; pMSC cannot be easily differentiated into insulin producing cells. Alternatively, pMSCs may participate in protecting β-cells from inflammation or inducing new vessel formation. The regenerative and immunomodulatory properties of MSCs are widely established. Because of these properties, several studies reported that MSCs have beneficial effects in experimental models of diabetes [79,80,94,95]. In cell culture, MSCs demonstrated the capacity to extend islet survival and maintain islet function [83-85]. Transplantation of MSCs also facilitated islet engraftment, suppressed immune rejection of allogeneic islets, and reversed diabetes *in vivo* [86-92]. These findings are exciting for the Edmonton islet transplant protocol; MSCs may be adapted as an adjunctive therapy to improve and prolong islet function as well as to protect human islets from immune rejection without the requirement for chronic immunosuppression. Certainly, whether allogeneic or autologous MSCs

are better suited for transplantation remains to be determined [94,99]. The long term safety profile of MSCs, including the potential to differentiate into unwanted stromal cell lineages, needs to be addressed. Overall, MSCs possess several attractive attributes to improve islet survival after transplantation and to reduce toxicity from immunosuppression.

1.5. SUMMARY

According to the Canadian Diabetes Association clinical practice guidelines, β-cell replacement therapy (islet transplantation) is still not an acceptable standard treatment for type 1 diabetes mellitus [9]. Presently, insulin therapy is safe and is the mainstay treatment, even though islet transplantation offers greater metabolic control. The limitations of islet transplantation include the lack of a readily available supply of donor islets, the use of chronic immunosuppression and the progressive loss of insulin independence after transplantation. Regenerative medicine may offer potential solutions for β-cell replacement; but, practical clinical translation has not been achieved. To resolve the issue of insufficient β-cell supply, growing β-cells with patients' own tissues for transplant has emerged as the preferable approach. Currently, these studies continue to be bench side pursuits. To relieve transplant recipients from chronic immunosuppression and extend long term graft function, the application of MSCs is a promising cell therapy. Numerous clinical trials utilizing MSCs have been initiated for immunotherapies of sepsis and graft versus host disease. As we venture into a new realm of medicine with stem cell technologies, safety must be

a primary consideration of all research as these potential therapies may have unforeseen medical, ethical and legal consequences. Stem cells have a highly proliferative capacity, leading to the risk of neo-plastic transformation. Thus, the long term consequences of stem cell treatments remain unknown. The implementation of stem cell therapies will require multiple multi-centered clinical trials. The ethical use of donor tissues, patient privacy, disclosure and numerous newly emerging issues surrounding stem cell technologies will require legislation to govern proper clinical application. Nevertheless, this new perspective in medical research, focusing on regeneration and replacement, continues to offer hope for curative therapies in the future.

1.6 OBJECTIVES AND GENERAL OUTLINE OF THESIS

The objective of this study is to examine the immunoprotective effect of human mesenchymal stem cells on human islets in cell culture as well as in a preclinical model of islet transplantation. Various factors contribute to the progressive failure of islet graft function including allograft rejection, post-transplant inflammation, immunosuppressive drug toxicity and lack of sufficient progenitor cells to mediate β cell turnover. Immediately after transplantation, however, loss of considerable islet mass is primarily caused by inflammation. This inflammatory response is characterized by elevated levels of interferon γ, tumour necrosis factor α and interleukin 1β. Inhibiting the activity of these pro-inflammatory cytokines with anti-inflammatory drugs can prevent injury to transplanted islets but requires systemic immunoregulation. Consequently, graft

specific immunoprotection is a desirable alternative. Cell based therapy with an immunoregulatory cell such as the mesenchymal stem cell (MSC) has emerged as a promising strategy to achieve localized immunosuppression. MSCs are reported to reduce inflammation after transplantation and to initiate cell repair at sites of tissue damage.

The scope of Chapter 2 is to develop a strategy that protects islets from pro-inflammatory cytokines in cell culture. The protective effects of MSCs derived from bone marrow and pancreatic tissue on human islets was examined. Skin fibroblasts, which are morphologically identical to MSCs, were used as a control cell to confirm that the effect is MSC dependent. Other studies have reported the beneficial effects of MSCs on rodent islets and identified several growth factors that can augment islet function. Among these factors, we explored the role of HGF in MSC mediated islet protection.

In rodent islet and MSC co-culture studies, the production of beneficial factors by MSCs has been associated with improved islet survival and function. In our islet:MSC co-culture, we observed physical interaction between islets and MSCs, which suggests that cell contact can participate in protection. Thus, the role of secreted factors and cell to cell contact is further explored in Chapter 3. The cytoprotective effect of secreted factors is determined by assessing islet function in culture media conditioned by MSCs. An increase in the amount of secreted factors may elicit a more potent protective effect. Because hypoxia can increase MSC activity, culture medium from MSCs cultured in a hypoxic chamber was also tested on cytokine treated human islets. To test the role of cell

contact, islets and MSCs were cultured in separate compartments via alginate microencapsulation of human islets. This method prevents direct cell contact but allows the diffusion of soluble factors between islets and MSCs.

The third objective, in Chapter 4, is to examine the function of human islet and MSC co-transplants in a pre-clinical mouse model of diabetes. Localized and systemic delivery of MSCs to the islet transplant graft are explored. For localized MSC transplantation, islets and MSCs are aggregated and placed under the renal capsule. Localized transplantation is a method for graft specific immunotherapy; but large grafts with co-transplants can be a restriction for this approach as the transplant site may not be able to accommodate the entire volume of tissue. For a systemic approach, MSCs are injected into the tail vein after islet transplantation. MSCs can migrate to sites of inflammation but long term engraftment of MSCs is minimal.

Overall, these studies examine the therapeutic potential of mesenchymal stem cells in islet cell culture as well as in an islet transplant model. Inflammation is recognized as the main cause of impaired islet graft function in the immediate post-transplant period. The focus is to investigate strategies to protect islets from inflammation mediated islet dysfunction. To mimic the inflammatory environment after islet transplantation in cell culture, the function of islets and MSCs co-culture is assessed in the presence of pro-inflammatory cytokines. The clinical translation of this method is then determined in a mouse model of diabetes. These studies explore the potential application of MSCs as a

cell therapy to improve clinical islet transplant outcomes by prolonging graft function.

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CHAPTER 2

HUMAN MESENCHYMAL STEM CELLS PROTECT HUMAN ISLETS FROM PRO-INFLAMMATORY CYTOKINES *

2.1 INTRODUCTION

Islet transplantation is an attractive alternative treatment to daily insulin injections for patients with type 1 diabetes [1]. Following the Edmonton Protocol, nearly 90% of islet transplant recipients remained insulin independent at one year [2]; however, only 10% of the recipients were insulin independent at five years' post transplant [3]. This loss of graft function may be attributed to various factors including toxicity of immunosuppressive drugs [3], immune rejection [3], and inadequate supply of islet precursor cells for β cell replacement [4]. However, immediately after transplantation, inflammation plays a significant role in the loss of islet function [5,6]. This inflammatory response is characterized by elevated interferon-γ (IFN- γ), tumor necrosis factor-α, (TNF-α) and interleukin 1β (IL-1β) [5,7]. Rodent and human islet cells exposed to these pro-inflammatory cytokines lose glucose responsiveness and express increased markers of apoptosis [8,9]. In addition to β-cell cytotoxicity, these cytokines exacerbate the inflammatory response by recruiting and activating immune cells such as macrophages [5,10].

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Decreasing cytokine expression, inhibiting cytokine activity or inhibiting macrophage activity improves the function of transplanted islets [5,7,10]. Thus, minimizing inflammation at the transplant site during engraftment may help to prolong islet graft function and maintain long-term insulin independence.

Control of islet graft inflammation may be achieved by co-transplantation of islets with multipotent mesenchymal stromal cells, also known as mesenchymal stem cells (MSCs) [11]. These stromal cells are connective tissue derived stem cells with immunomodulatory and regenerative properties [12,13]. They also secrete anti-inflammatory proteins and suppress the activity of various immune cells such as alloantigen activated T and B lymphocytes [12,13]. Moreover, MSCs can secrete growth factors that improve tissue survival, stimulate angiogenesis and facilitate tissue engraftment in animal models of myocardial infarction, diabetes and graft versus host disease [12,13].

In cell culture, the trophic effects of MSCs have also been reported with rodent and human islets [14-18]. However, the capacity of human MSCs to protect human islets from pro-inflammatory cytokines has not been studied. Therefore, in this study, we examined the cytoprotective effect of bone marrow and pancreatic derived [19] MSCs on human islets *in vitro*. We co-cultured human islets with human MSCs and measured islet cell survival and function after exposure to a pro-inflammatory cytokine cocktail. We report that MSC aggregates preserve glucose stimulated insulin secretion (GSIS), prevent islet β-cell apoptosis, and identify possible secreted MSC factors that mediate this

protection. Thus, co-administering MSCs may be beneficial in prolonging islet graft survival.

2.2 MATERIALS AND METHODS

Ethics Statement

Human pancreases were procured from cadaveric donors after written informed research consent was provided by donor relatives. Written ethical approval for this research study was provided by the University of Alberta's Health Research Ethics Board – Biomedical panel (study ID: Pro00001416).

2.2.1 Preparation of Human Islets

Human pancreases (ages ranging from 16 to 71, n=23) were processed according to islet isolation protocols previously described by our group [1,4]. Islet enriched fractions (10-30% dithizone positive) were cultured in Roswell Park Memorial Institute (RPMI) 1640 (Gibco, Carlsbad CA) medium supplemented with 0.5% w/v fraction V bovine serum albumin (BSA, Sigma-Aldrich, Oakville, Canada) and 1.0% v/v insulin-transferrin-selenium (ITS, Sigma-Aldrich).

2.2.2 Preparation of Human Bone Marrow

To prepare bone marrow derived mesenchymal stem cells (bMSCs), human bone marrow was extracted from six patients aged 24, 42, 45, 46, 62, and

69 years (Division of Orthopedic Surgery, University of Alberta) following informed consent [20]. For expansion, cells were plated in Modified Essential Medium alpha (MEMα, Cellgro Manassas, VA) supplemented with 2.5 ng/mL basic fibroblast growth factor (bFGF, Millipore, Billerica, MA), 10% fetal bovine serum (FBS, Gibco), 1 mM sodium pyruvate (Gibco), 10 mM HEPES (Gibco), 100 U penicillin/1000 U streptomycin (Biowhittaker, Walkersville, MD) at a density of 166,000 cells per cm^2. Non-adherent cells were removed by changing the medium every 2-3 days. Once confluent, the cell monolayer was washed with versene and was detached with 0.05% v/v trypsin-EDTA (Invitrogen, Carlsbad CA). Cells were counted and re-seeded into supplemented MEMα culture medium at a density of 5000-10000 cells/cm^2.

2.2.3 Preparation of Pancreatic Derived MSCs

To prepare human pancreatic derived mesenchymal stem cells (pMSCs), human pancreatic tissue from islet depleted fractions were obtained from seven donors aged 36, 44, 49, 49, 52, 57, and 64 years and were cultured in RPMI 1640 with 0.5% w/v BSA and 1.0% v/v ITS for 24 to 48 hours [19]. In our original study [19], pMSCs expressed surface markers characteristic of MSCs and differentiated into osteocytes, adipocytes and chondrocytes. To expand pMSCs, these cell preparations were cultured for 5 to 8 days in RPMI 1640 with 10% FBS, 20 ng/mL epidermal growth factor (EGF, R&D Systems, Minneapolis, MN), 20 ng/mL bFGF, 1 mM sodium pyruvate, 10 mM HEPES, 100 U penicillin/ 1000 U streptomycin, and 71.5 μM β-mercaptoethanol (Sigma-Aldrich). Once

75-90% confluent, the cell monolayer was enzymatically detached with 0.05% v/v trypsin-EDTA. Cells were re-plated in supplemented RPMI medium described above at a density of 2500-5000 cells per cm^2. Both bone marrow and pancreatic derived MSCs from passages 2-6 were utilized for this study. All cell cultures were maintained at 37^oC with 5% CO_2 in a humidified incubator.

To confirm that both bMSCs and pMSCs express the classical MSC surface antigens, cells from passages 2-6 were stained with MSC markers based on the position statement from the International Society for Cellular Therapy (ISCT) [21]. Cells were fixed in 4% w/v paraformaldehyde for 1 hour and washed with phosphate buffer saline (PBS) before primary antibody staining. Cells were stained for MSC markers CD29-PECy5 (Caltag, Carlsbad CA), CD44-FITC (Chemicon, Billerica, MA), CD73-PE, CD90-PE (BD Biosciences, Mississauga, Canada), and CD105-PE (Biolegend, San Diego, CA) as well as hematopoietic lineage markers CD11b-FITC (Abcam, San Francisco CA), CD19-PE (Abcam), CD34-FITC and CD45-PE (Caltag) for 30-60 minutes (4^oC, protected from exposure to light). MSC marker expression was analyzed on a BD FACScalibur flow cytometer. For isotype controls, cells were also stained for IgG_1-PE (Cedarlane, Burlington, Canada), IgG_1-FITC (Cedarlane) and IgG_1-PECy5 (Caltag).

2.2.4 Preparation of Human Dermal Fibroblasts

Human dermal fibroblasts were prepared and expanded from normal skin samples of donors aged 35 and 60 in order to serve as a negative control cell

population (Division of Plastic Surgery, University of Alberta) [22]. Fibroblasts were cultured in Dulbecco's Modified Eagle Medium high glucose (DMEM 25 mM glucose, Gibco) and detached using 0.05% v/v trypsin-EDTA. For sub-culturing, cells were split at a ratio of 1:6 and fibroblasts at passages 3-8 were used in this study.

2.2.5 Exposure of Human Islet:MSC Co-cultures to Pro-inflammatory Cytokines

Bone marrow (bMSC) and pancreatic (pMSC) derived MSCs were enzymatically detached from culture plates, counted, and added (0.5, or 1.0 x 10^6 cells) to a 100 mm low adherence culture dish (Corning) with 500 human islets in a total volume of 10 mL. Controls included islets cultured alone (± cytokines) and islets co-cultured with human dermal fibroblasts (± cytokines). The culture medium consisted of DMEM low glucose (5.6 mM glucose, Gibco) with 1% FBS, 20 ng/mL EGF, 20 ng/mL bFGF, 10 mM HEPES, 100 U penicillin/1000 U streptomycin, and 71.5 μM β-mercaptoethanol. After 24 hours, these islet:MSC co-cultures and islet:fibroblast co-cultures were exposed to a previously described cocktail of pro-inflammatory cytokines [23] including 1560 ng interferon-γ, 250 ng tumor necrosis factor-α, and 0.4625 ng interleukin 1β (specific activities $2.4x10^6$ U/mg, $5\text{-}2x10^7$ U/mg, $1.16\text{-}0.54x10^9$ U/mg respectively; Biolegend) in 10 mL volume for 48 hours.

2.2.6 Characterization of Islet:MSC Co-cultures

A static incubation assay [24] was used to determine glucose responsiveness in islet controls and in co-cultures following the 48 hour cytokine exposure. Islets, islet:MSC co-cultures and islet:fibroblast co-cultures were collected and washed twice by gravity sedimentation over 30 minutes. Preparations were then divided into representative aliquots and incubated at 37^{o}C for 2 hours in 1.5 mL RPMI supplemented with 2.0 mM L-glutamine, 0.5% w/v BSA and either 2.8 mM (low) or 20.0 mM (high) glucose. Tissue and medium were then separated by centrifugation and assayed for their respective insulin contents by a human insulin immunoassay (Meso Scale Discovery, Gaithersburg, MD). The insulin content of that secreted in the medium was normalized to that of the total cellular insulin content [24]. To assess total cellular insulin content, intact islets from representative aliquots were lysed then centrifuged to remove cellular debris. Cellular insulin content was measured by a human insulin immunoassay. Stimulation indices were calculated by dividing the amount of insulin released at 20.0 mM glucose by that released at 2.8 mM glucose and insulin release (% insulin content) is reported as insulin secreted at 2.8 mM or 20.0 mM glucose divided by total cellular insulin content.

2.2.7 Analysis of Islet β-cell Apoptosis by Immunohistochemistry

Cytokine induced β cell damage was assessed by the co-expression of insulin and TUNEL, a marker for cell apoptosis, from human islets. After culture, paraffin sections of islets were prepared for double immunofluorescence (IF)

analysis by fixation with 4% w/v paraformaldehyde (BDH Laboratory Supplies, Poole, England) and embedding in 2% w/v low melting point agarose (Sigma-Aldrich). Paraffin sections were processed and immunostained. After rehydration, antigen retrieval for tissue samples was performed in sodium citrate buffer (pH 6.0). The samples were then blocked with 20% normal goat serum (NGS, Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA) for 1 hour in the dark. For apoptosis, an APO-BrdU TUNEL Assay Kit (Invitrogen) was utilized, in which an AlexaFluor 488 labeled anti-BrdU antibody was used for detection. The same tissues were then stained with a guinea pig anti-insulin antibody (Dako, Mississauga, ON, Canada) diluted at 1/1000 in 5% NGS followed by a secondary AlexaFluor 594 mouse anti-guinea pig antibody (Molecular Probes, Eugene, OR, USA). Slides were cover slipped with Prolong Gold Anti-fade (Invitrogen) to preserve fluorescence. Negative controls included sections of the same tissues incubated without insulin primary antibodies. For the TUNEL assay, TUNEL positive and TUNEL negative control cells were stained to confirm TUNEL positivity. Slides were visualized with an Axioscope II microscope equipped with an AxioCam MRC and analyzed with Axiovision 4.6 software (Carl Zeiss, Gottingen, Germany).

2.2.8 Analysis of Culture Supernatant for Cytoprotective Factors

To assess cytoprotective factors secreted by MSCs in islet:MSC co-cultures exposed to cytokines, factors were measured in the cell culture supernatant and analyzed by custom immunoassay kits (interleukin 6 (IL-6) /

interleukin 10 (IL-10), hepatocyte growth factor (HGF) / vascular endothelial growth factor (VEGF), matrix metalloproteinase 2 (MMP2) / matrix metalloproteinases 9 (MMP9), Meso Scale Discovery). To determine base line levels, MSCs were cultured alone as either a cell monolayer or as cell aggregates in tissue cultured or low adherent 6 well plates, respectively, at a density of 0.2 x 10^6 cells/2.0mL. Supernatant was collected from 1 and 3 day MSC cultures, and cell debris was removed from the supernatant and collected by centrifugation (10, 000xg, 10 minutes, 4°C). Supernatant from islet:MSC co-cultures (the same used to measure insulin secreted content) was collected following exposure to cytokines and also centrifuged to remove and collect cell debris. To normalize protein content secreted into the medium to cellular DNA, cells collected from corresponding supernatant samples were assessed for DNA content. To measure DNA content, cell pellets were washed twice with citrate buffer (150 mM NaCl, 15 mM citrate, 3 mM EDTA, pH 7.4), sonicated in DNA lysis buffer (0.5% v/v Triton x-100 in pH 7.5 Tris HCl-EDTA) and aliquots of 25µL and 50µL were assayed in duplicate with Pico Green reagent (Molecular Probes, Carlsbad, CA) and fluorescence was detected at 485 ex. /527 em. nm. [24].

2.2.9 Effect of Hepatocyte Growth Factor on Human Islets Exposed to Pro-inflammatory Cytokines

Islet cultures (500 islets in 10mL of culture medium used for islet:MSC co-cultures) were prepared as described above with recombinant human hepatocyte growth factor (10 ng/mL rhHGF, R&D Systems, Minneapolis, MN). After 24 hours, the culture medium of the islet cultures was changed.

The cultures were then treated with a second dose of rhHGF and exposed to pro-inflammatory cytokines [23], including 1560 ng interferon-γ, 250 ng tumor necrosis factor-α, and 0.4625 ng interleukin 1β (specific activities 2.4 x 10^6 U/mg, 5-2 x 10^7 U/mg, 1.16-0.54 x 10^9 U/mg respectively; Biolegend) for 48 hours. Controls included islets cultured alone and islets exposed to cytokines without HGF.

2.2.10 Statistical Analysis

Data are presented as mean ± SEM. Comparisons of mean values were performed with one-way ANOVA and/or Kruskal Wallis multiple comparisons tests with the level of significance set at $\alpha=0.05$. A Bonferroni or corrected Bonferroni analysis was conducted for data considered significantly different between treatment groups. All statistical analyses were performed with STATA 11 (StataCorp LP, College Station, TX).

2.3 RESULTS

2.3.1 Characterization of Bone Marrow and Pancreatic Derived MSCs

MSC surface antigen expression [19] was analyzed by flow cytometry for passages 2-6. Both bone marrow (n=3) and pancreatic (n=5) derived MSCs expressed high levels of CD29, CD73, CD90 and CD105 (>90%). However, these cells did not express hematopoietic markers CD11b, CD19, CD34 and CD45 (<5%, Table 2-1).

2.3.2 MSCs Preserve Islet Function in Islet:MSC Co-cultures Exposed to Pro-inflammatory Cytokines

Glucose stimulated insulin secretion (GSIS) was used to assess human islet function in a two hour static incubation assay at 2.8 mM and 20.0 mM glucose. Islets without cytokine exposure exhibited a glucose induced stimulation index (SI) of 2.1±0.2 (n=7) with corresponding insulin release at 2.8 mM and 20.0 mM glucose of 2.3±0.3% and 4.6±0.4% (n=7). Cytokine exposure significantly altered GSIS. Exposure to cytokines significantly increased insulin release at 2.8 mM and 20.0 mM glucose, respectively (11.6±1.9% and 14.3±3.1%, $p<0.05$), resulting in a reduced SI of 1.2±0.1 ($p<0.05$ vs. no cytokine). In contrast, islets co-cultured with MSCs maintained GSIS, and this protective effect was also dependent on MSC dose. In particular, islets co-cultured with 1.0×10^6 bMSCs had significantly improved GSIS compared to cytokine treated islets (SI=2.0±0.2 and percentage insulin release at 2.8 mM and 20.0 mM glucose of 3.6±1.6% and 6.8±0.9%, n=6, $p<0.05$). Similar effects were also observed with pMSCs but the effect was more robust with all doses of bMSC aggregates (Table 2-2). On the other hand, this protective effect was not observed when islets were co-cultured with dermal fibroblasts. Insulin release was elevated at both 2.8 mM and 20.0 mM glucose, and the stimulation index was comparable to cytokine exposed islets (Table 2-2). While increasing fibroblast numbers marginally decreased percentage insulin release, the values were not significantly different from cytokine treated islets. Recovery of total cellular insulin content was significantly reduced after cytokine exposure. However, recovery of insulin content from islets co-cultured with 1.0×10^6 bMSCs was not significantly

reduced compared to untreated controls (Table 2-2). For each co-culture experiment, independent islet samples were assessed. In addition, to determine the effect of MSCs on islets without cytokines, islets were co-cultured with 1.0 x 10^6 bMSCs or 1.0 x 10^6 pMSCs as independent conditions. In the absence of cytokines, GSIS from islet:MSC co-culture was not different from islet controls. In summary, the SI for islets co-cultured with bMSCs was 2.6±0.4 compared to islets alone, 2.1±0.2; and the SI for islets co-cultured with pMSCs was 2.4±0.2 compared to islets alone, 2.2±0.4.

2.3.3 Bone Marrow Derived MSCs Prevent Cytokine Induced Islet β-cell Apoptosis

TUNEL identifies DNA fragmentation within a cell and is an indicator of a cell undergoing apoptosis. To determine islet β cell apoptosis, tissues were co-stained for insulin and TUNEL. Islets, islets + cytokines, islets + bMSCs, and islets + bMSCs + cytokines were compared. Islets cultured without cytokines exhibited few TUNEL positive cells (Figure 1B). After pro-inflammatory cytokine exposure, insulin and TUNEL co-expression was observed in islets cultured alone (Figure 1F). The number of insulin positive cells from cytokine treated islets was also decreased compared to untreated islet controls (Figure 1D). Interestingly, other cells within the islet that did not stain for insulin were TUNEL positive. Changes to islet morphology were observed, as insulin positive cells appear less organized than untreated controls (Figure 1D, 1F). In the islets + bMSCs control, no TUNEL staining was observed (data not shown). Insulin staining and the islet morphology were similar to untreated islet controls.

Cytokine exposure, however, did not induce TUNEL expression from islets co-cultured with 1.0 x 10^6 bMSCs (Figure 1H, 1I). The number of insulin positive cells was comparable to control tissues, and the organization of insulin positive cells in the islet did not appear to be affected.

2.3.4 Bone Marrow and Pancreatic Derived MSCs Secrete Cytoprotective Factors

Basal secretion from bMSCs and pMSCs was determined in MSC cultures without islets. MSCs secrete a variety of soluble factors that are involved in cell survival, angiogenesis and immunoregulation. Of those factors assayed, four factors (IL-6, HGF, VEGF, and MMP2) were detectable in cultures of bMSCs and pMSCs without islets or cytokines (Table 2-3). Control samples were the MSC culture medium without MSCs. The contribution of growth factors from culture media was minimal (<0.5% of measured values) except with the IL-10 and MMP9 assays (between 1-10% of measured values). During expansion as a cell monolayer, production of HGF, VEGF and MMP-2 increased with culture time in both cell populations. On days 1 and 3, bMSC monolayers secreted higher levels of HGF (17.5±3.6 pg/ng vs. 2.2±0.7 pg/ng), VEGF (32.4±2.9 pg/ng vs. 9.4±2.5 pg/ng) and MMP2 (360.7±34.3 pg/ng vs. 114.1±9.3 pg/ng) than pMSC monolayers. Expression of IL-6 from bMSCs demonstrated increased production of IL-6 with cell expansion (13.1±1.2 pg/ng and 22.7±0.2 pg/ng), while pMSC secretion of IL-6 remained constant (16.1±6.5 pg/ng and 17.4±7.4 pg/ng) between days 1 and 3 in culture, respectively (Table 2-3).

When cultured as cell aggregates, MSCs exhibited a different secretion profile. HGF, VEGF, MMP2 and IL-6 remained detectable. However, production of these cytoprotective factors from aggregated bMSCs was reduced in comparison to monolayer bMSCs (Table 2-3). In addition, between days 1 and 3, expression of IL-6 (0.5±0.1 pg/ng and 0.3±0.1 pg/ng), HGF (1.5±0.4 pg/ng and 1.5±0.4 pg/ng) and MMP2 (47.2±6.1 pg/ng and 51.7±5.7 pg/ng) did not change. In contrast, pancreatic MSCs demonstrated greater HGF and MMP2 production as cell aggregates in comparison to monolayer cells. Production of IL-6 and VEGF was similar between aggregated and monolayer pMSCs at both days 1 and 3.

2.3.5 Pro-inflammatory Cytokine Stimulation Increases Cytoprotective Factor Release

Levels of cytoprotective factors were also determined in islet:MSC co-cultures following pro-inflammatory cytokine exposure. IL-10 production remained low in all conditions tested. Compared to islet controls, islets exposed to cytokines had an increase in the expression of all cytoprotective factors we assessed. Islet:MSC co-cultures exposed to pro-inflammatory cytokines resulted in a further increase of these factors except MMP9 (Table 2-4). HGF and MMP2 levels remained low in islets with or without cytokines, while MSC aggregates alone expressed relatively high levels. In islet:MSC co-cultures, the increase in HGF and MMP2 production likely resulted from contributions by MSC aggregates. MMP9 levels were undetectable from MSC aggregates with or without cytokines. With islets, MMP9 expression was high in cytokine treated islets (25.6±6.8 pg/ng) compared to untreated islets (12.5±2.5 pg/ng). After co-

culture, MMP9 levels decreased in a dose dependent manner (15.1±3.8 pg/ng, 8.5±2.4 pg/ng, Table 2-4). A very similar pattern was observed when human islets were co-cultured with pancreatic derived MSCs (data not shown).

2.3.6 Hepatocyte Growth Factor Preserves Islet Function after Pro-inflammatory Cytokine Exposure

When islets treated with cytokines were exposed to 2.8 mM and 20.0 mM glucose, they exhibited a loss of glucose responsiveness with a stimulation index (SI) of 1.1±0.1 (n=5) (Table 2-5). Percentage insulin release at 2.8 mM and 20.0 mM glucose (10.3±1.8% and 10.9±1.5% respectively, n=5) was also significantly elevated compared to untreated islets (2.8±1.4% and 6.0±0.8% respectively, n=5). Addition of HGF to the culture medium, however, preserved the glucose responsiveness of cytokine treated islets (SI=1.8±0.2, $p<0.05$), but insulin release at 2.8 mM and 20.0 mM glucose remained elevated (8.0±1.8% and 14.1±2.6% respectively, n=4) compared to untreated islets (Table 2-5). Recovery of insulin content from cytokine treated islets did not improve with the addition of HGF in culture compared to islets treated with cytokines only. The effect of HGF on islets without cytokines was also assessed. No differences in GSIS were observed as the SI for islet with HGF was 2.3±0.5 compared to the SI for islets alone, 2.4±0.4.

2.4 DISCUSSION

During islet engraftment, up to 60% of islet tissue is lost within the first 72 hours after transplantation [5,6]. The detection of inflammation in and around the islet graft with syngeneic and autologous donors suggests that a non-specific immune response is the major factor for loss of islets [5,6,25]. This inflammatory response can be characterized by immune cell infiltration and elevated levels of IFN-γ, TNF-α, and IL-1β [5-7,10,26]. Because the achievement of insulin independence with the Edmonton Protocol depends on transplanting a sufficient islet mass, preserving islet mass and function can prevent early graft failure and may also decrease the requirement for multiple islet donors [3,5]. Although chemical and pharmacologic inhibition of these cytokines can improve islet graft function [7,10,26], islet graft specific immunosuppression would be more desirable and could be achieved by treatment with an immunoregulatory cell such as the MSC. Described as adult connective tissue derived stem cells with the capacity to differentiate into osteocytes, adipocytes and chondrocytes, MSCs are typically expanded as a cell source for tissue replacement strategies [11-13]. However, the reduction in inflammation, fibrosis and cell death with MSC therapy led to the recognition that MSCs could also produce potent protective factors for tissue repair and immunomodulation [27-30]. Here we have proposed that the cytoprotective properties of MSCs could protect human islets from pro-inflammatory cytokines. To investigate this hypothesis, we devised a co-culture method of islets and MSCs and tested the effect on islet glucose stimulated insulin secretion (GSIS) after cytokine exposure.

Previously, the favorable effects of islet:MSC co-culture for islet function have been predominantly reported in cultures where islets and MSCs are physically separated [14,15]. However for co-transplantation, islets and MSC are not separated in distinct compartments [31-33]. To better understand islet:MSC interactions for transplantation, MSCs and islets need to be cultured together in the same compartment. One approach for cell contact based co-culture is to coat islets with a layer of MSCs to provide a protective barrier to the immune response [16] or to facilitate tissue engraftment [17]. Duprez *et al*. demonstrated that MSCs could coat islets; and coating was enhanced with increased MSC numbers and time in culture [16]. In our co-culture design, we did not observe this interaction. Instead, MSCs formed aggregates, which could physically interact with islets. The formation of MSC aggregates in co-culture with human islets demonstrates that direct co-culture is a promising approach to develop islet graft specific cell therapies.

To measure the beneficial effects of this co-culture strategy, we tested islet function (GSIS) of islet:MSC aggregates exposed to pro-inflammatory cytokines. Although MSC monolayers are beneficial to islet survival and function [14,15], several authors have reported that direct co-culture does not improve islet function [16,17]. Here we report that aggregation of MSCs with human islets can protect human islets from pro-inflammatory cytokines, but fibroblasts are not protective for islet function. This protective effect was not restricted to bMSCs, but was also observed with pMSC. Thus, preservation of GSIS in cytokine

treated human islets is MSC dependent. Examination for the percentage insulin release reveals that cytokine exposure significantly increases both basal and stimulated insulin secretion ($p<0.05$) compared to untreated control islets. However, the release of insulin is not glucose dependent, which suggests that increased insulin secretion may be due to the cytotoxic effects of cytokines such as disruption of cell membrane integrity [5]. Hostens *et al*, also reported that cytokine exposure could increase insulin release when reported as a percentage of total cellular insulin [34]. They concluded that their cytokine treatment altered the functional state of the β cell. In contrast, co-culture of islets with MSCs prior to cytokine exposure reduces percentage insulin release and maintains glucose responsive insulin secretion (Table 2-2). Particularly, increasing MSC numbers to 1.0×10^6 significantly improved islet function ($p<0.05$), and decreased percentage insulin secretion at 2.8 mM and 20.0 mM glucose to levels that were comparable to untreated islets. Although others have reported that direct islet:MSC interactions do not produce beneficial effects [16,17], the difference in MSC-islet interaction in our co-culture approach (minimal islet:MSC coating) is a possible explanation for the protective effects that we observed. Nevertheless, this protection is limited because cellular insulin content was decreased in all conditions exposed to cytokines. The difference in basal insulin secretion of control and co-culture islets suggests that MSCs may protect the glucose sensing but may not affect the insulin biosynthetic function of human islets. While we have not explored the mechanisms for this loss in insulin content, the disruptive

effects of IFN-γ, TNF-α, and IL-1β on insulin biosynthesis have been previously reported [5,8,9]. Interestingly, the recovery of cellular insulin content was greatest with bMSCs.

In addition to impairing glucose responsiveness, IFNγ, TNFα and IL-1β can be cytotoxic to β cells. As bMSCs exhibited protective effects on islet function, we assessed the ability of bMSCs to prevent β cell apoptosis in our islet:MSC co-cultures. We observed that most β cells in the cytokine treated islets group were positive for TUNEL, confirming the cytotoxicity of the pro-inflammatory cytokine cocktail. A decrease in the number of insulin positive cells was observed, correlating with our data demonstrating 34.7±3.2% cellular insulin recovery in the presence of cytokines. In contrast, when islets were co-cultured with MSCs, insulin expression was maintained and fewer TUNEL positive cells were present after cytokine treatment. Therefore we conclude that MSCs could prevent islet β cell apoptosis in the presence of cytokines. Islet structure is also important for islet function. We observed that islet morphology was altered after cytokine exposure but the morphology of islets from co-culture and controls remained intact, correlating with the reported effects on islet function. These results suggest that the mechanisms underlying the protective effect of MSCs on β cells involve the mitigation of cell death pathways and the retention of native islet morphology. Identifying the signals from islet:bMSC co-cultures may be a strategy to enhance this protection.

As MSCs but not fibroblasts from different tissue sources induce similar effects on human islet function, a common MSC specific secreted factor may

mediate these effects. We investigated the cytoprotective factors IL-6, IL-10, HGF, VEGF, MMP2, and MMP9 because of their reported beneficial effects on islets [14,15,35] and in islet transplantation [31,33,36,37]. HGF, for instance, can signal pathways regulating cell survival and insulin secretion [15]. IL-6, on the other hand, prevented the functional impairment of IFN-γ, TNF-α, and IL-1β treated mouse islets [38]. Basal secretion levels from monolayer and aggregated MSCs were initially tested. IL-6, HGF, VEGF and MMP2 were consistently detected from both pancreas and bone marrow MSC monolayers, but IL-10 and MMP9 levels were low. Expression of these factors was also greater from bMSCs than pMSCs. Berman *et al*. reported similar findings, as monkey MSCs expressed IL-6, HGF and VEGF genes; whereas IL-10 gene expression remained low during monolayer expansion [37]. The three dimensional microenvironment of aggregated MSCs is unique from the two dimensional MSC monolayer. We wanted to determine whether basal production of soluble factors was markedly different with aggregated MSCs. In general, aggregated MSCs also express IL-6, HGF, VEGF and MMP2. An intriguing trend emerged in which bMSC aggregates had markedly decreased cytoprotective factor expression but pMSCs exhibited increased expression of these same factors (Table 2-3). This difference in cytoprotective factor production suggests that MSC function is dependent on tissue source and culture method as a cell monolayer or cellular aggregates.

To better define the factors mediating protection of islets, we examined the production of these cytoprotective factors from islet:MSC co-cultures. As bMSCs exhibited the greatest protective effect, we selected the islet:bMSC co-

cultures to determine expression profiles. HGF and MMP2 were MSC specific, and production increased with increasing numbers of MSCs (Table 2-4). We also observed that while MMP9 levels decreased, islet function improved with increasing MSC numbers, which suggests that bMSCs may protect by mitigating excessive MMP9 expression. Others reported that islets cultured in medium composed of IL-6, TGF-β, HGF and VEGF had significantly improved function [15]. In our co-culture system HGF, MMP2 and MMP9 appeared to be important MSC dependent factors associated with improved islet function after cytokine treatment. Addition of HGF to cytokine treated islets resulted in preservation of glucose responsiveness based on the stimulation index ($p<0.05$). Basal and stimulated insulin release, on the other hand, was elevated compared to untreated islets ($p<0.05$). In addition, recovery of insulin content was not improved with HGF (Table 2-5); whereas, islets co-cultured with bMSCs demonstrated better insulin content recovery than cytokine treated islets (Table 2-2). As HGF alone was unable to completely reproduce the effects of bMSC, we did not proceed to inhibit the activity of HGF in co-culture. Based on these results, we believe that other factors in addition to HGF such as MMP2, MMP9 and TGF-β may be important for cytoprotection of islet function.

2.5 CONCLUSION

In summary, MSC aggregates preserved GSIS and prevented islet β cell apoptosis of pro-inflammatory cytokine treated islets. The absence of this protective phenomenon with human dermal fibroblasts strongly suggests that preservation of GSIS is MSC dependent. Assessment of the secreted factors from MSCs demonstrates that HGF, MMP2 and MMP9 are possible secreted factors mediating protection. However, addition of rhHGF to islet cultures reveals that HGF alone cannot replace the beneficial effects of MSCs. Although the mechanism of protection is unclear, future studies can address the ability of MSCs to attenuate inflammation mediated β-cell graft dysfunction. Replicating these results *in vivo* will help develop a strategy to administer MSCs for clinical islet transplantation and to prolong graft survival and function.

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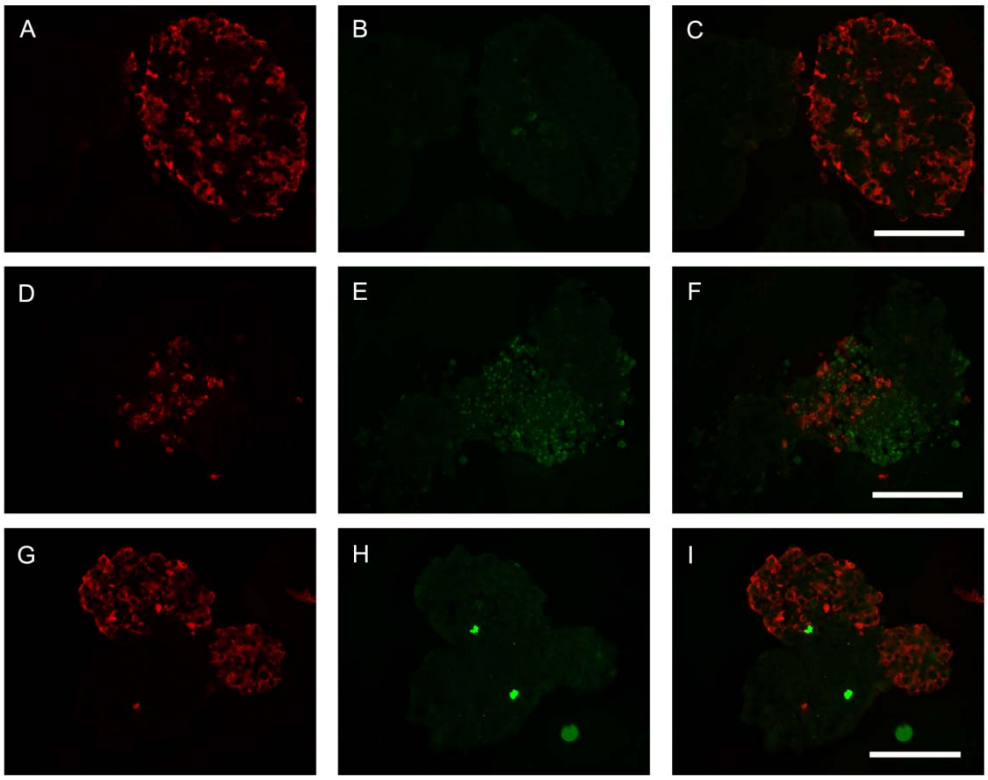
FIGURE LEGEND

Figure 2-1: Protection of Human Islets from Cytokine Induced Apoptosis by Bone Marrow Derived MSCs



A-C) 500 Islets, D-F) 500 Islets + cytokines, G-I) 500 Islets + 1.0 x 10^6 bMSCs, + cytokines. Tissues were stained for insulin (A,D,G) in red and TUNEL (B,E,H) in green. The merge of the red and green images are presented in panels C, F, and I. Islets cultured without cytokines demonstrated minimal TUNEL positive cells. After cytokine exposure, the number of TUNEL positive cells increased; TUNEL and insulin co-expression was also increased with cytokine treatment. Alteration of native islet organization was observed. After cytokine exposure, co-expression of insulin and TUNEL did not increase in the islets + bMSCs group; cytokines – cocktail of IFNγ, TNFα and IL-1β described in materials and methods. Scale bar represents 100 μm.

Table 2-1: Characterization of Cell Surface Antigens on Human Bone Marrow and Pancreatic Derived Mesenchymal Stem Cells

Epitopes	bMSC (n=3)	pMSC (n=5)
MSC Markers		
CD29	99.5±0.2	99.6±0.2
CD44	77.4±4.9	76.7±12.5
CD73	99.8±0.0	99.9±0.1
CD90	96.4±0.6	97.4±3.0
CD105	98.4±0.5	99.5±0.3
Non MSC Markers		
CD11b	0.9±0.1	0.7±0.2
CD19	0.9±0.1	0.8±0.2
CD34	1.2±0.1	1.2±0.1
CD45	1.5±0.2	4.3±3.8

Values are expressed as mean ± SEM from MSCs between passages 2 and 6. bMSCs, bone marrow mesenchymal stem cells; pMSCs, pancreatic mesenchymal stem cells.

Table 2-2: Effect of Cytokine Exposure on Human Islet Total Cellular Insulin Content and Insulin Secretory Capacity

Culture Conditions	% Recovery Cellular Insulin Content	Insulin Release (% insulin content) 2.8 mM Glucose	20.0 mM Glucose	Stimulation Index
Islet (n=7)	100	2.3±0.3	4.6±0.4	2.1±0.2
Islet + cytokine, (n=7)	34.7±3.2*	11.6±1.9*	14.3±3.1*	1.2±0.1*
Islet + 0.5 x 10^6 bMSC + cytokine (n=7)	52.8±5.1*	5.1±0.8†	9.0±1.4	1.8±0.2
Islet + 1.0 x 10^6 bMSC + cytokine (n=6)	68.0±5.3	3.6±1.6‡	6.8±0.9‡	2.0±0.2‡
Islet, (n=7)	100.0	2.8±0.3	5.8±0.9	2.2±0.4
Islet + cytokine (n=7)	28.7±5.5*	14.2±1.9*	15.4±1.8*	1.1±0.1*
Islet + 0.5 x 10^6 pMSC + cytokine (n=7)	35.6±7.4*	8.6±0.7§	13.6±1.5	1.6±0.2
Islet + 1.0 x 10^6 pMSC + cytokine (n=6)	33.2±7.3*	6.9±0.8‖	10.3±1.2	1.5±0.2
Islet, (n=4)	100.0	2.0±0.4	4.3±0.4	2.3±0.3
Islet + cytokine, (n=4)	32.2±3.9*	12.8±3.3*	16.9±5.4*	1.3±0.1*
Islet + 0.5 x 10^6 fibro + cytokine (n=3)	31.7±7.7*	9.9±1.5*	12.6±0.5*	1.3±0.2*
Islet + 1.0 x 10^6 fibro + cytokine (n=4)	40.3±5.9*	8.5±1.0*	13.6±2.7*	1.4±0.1*

Results are reported as % recovery of total cellular insulin content relative to untreated controls (islets alone). Islet function is assessed by a static glucose stimulated insulin release assay. The stimulation index (SI) is calculated as a ratio of insulin release at high glucose versus low glucose. Insulin release (% insulin content) is reported as insulin secreted at 2.8 mM glucose or 20.0 mM glucose divided by cellular insulin content for corresponding islets. Values are expressed as mean ± SEM.

* $p < 0.05$ for islet vs. all conditions
† $p < 0.05$ for islet + cytokine vs. islet + 0.5 x 10^6 bMSC + cytokine,
‡ $p < 0.05$ for islet + cytokine vs. islet + 1.0 x 10^6 bMSC + cytokine
§ $p < 0.05$ for islet + cytokine vs. islet + 0.5 x 10^6 pMSC + cytokine
‖ $p < 0.05$ for islet + cytokine vs. islet + 1.0 x 10^6 pMSC + cytokine.
(bMSCs) represents bone marrow derived mesenchymal stem cells, (pMSCs) represents pancreatic derived mesenchymal stem cells and (fibro) represents dermal fibroblasts.

Table 2-3: Basal Secretion of Growth Factors and Cytokines from Human Bone Marrow and Pancreatic Derived Mesenchymal Stem Cells

			(pg protein/ng DNA)					
Conditions	**MSC**	**Days Cultured**	**IL-6**	**IL-10**	**HGF**	**VEGF**	**MMP2**	**MMP9**
Monolayer	b	1	13.1±1.2	0.0±0.0	2.1±0.2	12.0±2.1	114.4±6.7	0.3±0.2
Monolayer	b	3	22.7±0.2	0.1±0.0	17.5±3.6	32.3±3.9	360.7±34.3	0.5±0.2
Monolayer	p	1	16.1±6.5	0.0±0.0	0.5±0.1	2.6±0.3	81.7±2.7	0.2±0.1
Monolayer	p	3	17.4±7.4	0.0±0.0	2.2±0.7	9.4±2.5	114.1±9.3	0.0±0.0
Aggregate	b	1	0.5±0.1	0.0±0.0	1.5±0.4	1.5±0.3	47.2±6.1	0.2±0.0
Aggregate	b	3	0.3±0.1	0.0±0.0	1.5±0.4	3.4±0.8	51.7±5.7	0.2±0.1
Aggregate	p	1	9.1±3.3	0.1±0.0	3.0±0.5	2.8±0.5	112.3±4.8	0.1±0.0
Aggregate	p	3	25.6±7.3	0.1±0.0	17.9±3.2	12.6±2.6	312.0±16.2	2.3±1.1

Levels of cytoprotective factors were measured from conditioned culture media, where bone marrow and pancreatic MSCs were cultured alone as either a cell monolayer or cellular aggregates. Values obtained from cell cultures were subtracted from background levels (<0.5% of measured values for IL-6, HGF, VEGF, MMP2 and 1-10% of measured values for IL-10, MMP9) measured in culture media alone. All values are normalized to the DNA contents of each culture condition. Values are expressed as mean ± SEM (n=3). (b) represents bone marrow and (p) represents pancreas derived MSCs.

Table 2-4: Secretion of Growth Factors and Cytokines from Human Islet and Bone Marrow MSC Co-cultures

	(pg protein/ng DNA)					
Culture Conditions	**IL-6**	**IL-10**	**HGF**	**VEGF**	**MMP2**	**MMP9**
Islet	9.6±2.7	0.0±0.0	0.1±0.0	2.8±0.5	3.4±0.5	12.5±2.5
Islet + cytokine	21.1±4.4	0.1±0.0	0.1±0.0	5.5±0.4	5.7±0.9	25.6±6.8
Islet + 0.5 x 10^6 bMSC + cytokine	33.8±9.5	0.1±0.0	3.2±1.0*	8.6±1.5	95.8±18.7*	15.1±3.8
Islet + 1.0 x 10^6 bMSC + cytokine	24.2±5.3	0.1±0.0	3.4±1.0*	7.7±0.9	94.5±19.8*	8.5±2.4
0.5 x 10^6 bMSC	1.4±0.2	0.0±0.0	6.0±2.6*	6.8±0.6	144.2±21.8*	0.3±0.2*
0.5 x 10^6 bMSC+ cytokine	44.6±4.8	0.2±0.0	4.4±1.0*	3.7±0.4	165.4±12.7*	0.2 ±0.1*

Levels of cytoprotective factors were also measured from conditioned media from islets cultured alone, with cytokines and with bone marrow MSCs with and without cytokines. Values obtained from cell cultures were subtracted from background levels (<0.5% of measured values for IL-6, HGF, VEGF, MMP2 and 1-10% of measured values for IL-10, MMP9) measured in culture media alone. All values are normalized to the DNA contents of each culture condition. Values are expressed as mean ± SEM (n=5). * $p < 0.05$ for islet + cytokine vs. all conditions.

Table 2-5: Effect of Hepatocyte Growth Factor (HGF) on Human Islet Total Cellular Insulin Content and Insulin Secretory Capacity after Exposure to Pro-inflammatory Cytokines

Culture Conditions	% Recovery	Insulin Release (% insulin content)		Stimulation Index
	Cellular Insulin Content	2.8 mM Glucose	20.0 mM Glucose	
Islet (n=5)	100	2.8±0.8	6.0±1.4	2.4±0.4
Islet + cytokine (n=5)	37.4±6.5*	10.3±1.8*	10.9±1.5	1.1±0.1*
Islet + HGF (10ng/mL) + cytokine (n=4)	33.7±9.6†	8.0±1.8	14.1±2.6	1.8±0.2‡

Results are reported as % recovery of total cellular insulin relative to untreated controls (islets alone). Islet function is assessed by a static glucose stimulated insulin release assay. The stimulation index (SI) is calculated as a ratio of insulin release at high glucose versus low glucose. Insulin release (% insulin content) is reported as insulin secreted at 2.8 mM glucose or 20.0 mM glucose divided by insulin content for corresponding islets. Values are expressed as mean ± SEM.

* $p < 0.05$ for islet vs. islet + cytokine
† $p < 0.05$ for islet vs. islet + HGF (10ng/mL) + cytokine
‡ $p < 0.05$ for islet + cytokines vs. islet + HGF (10 ng/mL) + cytokine.

CHAPTER 3

PROTECTIVE EFFECT OF MESENCHYMAL STEM CELLS ON HUMAN ISLETS IS ENHANCED BY CELL CONTACT AND HYPOXIA

3.1 INTRODUCTION

Islet transplantation, the replacement of damaged insulin producing β-cells with donor islets, has emerged as a potential cure for patients with type 1 diabetes [1-3]. However, long term graft function is poor as most islet recipients treated with the Edmonton Protocol lose insulin independence within five years [2]. Although immunosuppressive drug toxicity and chronic immune rejection are detrimental to islet grafts, inflammation is considered the primary barrier to engraftment immediately after transplantation [4-6]. In experimental islet transplant models with syngeneic donor tissue, 50-70% of islet death occurs within the first 72 hours post-transplant [5,6]. Inflammation is present within these islet grafts and is characterized by elevated interferon-γ (IFN-γ), tumor necrosis factor-α (TNFα), and interleukin 1β (IL-1β) [5,6]. Decreasing cytokine expression, inhibiting cytokine function or inhibiting macrophage activity improved the survival and function of transplanted islets, which demonstrates that inflammation plays an important role in islet destruction [5,7]. As a result, anti-TNF-α strategies have been routinely adopted in current clinical islet transplantation to control post-transplant inflammation [8]. But, undesirable side effects or insufficient local immunosuppression can occur with systemic drug

administration. To attain long term islet transplant success, graft specific protection from inflammation or promoting cell repair may be more favorable solutions than systemic immunoregulation.

Regenerative medicine holds the potential to restore the body to health by promoting cell repair with stem cells or alleviating the shortage of donor organs [9]. In addition, the approach utilizes stem cells that naturally exist in the human body to promote endogenous regeneration of tissues or organs in patients or generation of tissues outside the patient's body for transplantation. Among the many types of stem cells, mesenchymal stem cells (MSCs) are relatively unique due to their regenerative and immunomodulatory properties [10-12]. MSCs stimulate tissue repair and reduce inflammation in animal models of corneal injury, myocardial infarction and sepsis [10,12]. A number of factors secreted from MSCs have been implicated in cell survival and tissue engraftment including hepatocyte growth factor, fibroblast growth factor, and vascular endothelial growth factor [10,11]. But, production of soluble factors by MSCs is not the only mode of action; MSCs can transfer cellular subunits such as mitochondria and interact with various immune cells to decrease cell activity based on contact dependent or independent pathways [12]. When activated by inflammatory cytokines, MSCs presented the co-inhibitory signal, ligand for Programmed cell death 1(PD-L1), to inhibit T and B lymphocyte activation [11]. MSCs also released molecules such as interleukin 10, transforming growth factor β1, prostaglandin E2 and indoleamine-2,3-dioxygenase that downregulate T

lymphocyte activity [11]. Due to these properties, mesenchymal stem cells may be a useful cell therapy to enhance clinical islet transplantation.

Several reports have described the superior outcomes of pancreatic islet cell culture [13-17] and experimental islet transplantation in the presence of MSCs; but the role of cell contact in these benefits is unclear [18-23]. In direct contact cell culture, human mesenchymal stem cells prolonged islet cell survival and improved islet cell function [13,16]. In other studies, MSC-conditioned medium was also able to enhance insulin secretion from human islets [14,15]. Factors secreted by MSCs rather than cell contact mechanisms were responsible for these beneficial effects in cell culture, which suggests that the administration of bioactive factors may replace the use of MSCs for islet therapy.

On the other hand, cell contact may play a role in islet revascularization. Human islets in direct contact with human bone marrow derived mesenchymal stem cells (bMSCs) displayed greater endothelial cell coating than islets cultured with endothelial cells alone [17]. After co-transplantation of rodent islets and MSCs into diabetic rodents, MSCs were co-localized with markers for endothelial cells around the islet graft to show that MSCs contributed to angiogenesis by differentiating into endothelial cells or forming vessel stabilizing cells known as pericytes [18-20]. Rodent MSCs decreased the islet mass that was required to reduce hyperglycemia by stimulating islet angiogenesis and providing stromal support [18-20].

Another method of delivering MSCs is systemic administration. Injection of MSCs reveals that interaction between MSCs and islets does occur. Lee ***et al.***

reported that intracardiac infusion of human mesenchymal stromal cells, also known as mesenchymal stem cells, promoted repair of endogenous pancreatic islet cells in diabetic mice [24]. Reduction in blood glucose concentrations as well as an increase in islet mass and insulin content were observed with MSC-treated mice but not with the untreated mice [24]. Human insulin was not detected but human DNA was present in the pancreases and kidneys of MSC treated mice, which suggest that MSCs migrated to the site of injured tissues and some MSCs engrafted into pancreatic islet cells [24]. This finding demonstrates that cell contact dependent pathways are important modes of action for islet regeneration. Therefore, MSCs or their secreted products may have a role in clinical islet transplantation.

In our experimental design for a graft specific anti-inflammatory therapy, we aggregated human islets with human MSCs. The function of the islet:MSC aggregates was tested after treating with pro-inflammatory cytokines [13]. Our results demonstrated that the anti-inflammatory properties of MSCs were sufficiently potent to protect human islets from pro-inflammatory cytokines [13]. Several factors associated with protection were identified including hepatocyte growth factor (HGF) as well as matrix metalloproteinases (MMP) 2 and 9 [13]. When HGF was administered alone, HGF partly preserved islet function but did not replace the protective effect of MSCs [13]. In the islet:MSC co-cultures, MSCs directly interacted with islets [13]. This interaction increased in the presence of inflammatory cytokines [13]. Therefore, understanding the effect of

cell contact between MSCs and islets is critical to developing therapeutic applications of MSCs for islet transplantation.

Based on the physical interaction between islets and MSCs in our co-cultures, we hypothesized that the protection conveyed by human MSCs on islet function was dependent on cell to cell contact. To determine the role of mesenchymal stem cell contact on the protection of islets, we have devised direct and indirect co-cultures of human islets with MSCs. For direct contact, human islets were aggregated with human bone marrow derived mesenchymal stem cells (bMSCs). The co-cultured islets were exposed to cytokines. For indirect contact, three different methods were used for cell cultures. Human islets were encapsulated before co-culturing with MSCs in culture dish. In our second approach, human islets were cultured in bMSC-conditioned medium. For the third condition, hypoxic bMSC-conditioned medium was prepared before culturing with human islets. For all co-cultures, islet function and total cellular insulin content were determined after exposure to the same amount of pro-inflammatory cytokines. The interaction between bMSCs and islets was characterized by microscopy.

3.2 MATERIALS AND METHODS

3.2.1 Preparation of Human Islets

Human pancreases were procured from cadaveric donors and were processed by the clinical islet laboratory (University of Alberta and Alberta

Health Services) or Alberta Diabetes Institute IsletCore islet isolation protocols [13]. Written informed research consent was signed by donor relatives. Islet enriched fractions (15-50% dithizone positive) were cultured in Roswell Park Memorial Institute (RPMI) 1640 (Gibco, Carlsbad CA) medium supplemented with 0.5% w/v fraction V bovine serum albumin (BSA, Sigma-Aldrich, Oakville, Canada), 1.0% v/v insulin-transferrin-selenium (ITS, Sigma-Aldrich) and 100 U penicillin / 1000 U streptomycin (Biowhittaker, Walkersville, MD).

3.2.2 Preparation of Human Bone Marrow derived Mesenchymal Stem Cells (bMSCs)

Human bone marrow (HBM) was extracted from four patients at the age of 24, 42, 46 and 69 years at the Division of Orthopedic Surgery, University of Alberta, with signed informed consent. To isolate mesenchymal stem cells (MSCs), HBM cells were cultured in Modified Essential Medium alpha (MEMα, Cellgro Manassas, VA) supplemented with 2.5 ng/mL basic fibroblast growth factor (bFGF, Millipore, Billerica, MA), 10% fetal bovine serum (FBS, Gibco), 1 mM sodium pyruvate (Gibco), 10 mM HEPES (Gibco), 100 U penicillin/1000 U streptomycin (Biowhittaker, Walkersville, MD) at a density of 166,000 cells per cm^2. Non-adherent cells were removed by changing the medium every 2-3 days. Once confluent, the cell monolayer was washed with versene and was detached with 0.05% trypsin-EDTA (Invitrogen, Carlsbad CA). Cells were counted and re-seeded into MEMα culture medium at a density of 5,000-10,000 cells/cm^2. All cell cultures were maintained at 37^{o}C, 5% CO_2 in a humidified incubator [25].

To confirm that these cell preparations express the classical MSC surface antigens, cells from passages 2-6 were stained with MSC markers based on the position statement from the International Society for Cellular Therapy [ISCT, 26]. Cells were fixed in 4% w/v paraformaldehyde for 1 hour and washed with PBS before primary antibody staining. Cells were stained for MSC markers CD29-PECy5 (Caltag, Carlsbad CA), CD44-FITC (Chemicon, Billerica, MA), CD73-PE, CD90-PE (BD Biosciences, Mississauga, Canada), and CD105-PE (Biolegend, San Diego, CA) as well as hematopoietic lineage markers CD11b-FITC (Abcam, San Francisco CA), CD19-PE (Abcam), CD34-FITC and CD45-PE (Caltag) for 30-60 minutes at 4^{o}C, protected from exposure to light. MSC marker expression was analyzed on a BD FACScalibur flow cytometer. For isotype controls, cells were also stained for IgG_1-PE (Cedarlane, Burlington, Canada), IgG_1-FITC (Cedarlane) and IgG_1-PECy5 (Caltag).

3.2.3 Direct Contact Co-culture of Human Islets and Human bMSCs

For direct co-culture, bone marrow derived mesenchymal stem cells (bMSCs) were enzymatically detached from culture plates and 1.0×10^6 cells were added to a 100 mm low adherence culture dish (Corning) with 500 human islet equivalents in a total volume of 10 mL. The culture medium, known as supplemented DMEM, consisted of DMEM low glucose (5.6 mM glucose, Gibco) with 1% FBS, 20 ng/mL EGF, 20 ng/mL bFGF, 10 mM HEPES, 100 U penicillin/1000 U streptomycin, and 71.5 μM β-mercaptoethanol. After 24 hours, these bMSC islet co-cultures were exposed to a cocktail of pro-inflammatory

cytokines [13] including 1560 ng interferon-γ, 250 ng tumor necrosis factor-α, and 0.4625 ng interleukin 1β (specific activities 2.4 x 10^6 U/mg, 5-2 x 10^7 U/mg, 1.16 - 0.54 x 10^9 U/mg respectively; Biolegend) for 48 hours. Controls included the islets cultured alone (± cytokine exposure). After 72 hours in culture with bMSCs, samples were taken to determine total cellular insulin content and glucose stimulated insulin secretion.

To assess the physical interaction between bMSCs and islets, bMSCs were labeled with a fluorescent quantum dot before co-culture with human islets. These islet:bMSC co-cultures were separate from experiments assessing glucose responsiveness, but the co-cultures represent the same conditions. In brief, bMSCs were recovered from culture by enzymatic detachment, counted and labeled with Qtracker 655 reagent (83 μM, Invitrogen) prepared in bMSC culture medium for 1 hour and washed twice by centrifugation prior to re-culturing. Human islets (n=500) were then cultured with 1.0 x 10^6 Q-dot labeled bMSCs in conditions described above for co-culture (± cytokines). After 72 hours in culture, photomicrographs were taken with a Zeiss colibri microscope using phase contrast and LED fluorescence with a 595 nm monochromatic filter.

3.2.4 Indirect Contact Co-culture of Human Islets and Human bMSCs

3.2.4.1 Co-Culture of Encapsulated Human Islets with Aggregated bMSCs

To prepare islets for alginate microencapsulation, human islets were collected and washed in calcium free Hanks Buffered Saline Solution (HBSS, Sigma) to prevent alginate cross-linking. The tissue was re-suspended in HBSS

(Sigma) and mixed with an equal volume of 1.5% w/v alginate (MVG, Pronova Biomedical, Oslo, Norway). Alginate microcapsules were formed by extrusion of this alginate tissue mixture through an electrostatic spray device (5.7 - 6.0 kV) into a calcium chloride solution (120 mmol/L $CaCl_2$, 10 mmol/L HEPES, 0.01% Tween 20), yielding particles of 300-600 μm in diameter. Five hundred (500) encapsulated human islets were added to a 100 mm low attachment culture dish (Corning). Bone marrow derived mesenchymal stem cells (bMSCs), between passages two and six, were detached from a T-175 tissue culture treated flask. One million bMSCs were seeded onto the same dish with encapsulated islets in a total volume of 10 mL supplemented DMEM (37^{o}C, 5% CO_2 in a humidified incubator). After 24 hours in culture with MSCs, encapsulated human islets were exposed to pro-inflammatory cytokines for 48 hours [13]. At the end of culture, samples of the encapsulated islets were taken to determine total cellular insulin content and glucose stimulated insulin secretion. After 72 hours in culture, photomicrographs were taken with a Leica light microscope using phase contrast.

3.2.4.2 Culture of Human Islets in bMSC-Conditioned Medium

To prepare bMSC-conditioned medium, bone marrow derived mesenchymal stem cells were detached from culture flasks and added to a 100 mm low adherence culture dish for a total cell number of one million in a total volume of 10 mL of supplemented DMEM. The cells were cultured for 72 hours. The bMSC-conditioned medium was collected, filtered (0.45 μm mesh) and stored at minus 80^{o}C until ready for islet culture. For islet culture, bMSC-

conditioned medium was thawed and warmed to 37^{o}C and water bath sonicated to reconstitute any precipitate. After 24 hours in culture, human islets were exposed to pro-inflammatory cytokines for 48 hours. At the end of culture, samples of the islets were taken to determine total cellular insulin content and glucose stimulated insulin secretion.

3.2.4.3 Culture of Human Islets in Hypoxic bMSC-Conditioned medium

To prepare hypoxic bMSC-conditioned medium, bMSCs were enzymatically detached from tissue culture treated plates. One million cells were then re-suspended in a 100 mm low attachment plate with 10 mL of supplemented DMEM. The bMSCs were aggregated for 72 hours under hypoxic (3% O_2) or normoxic (21% O_2) conditions. The culture medium was collected and passed through a mesh (0.45um) to separate the culture supernatant from the cell aggregates. The supernatant was then stored at minus 80^{o}C. For culture with human islets, the frozen medium was warmed to 37^{o}C, vortexed and water bath sonicated to reconstitute any precipitate before culture. After 24 hours in culture, human islets were exposed to pro-inflammatory cytokines for 48 hours. At the end of culture, samples of the islets were taken to determine total cellular insulin content and glucose stimulated insulin secretion.

3.2.5 Characterization of Contact and Non-Contact bMSC Islet Co-cultures as well as Islet Cultures in bMSC-Conditioned Medium

To determine glucose responsiveness, bMSC islet co-cultures were assessed using a static incubation assay [13,27]. bMSC islet co-cultures were

collected and washed twice by gravity sedimentation over 30 minutes. Preparations were then divided into representative aliquots and incubated at 37^{o}C for 2 hours in 1.5 mL RPMI supplemented with 2.0 mM L-glutamine, 0.5% w/v BSA with 2.8 mM and 20.0 mM glucose respectively. Culture supernatant was collected, stored at minus 20^{o}C and measured for insulin at a later time by a human insulin immunoassay (Meso Scale Discovery, Gaithersburg, MD). Representative aliquots were also taken to assess cellular insulin content [13,27]. For the encapsulated islets, the alginate microcapsule was dissolved with a dissociation medium (135 mM NaCl, 2.7 mM KCl, 1 mM Na_2HPO_4, 0.44 mM KH_2PO_4, 0.8 mM $MgSO_4 \cdot 7H_20$, 2.8 mM D-glucose, 10 mM HEPES, 1 mM EGTA, 4.2 mM $NaHCO_3$, 0.5% w/v fraction V BSA, 100 U penicillin/1000 U streptomycin; pH 7.4) prior to dividing the samples for cellular insulin content determination. To assess cellular insulin content, islet samples were re-suspended in 2 mM acetic acid containing 0.25% w/v BSA, sonicated, and centrifuged (10 minutes, 800 g, 4^{o}C) to remove cellular debris. A 50 µL sample of the supernatant was vacuum dried and was reconstituted in phosphate buffer with 0.5% w/v BSA (pH 7.4). Insulin content was measured using a human insulin immunoassay (Meso Scale Discovery).

3.2.6 Statistical Analysis

Data are presented as mean ± SEM. Comparisons of mean values were performed using a Kruskal Wallis multiple comparison tests with the level of significance set at α=0.05. A Mann Whitney U test with Bonferroni or corrected

Bonferroni analysis was conducted for data considered significantly different between treatment groups. All statistical analyses were performed with STATA 11 (StataCorp LP, College Station, TX).

3.3 RESULTS

3.3.1 Protective Effect of bMSCs on Encapsulated Human Islet Function

To determine the effect of bMSCs on human islets without cell-cell contact, human islets were alginate microencapsulated and were assessed by GSIS as well as total cellular insulin content. Encapsulated (EC; percentage insulin release of 3.3 ± 1.1% at 2.8 mM glucose and 5.8 ±1.5% at 20.0 mM glucose, SI = 2.0 ± 0.2) and non-encapsulated human islets (percentage insulin release was 3.2 ± 0.8% at 2.8 mM glucose and 6.9 ± 1.5% at 20.0 mM glucose; SI = 2.2 ± 0.2) exhibited similar GSIS values. After cytokine exposure, GSIS was disrupted in encapsulated human islets (SI = 1.2 ± 0.1 and percentage insulin release of 10.8 ± 3.4% at 2.8 mM glucose and 12.3 ± 3.8% at 20.0 mM glucose) and non-encapsulated human islets (Table 3-1). The increase in insulin release after cytokine exposure was similar to the increase reported with non-encapsulated islets exposed to cytokines. In the EC-islet:bMSC co-culture, insulin release at 2.8 mM glucose was lower (7.8 ± 2.3%) than the cytokine treated EC-islets alone (10.8 ± 3.4%). The resulting stimulation index was significantly greater than cytokine treated controls ($SI_{co\text{-}culture}$ = 1.8 ± 0.2 versus SI_{cyto} = 1.2 ±0.1, $p<0.05$, n=9). Whereas, when human islets were aggregated with bone marrow derived MSCs, islets exhibited preserved glucose sensitive insulin release (SI = 1.8 ± 0.1

and percentage insulin release at 2.8mM glucose of 4.5 ± 0.8% and at 20.0 mM glucose of 8.0 ± 1.7%) in comparison to cytokine-treated islets. After cytokine exposure, total cellular insulin content was decreased from EC-islets. Insulin content recovery from the co-cultured EC-islets was not superior but was similar to cytokine treated EC-islets (47.3 ± 7.0% versus 42.0 ± 4.7%). However, bMSC co-cultured islets had significantly improved insulin content recovery (62.5 ± 5.8%, $p < 0.05$, n=9) compared to cytokine treated islets (37.6 ± 4.6%).

3.3.2 Physical Interaction Between bMSCs and Islets in Co-culture

To investigate the physical interaction between EC-islets and bMSCs, photomicrographs were taken after pro-inflammatory cytokine exposure. Alginate microencapsulation and cytokine exposure did not distort the morphology of human islets. In co-culture with EC-islets, several MSC aggregates attached to and spread out on the alginate capsule (Fig 3-1D).

To investigate the physical interaction between islets and bMSCs, bMSCs were labeled with a red quantum dot before co-culture. Quantum dot labeling demonstrated that after 72 hours in culture with human islets, bMSCs formed cellular aggregates with minimal physical interaction with islets (Fig 3-2). When these islet:bMSC co-cultures were exposed to IFN-γ, TNF-α, and IL-1β, the bMSC aggregates physically interacted with the islets to a greater extent (Fig 3-2D).

3.3.3 Hypoxic bMSC-Conditioned Medium Preserves Human Islet Function

To determine the protective effect of conditioned medium from hypoxia cultured bMSCs, human islet function was assessed after treatment with pro-inflammatory cytokines using a glucose stimulated insulin secretion assay. Islet function, based on stimulation index, from islets + conditioned medium (3% O_2) was greater than cytokine exposed human islets ($SI_{CM\ hypoxia} = 2.0 \pm 0.1$ versus $SI_{Cyto} = 1.4 \pm 0.2$, $p < 0.05$, n=6). On the other hand, bMSC-conditioned medium collected from normoxia cultured bMSCs did not preserve islet function (SI = 1.4 ± 0.2, percentage insulin release at 2.8 mM glucose of 9.2 ± 2.3% and at 20.0 mM of 12.1 ± 2.2%). The stimulation index of islets in normoxic bMSC-conditioned medium was similar to cytokine treated islets. Total cellular insulin recovery from islets cultured with hypoxic (3% O_2) bMSC-conditioned medium (62.4 ± 6.6%, $p < 0.05$) was greater than both islets cultured in normoxic bMSC-conditioned medium (46.4 ± 4.3%) and cytokine treated islets only (35.0 ± 5.9%).

3.4 DISCUSSION

Islet transplantation continues to be an alternative treatment option for patients with severe type 1 diabetes [1-3]. When insulin therapy is no longer effective, β-cell replacement strategies such as, whole pancreas or islet transplantation is a recommended treatment [1-3]. Islet transplantation is safer and less invasive than whole pancreas replacement [2,3]. Islet transplantation also results in better glycemic control than daily exogenous insulin [1-3]. But, the drawback of islet transplantation is that insulin independence is not sustainable due to the progressive loss of islets [2]. For islet transplantation to be recognized and accepted as standard therapy for patients with type 1 diabetes, the current challenges of islet graft loss must be resolved. Inflammation is regarded as a crucial factor in graft loss that occurs immediately after islet transplantation [4,5]. To protect islet cells from inflammation, we cultured human islets in direct contact with mesenchymal stem cells (MSCs) [13]. MSCs preserved human islet function and decreased islet β-cell apoptosis after exposure to pro-inflammatory cytokines [13]. Based on the increase in physical interaction between MSCs and islets after cytokine exposure, we speculated that cell to cell contact was partly responsible for the protective effects. Using direct and indirect contact co-cultures, we examined the role of MSC contact with islets in the protection of islets from pro-inflammatory cytokines.

Factors secreted by MSCs can improve islet function and prevent islet cell death [13-16]. We have demonstrated that HGF can partly protect human islets from pro-inflammatory cytokines [13]. But, the combined effect of all secreted

factors from MSCs has not been determined. To assess the protective effects of indirect cell contact between islets and MSCs, islets were cultured in bMSC-conditioned medium. Islet function, after cytokine exposure, was determined using a static glucose stimulated insulin secretion assay. In this study, bMSC-conditioned medium was prepared from MSCs that were not pre-exposed to pro-inflammatory cytokines. The intention of preparing conditioned medium from unstimulated bMSCs was to ensure the test conditions for all islet variables were exposed to equal amounts of cytokines for consistency. If islets were cultured in cytokine-treated bMSC-conditioned medium, then the islets would be exposed to additional cytokines from the conditioned medium.

Based on results of experiments in which islets were exposed to cytokines, islet function and total cellular insulin content were not preserved in bMSC-conditioned medium. This lack of protection is likely due to the insufficient level of secreted factors from bMSCs in this conditioned medium to protect human islets. Hemeda *et al.* reported that treatment of MSCs with interferon-γ or tumor necrosis factor-α induced higher expression of IDO and granulocyte monocyte colony stimulating factor [13,28]. Similarly, in our cell culture, we have shown that treating bMSC aggregates with cytokines also elicits a distinct profile of soluble factors, different than bMSCs not treated with cytokines [13]. For instance, IL-6 was up-regulated in the presence of cytokines [13]. Inflammation is believed to prime MSCs to an anti-inflammatory phenotype [12,13]. *In vivo*, inflammation activates MSCs [12]. For example, injury increases homing of

MSCs to sites of tissue damage [12]. Thus, we believe that secreted factors from activated or stimulated MSCs would be more beneficial to human islets.

To evaluate the protective effects of bMSCs without cell contact, bMSCs and islets were cultured in separate compartments. As islets were separated from bMSCs by alginate microencapsulation, the effect of cytokine activated bMSCs was evaluated with this method. Human bMSCs exhibited a partial protective effect. Total cellular insulin content was not greater in the co-cultured encapsulated islets (EC-islets) than cytokine-treated EC-islets (Table 3-1). But, EC-islet function after co-culture with bMSC was significantly better than cytokine exposed EC-islets. Percentage insulin release at 2.8 mM and 20.0 mM glucose from co-culture EC-islets remained elevated compared to untreated EC-islets. Thus, the bMSC aggregates did protect encapsulated islet function. These observations suggest that cell contact between bMSCs and islets is not necessary to preserve islet function. But factors secreted by bMSCs do not preserve total islet cellular insulin content. Thus, this EC-islet:bMSC co-culture method is less effective than direct contact in protecting human islets from inflammation.

We utilized a third method to test bMSC indirect contact co-culture with human islets. In this approach, the effect of increasing secreted factor content on islet function was determined. Higher levels of cytoprotective factors could improve protection of human islets. To increase bMSC activity without an inflammatory stimulus, we cultured bMSCs in 3% oxygen (O_2). In the bone marrow niche, the oxygen tension, 3% O_2, is much lower than room air, 21 % O_2. At this natural oxygen tension, many benefits on MSC viability and function have

been reported, including improved angiogenic properties and greater proliferative capacity [29, 30]. Crisostomo *et al.* demonstrate that VEGF and FGF-2 (bFGF) were upregulated within 24 hours from bMSCs cultured at 1 % O_2 tension [29]. Upregulation of IL-6, VEGF, and bFGF were reported in another study, which demonstrated that MSCs can increase the production of angiogenic growth factor [30]. Because of the upregulation of these growth factors, we assessed whether this combination of secreted factors from human bMSCs after hypoxic conditioning would also be protective for human islets.

From our preliminary data, both islet insulin content and islet function from islet in hypoxic bMSC-conditioned medium were improved when compared to cytokine-treated islets (Table 3-2). Thus, secreted factors from hypoxic bMSCs were able to protect human islets from pro-inflammatory cytokines. These results suggest that a sufficient level of secreted factors from bMSC is required to protect islet function and improve insulin content recovery.

Based on our investigation of cell to cell contact and non-contact co-cultures, we conclude that the protective effect of bMSCs is enhanced by cell contact or hypoxic conditioning. When bMSCs were present in co-culture with islets, but separated by a barrier to prevent direct interaction between islets and bMSCs, bMSCs exhibit the ability to preserve islet function. But, islet insulin content remains reduced. Because islet function and insulin content recovery is greater in islet:bMSC co-culture than EC-islet:bMSC co-culture, direct cell to cell co-culture is more beneficial than indirect contact. While transplanting bMSCs together with islets may not be required to preserve islet function, transplanting

MSCs and islets into the same compartment is likely more beneficial. In addition, we report that bMSC-conditioned medium is not protective; our conditioned medium likely lacked the cyto-protective factors which were secreted by bMSCs after cytokine stimulation. When islets were cultured in medium collected from bMSCs in hypoxic culture conditions, both islet function and insulin content recovery were significantly better than pro-inflammatory cytokine treated islets. Activation and stimulation of MSCs is likely necessary for these protective effects. Based on the co-culture and conditioned media experiments, the levels of soluble factors secreted by bMSCs after cytokine stimulation or hypoxic conditioning are partly responsible for this protection. The cytoprotective factors released by MSCs may play an important role in reducing inflammation mediated damage. Thus for islet transplantation, MSCs would be most beneficial if they are transplanted in the proximity of graft tissue or if they can migrate to site of islet injury.

3.5 REFERENCES

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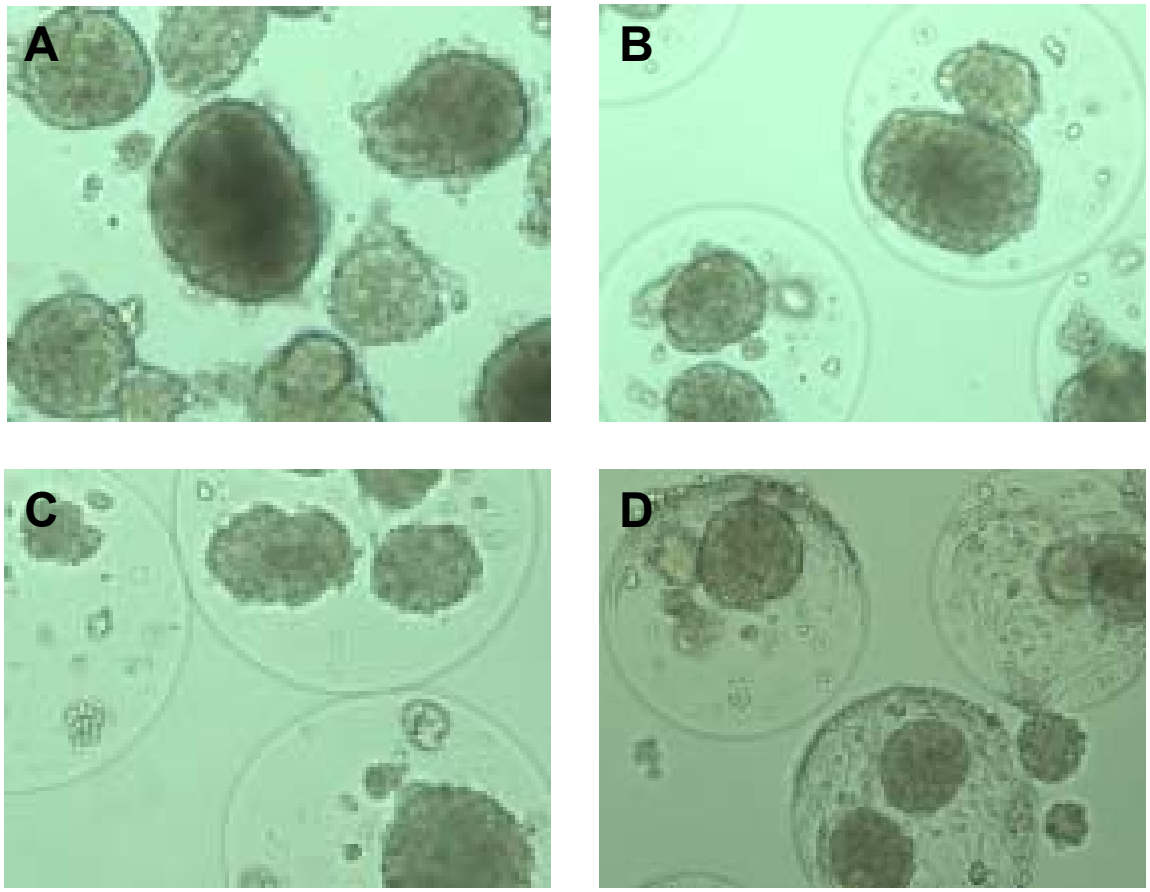
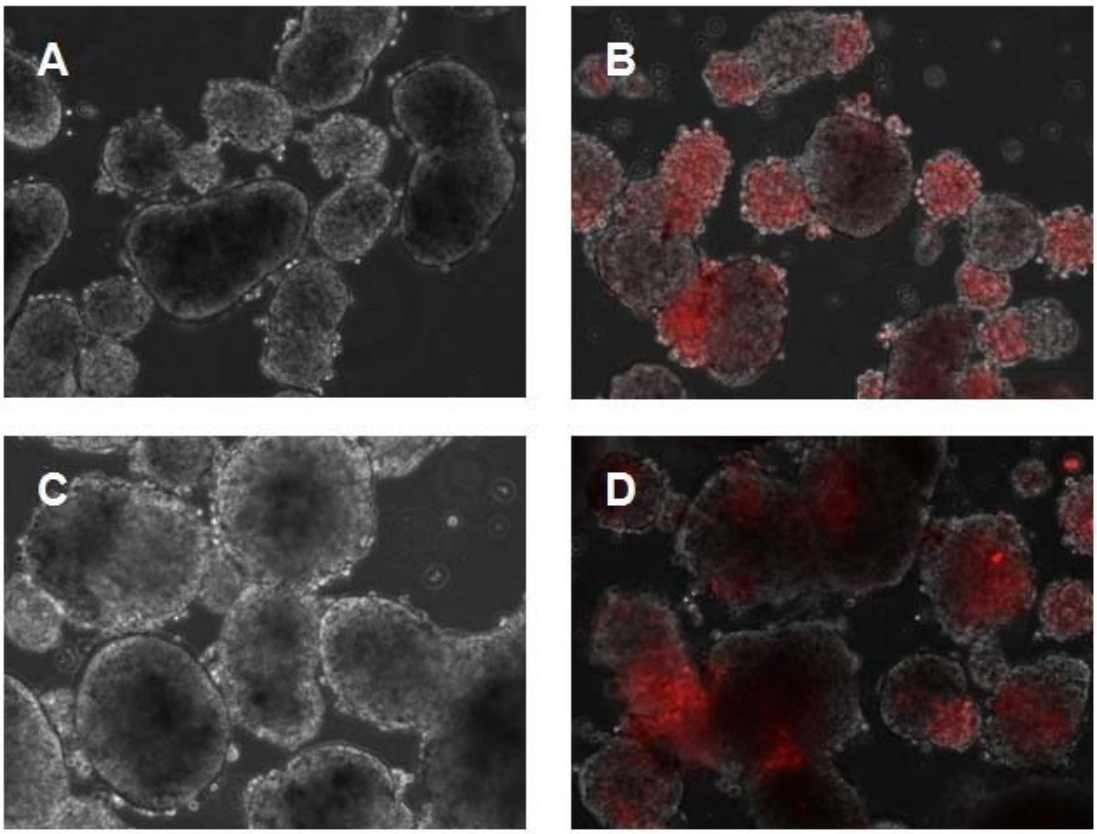
FIGURE LEGEND

Figure 3-1: Representative Photomicrographs of the Physical Interaction Between Encapsulated Human Islets and bMSCs



A) 500 IEQ human islets, B)500 IEQ encapsulated human islets, C) 500 IEQ encapsulated human islets + cytokines, D) 500 IEQ human islets + 1.0 x 10^6 bMSCs + cytokines. Alginate microencapsulation did not distort the morphology human islets and no gross changes in islet morphology were observed after cytokine exposure or MSC co-culture. MSCs attached to and spread out on the alginate microcapsule; cytokines – cocktail of IFN-γ, TNF-α and IL-1β described in materials and methods.

Figure 3-2: Representative Photomicrographs of the Physical Interaction Between Human Islets and Aggregated bMSCs



A) 500 IEQ human islets B) 500 IEQ human islets + 1.0 x 10^6 bMSCs, C) 500 IEQ human islets + cytokines, D) 500 IEQ human islets 1.0 x 10^6 bMSCs + cytokines. MSCs were labeled with quantum dots in red; cytokines – cocktail of IFN-γ, TNF-α and IL-1β described in materials and methods.

Table 3-1: Effect of Direct bMSC Contact on Human Islet Total Cellular Insulin Content and Insulin Secretory Capacity

Culture Conditions	% Recovery	Insulin Release (% insulin content)		Stimulation Index
	Cellular Insulin Content	2.8 mM Glucose	20.0 mM Glucose	
Islets	100.0	3.2±0.8	6.9±1.4	2.2±0.2
Islets + cytokines	37.6±4.6	12.8±3.2*	16.6±4.5	1.3±0.1*
Islets + 1.0 x 10^6 bMSCs + cytokines	62.5±5.9†	4.5±0.8	8.0±1.7	1.8±0.1
EC-islets	100.0	3.3±1.1	5.8±1.5	2.0±0.2
EC-Islets + cytokines	42.0±4.7	10.8±3.4	12.3±3.8	1.2±0.1‡
EC-Islets + 1.0 x 10^6 bMSCs + cytokines	47.3±4.3	7.8±2.3	13.7±3.8	1.8±0.2

Results are reported as % recovery of total cellular insulin content relative to untreated controls (EC-islets alone). Islet function is assessed by a static glucose stimulated insulin release assay. The stimulation index (SI) is calculated as a ratio of insulin release at high glucose versus low glucose. Insulin release (% insulin content) is reported as insulin secreted at 2.8 mM glucose or 20.0 mM glucose divided by cellular insulin content for corresponding islets. Values are expressed as mean ± SEM (n=9).

* $p < 0.05$ vs. Islet
† $p < 0.05$ vs. Islet + cytokines
‡ $p < 0.05$ vs. all EC-islet conditions
EC represents alginate microencapsulation
(bMSCs) represents bone marrow derived mesenchymal stem cells.

Table 3-2: Effect of Hypoxic bMSC-Conditioned Medium on Human Islet Total Cellular Insulin Content and Insulin Secretory Capacity

Culture Conditions	% Recovery	Insulin Release (% insulin content)		Stimulation Index
	Cellular Insulin Content	2.8 mM Glucose	20.0 mM Glucose	
Islets	100.0	2.8±0.4	7.5±1.0	2.7±0.3
Islets + cytokines	35.0±5.9	14.0±4.1*	20.8±6.4	1.4±0.2*
Islets + CM + cytokines	46.4±4.3	9.2±2.3†	12.1±2.2	1.4±0.2†
Islets + CM (3% O_2) + cytokines	62.4±6.6‡	4.7±0.8	9.4±1.4	2.0±0.1‡

Results are reported as percentage recovery of total cellular insulin content relative to untreated controls (islets alone). Islet function is assessed by a static glucose stimulated insulin release assay. The stimulation index (SI) is calculated as a ratio of insulin release at high glucose versus low glucose. Insulin release (% insulin content) is reported as insulin secreted at 2.8 mM glucose or 20.0 mM glucose divided by cellular insulin content for corresponding islets. Values are expressed as mean ± SEM (n=6).

* $p < 0.05$ vs. Islets
† $p < 0.05$ vs. Islets
‡ $p < 0.05$ vs. Islets + cytokines
(CM) represents normoxic bMSC-conditioned medium and (CM 3% O_2) represents hypoxic bMSC-conditioned medium.

CHAPTER 4

ISLETS TRANSPLANTATION WITH HUMAN MESENCHYMAL STEM CELLS

4.1 INTRODUCTION

In type 1 diabetes, loss of endogenous insulin production occurs due to autoimmune mediated destruction of the pancreatic β-cells [1]. Insulin therapy is the standard treatment, but dangerous hypoglycemia is a known common side effect [1]. In contrast to daily insulin injections, β-cell replacement enables restoration of precise physiological glycemic control and eliminates the occurrence of hypoglycemic episodes [2-4]. Among the two strategies for β-cell replacement, islet transplantation is a less invasive and safer alternative than whole pancreas transplantation. Currently, one of the main drawbacks of islet transplantation is the progressive loss of graft function, which leads to the loss of insulin independence [2-4]. To prevent islet dysfunction, the concerns of inflammation during islet engraftment, poor islet revascularization and toxic immuno-suppression need to be resolved [2,5,6]. This study focused on a regenerative medicine approach to improve islet graft function, in which stem cells are utilized for repair and regeneration of damaged tissues.

Mesenchymal stem cells (MSCs), also called mesenchymal stromal cells, have recently garnered great attention from medical scientists for its clinical applications to manage autoimmunity, improve transplantation and alleviate

inflammation [7-9]. MSCs decrease the activity of T and B lymphocytes that can minimize autoimmune reactions or allogeneic graft rejection *in vivo* [7-9]. In addition, MSCs can attenuate inflammation by secreting anti-inflammatory proteins as well as by reprogramming pro-inflammatory innate immune cells to promote wound healing [9]. MSCs also participate in revascularization by forming vessel stabilizing pericytes or secreting angiogenic factors [7,8]. Because of these regenerative and immunoregulatory characteristics, MSCs have been investigated as a cellular therapy for type 1 diabetes [10] as well as islet transplantation [11].

In experimental islet transplantation, several studies have reported the beneficial effects of mesenchymal stem cells on islet revascularization and islet graft function [12-17]. Although most strategies have focused on rodent islets, Berman *et al.* described a method to improve islet transplantation in non-human primates using bone marrow derived MSCs [17]. Therefore, the next step is to achieve success in utilizing human mesenchymal stem cells together with human islets for islet transplantation. Our objective is to assess the application of human islets and human MSCs in an immunodeficient diabetic mouse model. We hypothesized that human bone marrow derived mesenchymal stem cells could improve the engraftment of human islets.

Therefore, we investigated localized and systemic delivery of MSCs in islet transplantation. Aggregated MSCs were transplanted together with islets for local delivery. This method assessed a graft specific strategy to promote islet engraftment. For the systemic approach, MSCs were injected into a peripheral

vein after islet transplantation. For both methods, the outcomes of transplantation were assessed by weekly monitoring of blood glucose concentrations and by assessment of graft function after an oral glucose challenge. Grafts were characterized by insulin content, expression of endocrine hormones, extent of graft vascularization, and presence of amyloid deposits.

4.2 MATERIALS AND METHODS

4.2.1 Preparation of Human Islets

Human pancreases were procured from cadaveric donors and were processed by the clinical islet laboratory (University of Alberta and Alberta Health Services) or Alberta Diabetes Institute IsletCore islet isolation protocols [1,4]. Written informed research consent was signed by donor relatives. Islet enriched fractions (50-80% dithizone positive) were cultured in Roswell Park Memorial Institute (RPMI) 1640 (Gibco, Carlsbad CA) medium supplemented with 0.5% w/v fraction V bovine serum albumin (BSA, Sigma-Aldrich, Oakville, Canada), 1.0% v/v insulin-transferrin-selenium (ITS, Sigma-Aldrich) and 100 U penicillin/1000 U streptomycin (Biowhittaker, Walkersville, MD).

4.2.2 Preparation of Human Bone Marrow derived Mesenchymal Stem Cells

Human bone marrow (HBM) was extracted from three patients at the age of 17, 62, and 63 years old at the Division of Orthopedic Surgery, University of Alberta, with signed informed consent. To isolate human bone marrow

mesenchymal stem cells (bMSCs), HBM cells were cultured in Modified Essential Medium alpha (MEMα, Cellgro Manassas, VA) supplemented with 2.5 ng/mL basic fibroblast growth factor (bFGF, Millipore, Billerica, MA), 10% fetal bovine serum (FBS, Gibco), 1 mM sodium pyruvate (Gibco), 10 mM HEPES (Gibco), 100 U penicillin / 1000 U streptomycin (Biowhittaker, Walkersville, MD) at a density of 166,000 cells per cm^2. Non-adherent cells were removed by changing the medium every 2-3 days. Once confluent, the cell monolayer was washed with versene and was detached with 0.05% trypsin-EDTA (Invitrogen, Carlsbad CA). Cells were counted and re-seeded into MEMα culture medium at a density of 5,000-10,000 cells/cm^2. All cell cultures were maintained at 37^oC, 5% CO_2 in a humidified incubator.

To confirm that both bMSCs express the classical MSC surface antigens, cells from passages 2-6 were stained with MSC markers based on the position statement from the International Society for Cellular Therapy (ISCT) [21]. Cells were fixed in 4% w/v paraformaldehyde for 1 hour and washed with phosphate buffer saline (PBS) before primary antibody staining. Cells were stained for MSC markers CD29-PECy5 (Caltag, Carlsbad CA), CD44-FITC (Chemicon, Billerica, MA), CD73-PE, CD90-PE (BD Biosciences, Mississauga, Canada), and CD105-PE (Biolegend, San Diego, CA) as well as hematopoietic lineage markers CD11b-FITC (Abcam, San Francisco CA), CD19-PE (Abcam), CD34-FITC and CD45-PE (Caltag) for 30-60 minutes (4^oC, protected from exposure to light). MSC marker expression was analyzed on a BD FACScalibur flow cytometer. For

isotype controls, cells were also stained for IgG_1-PE (Cedarlane, Burlington, Canada), IgG_1-FITC (Cedarlane) and IgG_1-PECy5 (Caltag).

4.2.3 Transplantation

4.2.3.1 Localized Transplant of Human Islet and Human MSC Aggregates

To determine the effect of MSCs on human islets *in vivo*, human islets (1500 IEQ) and 1 x 10^6 bMSCs were transplanted into immunodeficient B6 Rag -/- mice (C57Bl/6 $Rag1^{tm1\text{-}mom/J}$, from the Jackson Laboratory, Bar Harbor, Maine). The recipient mice, 8-12 weeks old, were rendered diabetic with a single intraperitoneal dose of streptozotocin (180 mg/kg, Sigma) dissolved with citrate buffer (pH 4.5). To verify the diabetic status of the mice, blood was collected from the tail vein and blood glucose concentrations were determined using a OneTouch glucometer (LifeScan, Milpitas, CA). Mice were considered diabetic after two consecutive blood glucose readings greater than 20.0 mM.

Prior to transplantation, islets and bMSCs co-cultured were aggregated for 24 to 48 hours in supplemented DMEM medium (DMEM low glucose (5.6 mM glucose, Gibco) with 1% FBS, 20 ng/mL epidermal growth factor (EGF, R&D Systems, Minneapolis, MN), 20 ng/mL bFGF, 10 mM HEPES (Gibco), 100 U penicillin/1000 U streptomycin (Biowhittaker, Walkersville, MD), and 71.5 μM β-mercaptoethanol (Sigma-Aldrich)). The composite islet:bMSC tissues were collected and transferred into PE50 polyethylene tubing (Becton Dickenson, MD). The tissues were collected into a pellet by centrifugation and implanted into a space that was created under the left renal capsule.

4.2.3.2 Systemic infusion of Human MSCs

For systemic infusion of MSCs, islets were transplanted under the renal capsule. Immediately after human islets were transplanted, 1.2 x 10^6 human bMSCs (suspended in 200 uL hanks buffered saline solution) were infused as single cells via the tail vein into each mouse. Mice that exhibited signs of emboli including difficulty breathing, or asymmetric limb strength and loss of coordination were euthanized. To serve as a control, the same number of human islets (n = 1000 islet equivalents) were also transplanted into a separate group of diabetic B6 Rag -/- mice. Another is a group of diabetic B6 Rag -/- mice was treated with a dose of 2000 islet equivalents (IEQ) to confirm the function of the donor islets. The 2000 IEQ is defined as curative dose because this adequate amount of islets is able to reverse diabetic in mice [18].

4.2.4 Post-Transplantation Assessment

Each week, blood was collected from the tail vein and blood glucose levels were determined using a OneTouch glucometer. The main outcome was the achievement of euglycemia defined by two successive readings less than 11.0 mM glucose. For mice that achieved the primary outcome, graft function was assessed by an oral glucose tolerance test (OGTT). No food intake for these mice was allowed for 6 to 12 hours followed by an oral dose of dextrose (50% solution, 3mg/g body weight). Blood glucose readings were measured at 0, 15, 30, 60 and 120 minutes intervals after the oral glucose gavages. Graft function was

confirmed by a survival nephrectomy on week 8, in which the graft bearing kidney was removed and blood glucose values were recorded for an additional 2 to 5 days. The secondary outcome was graft composition, which was assessed by graft insulin content and graft histology.

4.2.5 Insulin Content of Human Islet Grafts

To prepare specimens for insulin content determination, grafts were collected from each group at survival nephrectomy. The engrafted left kidney was removed and non-grafted sections were cut away from the kidney. The grafts were immediately frozen in liquid nitrogen and stored at minus 80^{o}C until it is ready for processing the cellular insulin content. Graft tissues were homogenized, followed by sonication at 4^{o}C in 10 mL of 2 mM acetic acid containing 0.25% w/v BSA. After a 2 hour incubation at 4^{o}C, the tissue was re-sonicated, centrifuged at 8000 xg for 20 minutes. The supernatant was collected. The tissue pellet was re-extracted with an additional 5 mL of 2 mM acetic acid containing 0.25% w/v BSA. Insulin content was determined with an immunoassay (Meso Scale Discovery, Gaithersburg, MD).

4.2.6 Histology and Immuno-staining

To prepare transplanted grafts for histology, grafts were collected from each group at survival nephrectomy. The engrafted left kidney was removed and fixed with z-fix. Graft bearing kidneys were embedded in paraffin. Sections with 5 μm in thickness were cut with a microtome and placed on positively charge glass histobond slides. After rehydrating samples, the slides were quenched with

hydrogen peroxide and washed in distilled water followed by PBS. Samples were blocked with 20% v/v goat serum (Jackson Laboratory) for 15 minutes at room temperature before staining. Primary antibodies for use were polyclonal guinea pig anti-insulin (1:1000 dilution, Dako, Mississauga, ON, Canada), mouse anti-glucagon antibodies (1:5000 dilution, Dako), and monoclonal rabbit anti-von Willebrand factor (1:200 dilution, AbCam). All antibodies were diluted in 5% normal goat serum (Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA). Secondary antibodies were biotinylated goat anti-guinea pig and biotinylated goat anti-mouse. Avidin biotin complexes (Vector Laboratories) were bound for 30 minutes at room temperature before the addition of diaminobenzidine (Vector Laboratories).

When a brown precipitate was formed on positive control slides, the reaction was stopped with distilled water and counterstained with hematoxylin and eosin (Sigma). For immune-fluoresence, Alexa fluor conjugated secondary antibodies were utilized: 1:200 Alexa Fluor 488 anti-guinea pig (Molecular Probes, Eugene, OR), 1:200 Alexa Fluor 594 anti-mouse (Molecular Probes), and 1:200 Alexa Fluor 594 anti-rabbit (Molecular Probes). Slides were sealed with Prolong Gold Antifade and DAPI nuclear staining (Molecular Probes) to preserve fluorescence. Slides were visualized with an Axioscope II microscope equipped with an AxioCam MRC and analyzed with Axiovision 4.6 software (Carl Zeiss, Gottingen, Germany) as well as an AxioCam Colibri.

For double insulin and thioflavin S staining, sections were blocke with 2.0% normal goat serum and incubated with 1:100 guinea pig anti insulin (Dako)

for 60 minutes followed by texas red-goat anti-guinea pig secondary antibody (Jackson ImmunoResearch) for 60 minutes. Slides were then incubated with thioflavin S solution for 2 minutes and rinsed with 70% ethanol. A section of pancreas from a human islet amyloid polypeptide expressing rat was utilized as a positive control for islet specific amylin.

4.2.7 Statistical Analysis

Results are reported as mean ± SEM. Data were analyzed by a Kruskal Wallis multiple comparisons tested at a level of significance of 0.05. A Mann Whitney U test with Bonferroni corrections was performed for data considered significantly different. A Kaplan Meier survival analysis with log rank test was also performed to compare the percentage of euglycemic mice for each treatment group. All statistically analyses were performed using STATA 11 (StataCorp LP, College Station, TX).

4.3 RESULTS

4.3.1 Co-transplantation of Human Islets and Human MSCs under the Renal Capsule Demonstrates No Improvements in Islet Transplant Outcomes

To assess the localized effect of human bone marrow derived mesenchymal stem cells (bMSCs) on islet function *in vivo*, a marginal mass of human islets was transplanted with bMSCs aggregates into immunodeficient diabetic mice. After one week, 50% of mice transplanted with islets alone achieved normoglycemia. In comparison, 12.5% of mice co-transplanted with

islet:bMSC aggregates were normoglycemic at one week post-transplant. By the fiftieth day post transplant, a greater proportion of mice transplanted with islets alone had reversed diabetes than mice co-transplant group with islet:bMSC aggregates (87.5% versus 25%). Moreover, average blood glucose levels of the mice transplanted with islets only were significantly lower than the mice transplanted with islet:bMSC aggregates at each time point (Fig. 4-2A).
When normoglycemia was achieved, islet graft function was determined by an oral glucose tolerance test (OGTT). The co-transplant group exhibited reduced glucose tolerance versus the islet only control group (Fig 4-2C). No significant differences were detected in whole graft insulin content between the two groups.

4.3.2 Analysis of Graft Composition

Grafts from the co-transplant group and the islet only group were evaluated for morphology, vascularization and amyloid content. In the islet alone group, most of graft was composed of insulin positive aggregates. Islet grafts retained the morphology of islets in the native human pancreas. Insulin and glucagon positive cells were interspersed throughout each islet aggregate. In the co-transplant group, the volume of the graft appeared much larger than the islet only group. Most of the graft contained fibroblast-like cells. The morphology of the islets was distorted. Instead of intact spheroid aggregates, many islets were disorganized and became elongated structures. Numerous single insulin positive and glucagon positive cells were scattered throughout the co-transplanted graft,

which indicates fragmentation of transplanted islets. Islets were also smaller in size than the islet grafts without MSCs.

Blood vessel formation was detected by von Willebrand factor (VWF) expression, a marker for endothelial cells. In the native pancreas, VWF expression is present within and around the islets of Langerhans. In the islets only graft, VWF expression was present within the graft. In comparison, VWF staining was detected at the periphery of the islet aggregate in the islet:bMSC co-transplant group. Overall, vascularization was less extensive in the co-transplant group than the islet only group.

Amyloid was detected by thioflavin S expression. In the islets only graft, amyloid expression was present within the graft. In comparison, amyloid staining was not detected in the islet:bMSC co-transplant group.

4.3.3 Systemic Infusion of Human MSCs Transiently Improves Human Islet Transplant Outcomes

The effect of systemically delivered MSCs on islet grafts was determined by transplantation of human islets followed by intravenous injection of human bone marrow derived mesenchymal stem cells (bMSCs) into immunodeficient diabetic mice. Human islets, at a suboptimal dose of 1000 islet equivalents (IEQ), were transplanted alone under the renal capsule. In a second group, 1.2×10^6 bMSCs were injected into the tail vein after transplantation of human islets (n =1000 IEQ) under the renal capsule of immunodeficient diabetic B6 Rag -/- mice. A third group of diabetic B6 Rag -/- mice were injected with curative dose of 2000 IEQ to confirm that the human islets could reverse diabetes. Mice

transplanted with the suboptimal dose of human islets were able to lower blood glucose concentrations but only 33% of the mice in this group achieved euglycemia (Fig 4-4). All mice transplanted with 2000 IEQ reversed diabetes within seven days and maintained euglycemia throughout the post-transplant period.

After bMSC injection, the mice transplanted with human islets exhibited a greater reduction in blood glucose levels than mice that received islets only. At seven and fourteen days post transplant, blood glucose levels from the mice transplanted with 1000 IEQ plus the bMSCs injection were similar to the mice transplanted with the curative dose of 2000 IEQ. In the co-transplant group, a greater proportion of mice had achieved normoglycemia (two out of two mice) than control mice (one out of three mice). Reversal of diabetes in these mice was maintained over a period of two weeks. By the third week, the co-transplanted mice had returned to mild hyperglycemia (Figure 4-4). The co-transplanted group was re-infused with a second dose of MSCs (2×10^6 MSCs) on day 23 of post islet transplant. Blood glucose levels in the co-transplanted mice were decreased at weeks four to six; whereas the glycemia in the islet alone group remained elevated. At week six one out of two co-transplanted mice achieved normoglycemia and zero out of three in the islet alone group was euglycemic. Weight gain of the two mice in the co-transplant group were 0.8 g and 0.85 g but mice tranplanted with islets only exhibited weight gains of 0.3 g and 0.25 g in addition to a weight loss of 0.25 g.

Islet graft function was determined by an oral glucose tolerance test (OGTT). One mouse from the co-transplant group exhibited greater glucose tolerance versus the two out of three mice from the islet only control group (Fig 4-4).

4.4 DISCUSSION

After clinical islet transplantation, grafts are immediately exposed to inflammation, which leads to a decrease in islet engraftment and function [5,6,17]. Consequently, many islet recipients lose insulin independence and are required to return to insulin therapy [17]. To overcome these problems in islet engraftment, possible solutions are to decrease inflammation and improve graft vascularization [5,6]. Mesenchymal stem cells have been investigated for potential cell-based therapies that can control inflammation and improve engraftment [9]. In various studies using rodents and non-human primates, impressive results for co-transplantation of islets and MSCs have been reported [12-16]. In their experimental islet transplant models, MSCs improved islet function by promoting β-cell regeneration, decreasing β-cell apoptosis and preserving islet morphology [12-16]. MSCs also enhanced the revascularization of islet grafts through the secretion of angiogenic molecules such as vascular endothelial growth factor (VEGF) and hepatocyte growth factor (HGF) [12-16]. MSCs directly participated in revascularization by differentiating into endothelial cells or forming vessel stabilizing pericytes [12]. Their studies yielded promising results using animal islets together with animal MSCs. On the other hand, our

objective is to investigate and assess human islets and human mesenchymal stem cells in an immunodeficient mouse model of diabetes.

4.4.1 Local Delivery of Islets and MSCs to Renal Capsule is Unfavorable

The intention of localized delivery of MSCs to the islet graft is to evaluate a graft specific cell therapy for transplanted islets. To ensure that the bMSCs remained at the graft site, our study adapted a method of co-culturing islets and bMSCs prior to transplantation. For islet transplantation, previous investigations have proven that 2000 IEQ is an adequate amount of human tissue that can reverse diabetes in most mice [18]; this experiment used 1500 IEQ as a minimum islet mass expecting a lesser proportion of mice would reverse diabetes. Consequently, benefits of bMSCs on islet graft function would be observed as an improved rate of diabetes reversal with minimum mass islet transplantation.

After islet:bMSC co-transplantation, fewer mice achieved euglycemia (25% versus 87.5%, $p<0.05$ by log rank) and average blood glucose concentrations were higher than the islet only control group. In response to a glucose challenge, mice transplanted with islets and bMSCs had reduced glucose tolerance in comparison to the islet only control. The results of the glucose tolerance test indicate that islet graft function in the islet only group was superior to the islets:bMSC co-transplant under the renal capsule. This unexpected detrimental effect of bMSCs may be attributed to the compaction of islets in a limited space under the renal subcapsule. Merani *et al.* reported that islets maintained in a pellet form for 30 minutes exhibited significantly reduced

engraftment (38% versus 100%) [19]. A greater amount of islet cell apoptosis and a decrease in graft insulin content suggest that islet survival is markedly reduced after compaction [19]. In our co-transplant model, graft volume was approximately two to three times greater than islet alone group. The space under the renal capsule is restricted in size. As a result, the large volume of the co-transplant graft must be packed tightly, which may explain the poor outcome of compressing islets and bMSCs under the renal capsule.

In addition to islet compaction, other mechanisms are likely responsible for poor transplant outcomes. After islet:bMSC co-transplantation, differences in graft composition were observed. Islet function is dependent on islet vasculature and islet cell organization. Vascular density of islets in the native pancreas is substantially higher than surrounding parenchymal tissue to facilitate signaling between islet cells [6,12]. Restoring this pattern of vasculature in transplanted islets is critical to islet engraftment [6,12]. Transplantation of rodent islets with MSCs results in increased vascular density and improved graft function [12,13]. In the co-transplant group, endothelial cells were present only at the periphery of the islet graft; whereas, endothelial cells were detected within islets in the islet alone group. Islet graft vascularization was decreased in the presence of bMSCs. Interestingly, amyloid was present in the islet only group which suggests that the presence of MSCs may delay amyloid accumulation in the islet graft.

Islet cell architecture also influences islet function [15]; a change in the organization modifies the interaction between islet cells. Islets of normal size (150 μm to 250 μm) and morphology were observed in the islet only group.

Small islets, single islet cells and fragmented islets were much more frequent in the co-transplant group. The loss of endogenous islet morphology likely impairs islet function, as paracrine signaling is diminished in fragmented islets [15]. Insulin content was assessed to compare islet β-cell survival. No difference in insulin content between the co-transplant and islet alone treatment was observed. The similarity in insulin content implies that islet survival was not significantly different.

4.4.2 Systemic Delivery Demonstrates Beneficial Effects

To avoid graft compaction in the renal subcapsule, we explored the effect of systemic MSC administration. MSCs can exert beneficial effects by migrating to injury sites or secreting factors into the bloodstream [9]. After intravenous injection, MSCs can migrate to the pancreas [20-25]. Lee *et al.* showed that human MSCs are found only in the pancreases and kidneys of diabetic mice after intracardiac infusion [20]. One mechanism for this homing is through chemokines. MSCs express a set of receptors that respond to chemokines from pancreatic islets [26]. Inflammation or injury is another signal that activates MSC migration [9]. For instance, systemically delivered MSCs attenuate inflammation mediated damage after myocardial infarction, lung injury or chemical induced diabetes [9,20]. After islet transplantation, non-specific immune injury occurs due to elevated pro-inflammatory cytokines and macrophage infiltration [5,6]. Taken together, we hypothesized that systemically administered MSCs could repair islets after transplantation. Here we initiated a pilot study to assess the feasbility of intravenous injection of bMSCs after islet transplantation. Cell

injection poses the risk of pulmonary embolism and thus a small number of animals was utilized to test develop this transplant method.

Islet graft inflammation or chemokine production from transplanted islets could induce MSC migration. Inflammation would also activate MSC secretion of factors that initiate cell repair. We observed that transplanted islet function improves after systemic MSC delivery. Average blood glucose levels were lower in the islet + bMSC group compared to the islet only group. A greater proportion of mice treated with islet + bMSC achieved euglycemia after one week. The beneficial effect; however, was transient suggesting that bMSCs secrete factors to limit inflammation but do not participate significantly to improve islet engraftment.

A second dose of bMSCs also improved islet graft function. Because vascularization occurs with engraftment, vascular networks are not present in the islet graft during the first infusion of bMSCs. But, a second bMSC infusion may exert effects by migrating to the transplanted islets. As a result, multiple infusions may be required to achieve a long term effect. But a recent study demonstrated that multiple infusions of MSCs were not more effective than a single infusion in treating hyperglycemia in mice with diabetes [27].

Another group investigated the effect of multiple MSCs injections in a rat model of type 2 diabetes [28]. The MSCs transiently decreased hyperglycemia after each treatment [28]. The authors suggested that the improvement was associated with decreased peripheral insulin resistance and increased expression of protein involved in glucose metabolism (glucose transporter 4, phosphorylated

insulin receptor substrate 1 and protein kinase B) [28]. An alternative means to increase the long term benefit is to identify subpopulations of MSCs that have greater regenerative properties. Bell *et al.* have shown that MSCs that highly express aldehyde dehydrogenase (ALDH) are more potent in promoting endogenous islet regeneration and revascularization [29].

4.5 CONCLUSION

In summary, we described a method to deliver MSCs with islets that can temporarily improve islet transplant outcomes [30]. Although numerous studies have shown that localized delivery of islets and MSCs is beneficial in the renal capsule [13-15], our experimental results demonstrated that this site is not appropriate for islets and MSCs from human donors. The advantage of localized administration of MSCs is the increase in dose of MSCs that could be delivered directly to the graft. With systemic delivery, MSC migration to the transplant site is minimal because islet vascularization is delayed. Thus the initial MSC infusion likely reduced inflammation at the proximity of the site.

While the renal capsule is not appropriate, an alternative transplant site such as the omentum or hepatic portal vein can accommodate larger grafts. In particular, the portal vein is a clinically relevant site. After intraportal infusion, islets are subjected to a robust inflammatory and coagulatory response; islet engraftment is markedly reduced. Mesenchymal stem cells may be appropriate to suppress inflammation and promote islet engraftment. We have demonstrated evidence that human bone marrow derived MSCs protect human islets from pro-

inflammatory cytokines in cell culture [31]. Duprez *et al.* also evaluated the capacity of MSC to protect islets from human blood [32]. For clinical application, evaluating the function of islet after intraportal infusion may be a better site to investigate. An additional benefit is that the site can accommodate more tissue and islet compaction is less concerning.

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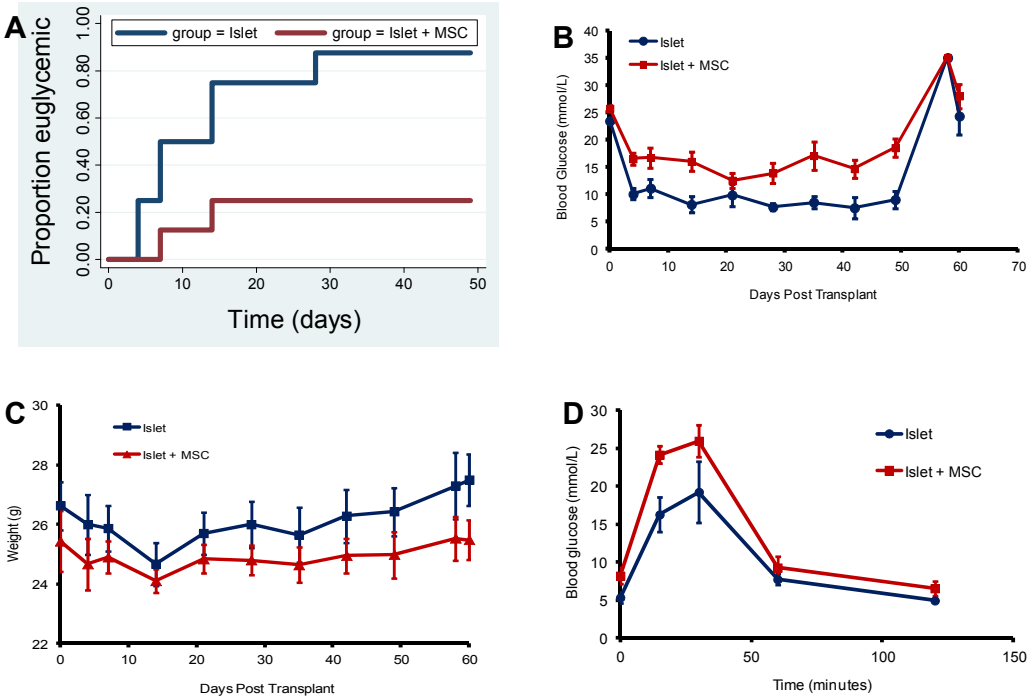
FIGURE LEGEND

Figure 4-1: Co-transplantation of Human Islets and Human MSCs Into the Renal Capsule Limits Islet Graft Function

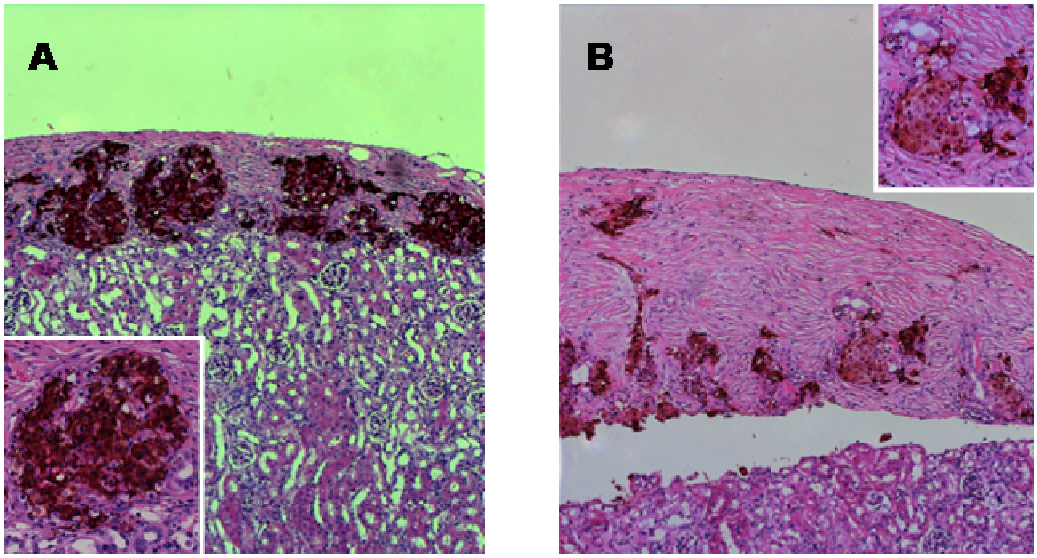


A) The proportion of mice that achieved euglycemia (<11.0 mmol/l blood glucose) in islet alone versus islet + MSC are reported as a Kaplan Meier survival curve and differences in survival were assessed by log rank analysis. B) The glycemia are reported as mean ± SEM. On week 8, survival nephrectomies were performed. C) The mice weights are mean ± SEM. D) Islet graft function for mice that achieved normoglycemia was assessed by an oral glucose tolerance test on week 8 prior to the survival nephrectomy. Values are mean ± SEM (n=8).

Table 4-1: Insulin Content of Islet and Islet + MSC Co-Transplant Grafts Under the Kidney Capsule of Diabetic Mice

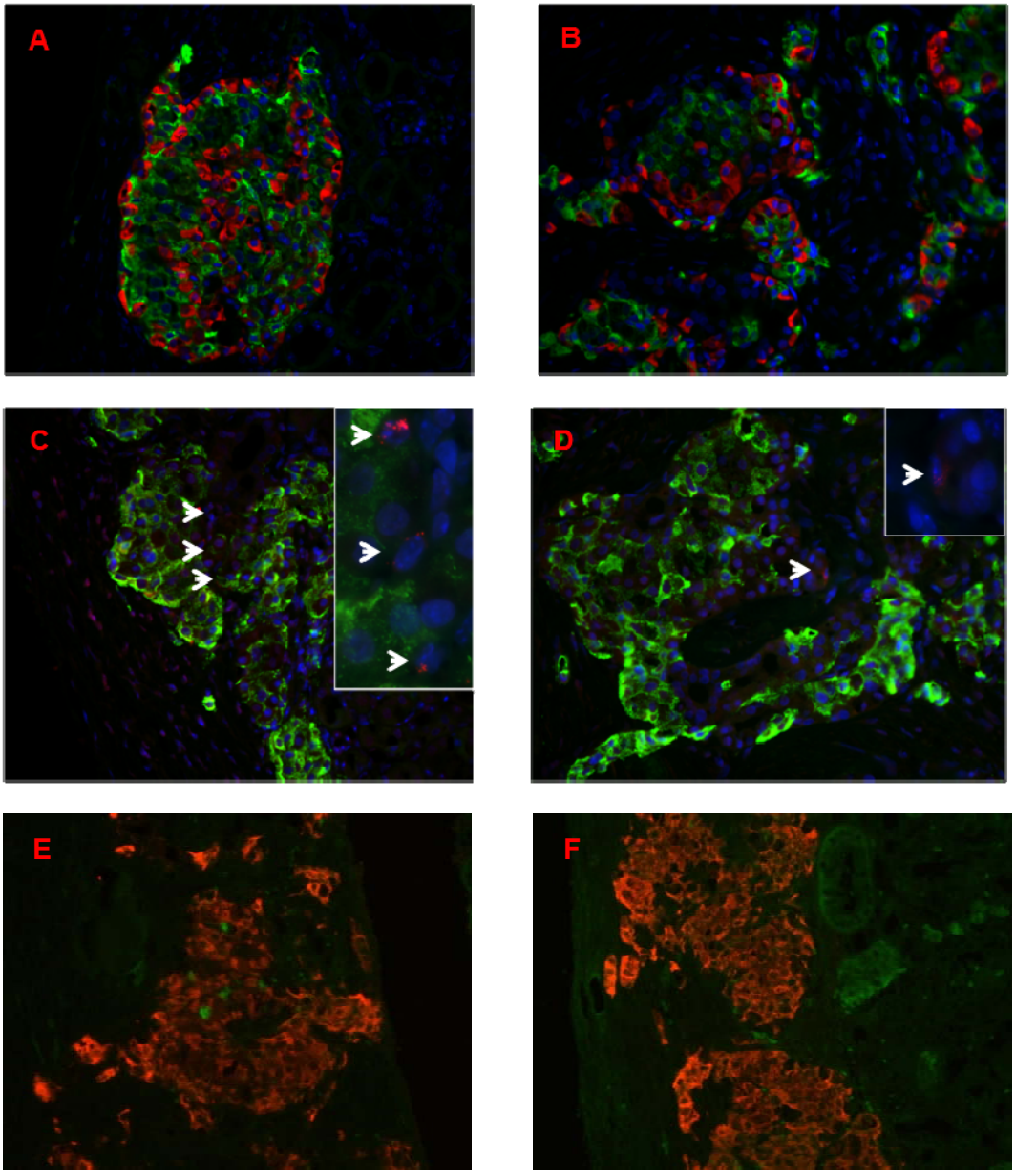
Groups	Graft Insulin Content (µg)
Islet	3.3, 1.3
Islet + MSC	3.3, 2.4

Figure 4-2: Histological Evaluation of Islet and Islet + MSC Grafts Under the Kidney Capsule of Diabetic Mice.



Overall morphology was determined after staining grafts for insulin and counter staining for hematoxylin and eosin. Magnification is x100. Inset image is x400. A) Islet transplant graft, B) Islet + MSC transplant graft

Figure 4-3: Immunofluorescent Staining of Islet and Islet + MSC Grafts Under the Kidney Capsule of Diabetic Mice.



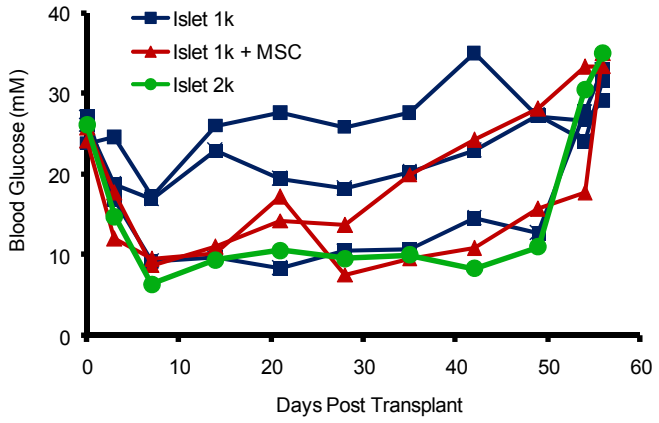
Islet organization was assessed by staining for insulin in green, glucagon in red and the nuclei with DAPI (blue). A) Islet transplant graft, B) Islet + MSC co-transplant graft. Magnification is x400. Islet vasculature is determined by staining for insulin in green and Von Willebrand's Factor in red, C) Islet transplant graft, D) Islet + MSC co-transplant graft. Islet amyloid is determined by staining for insulin in red and thioflavin S in green, E) Islet transplant graft, F) Islet + MSC co-transplant graft. Magnification is x100.

Table 4-2: Systemic Delivery of MSCs Transiently Reverses Diabetes in Marginal Mass Human Islet Transplantation but does not Result in Permanent Diabetes Reversal.

Groups (Total number)	Days Post Transplant								
	0	**3**	**7**	**14**	**21**	**28**	**35**	**42**	**49**
Islet (n=3)	0	0	1	1	1	1	1	0	0
Islet + MSC (n=2)	0	0	2	2	0	2	1	1	0

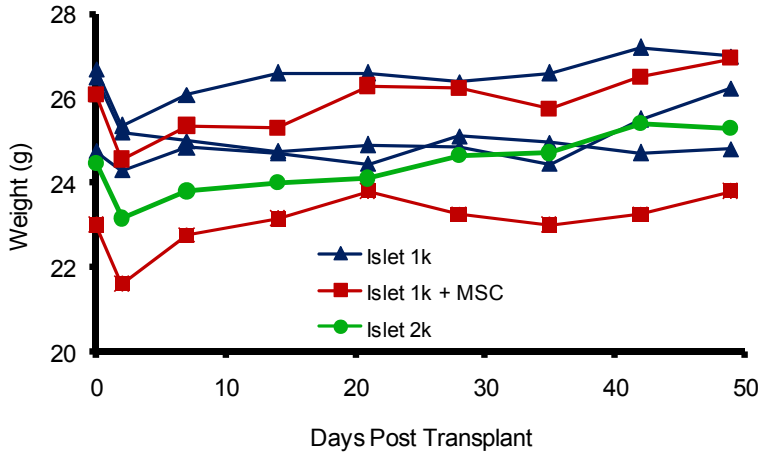
The number of mice that achieved euglycemia (<11.0 mmol/l blood glucose) in islet alone (1000 IEQ) versus islet (1000 IEQ) + MSC are reported. In the islet + MSC group, MSCs were injected into the mice at day 0 and day 23 after islet transplantation.

Figure 4-4: Systemic Delivery of MSCs Transiently Improves Human Islet Transplant Outcome

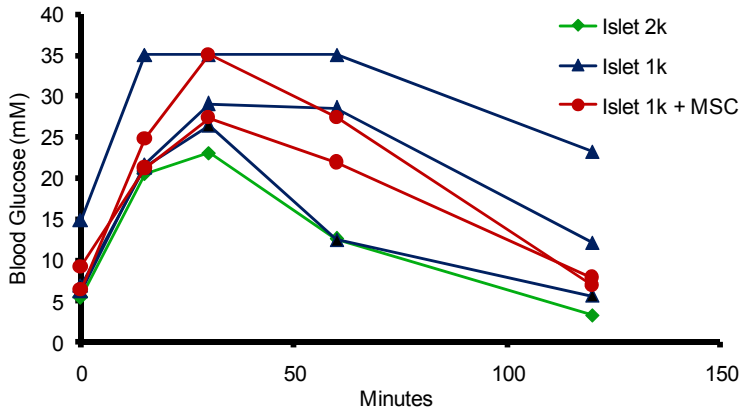


A) Each line represents the glycemia of from one mouse after human islet transplantation. Survival nephrectomies were performed on week 8.

Figure 4-4: Systemic Delivery of MSCs Transiently Improves Human Islet Transplant Outcome



B) Each line represents the weight of one mouse after human islet transplantation.



C) Each line represents the graft function of one transplanted mouse as assessed by an oral glucose tolerance test before survival nephrectomies.

CHAPTER 5

GENERAL DISCUSSION AND CONCLUSION

5.1 GENERAL DISCUSSION

Diabetes, a disorder of glucose metabolism, is a leading cause of morbidity and mortality around the world [1,2]. Of the 250 million individuals worldwide affected by diabetes, type 1 diabetes mellitus (T1DM) affects 5-10% of the population [1,2]. T1DM is characterized by autoimmune destruction of insulin producing islets [1,2]. On the other hand, Type 2 diabetes mellitus (T2DM) is caused by a defect in insulin processing [1,2]. T2DM makes up most of the diabetic cases (85 – 90%) [1,2]. Even though type 1 diabetes represents a relatively small proportion of all diabetic cases, the economic burden of T1DM is significantly greater than anticipated. T1DM accounts 25% of the total costs for all cases of diabetes when measured by medical expenditures and loss of workplace productivity [3]. Prevention of T1DM is difficult and attempts to prevent the onset of disease using immune tolerance induction have not been successful [4]. Currently, there is no cure to the disease, but patients can manage T1DM by daily insulin administration [1,2]. Even with insulin therapy, when control of blood glucose is poor, patients are at risk for renal failure, heart disease, and blindness. Strict glycemic control can be achieved with more frequent insulin injections, but dangerous hypoglycemic coma is a common side effect [1,2]. The

preferable approach is to restore pancreatic β cell function via whole pancreas or isolated islet transplantation [5,6]. In comparison to daily insulin administration, β-cell replacement achieves physiological glycemic control and alleviates hypoglycemic episodes [5,6]. Islet transplantation is more favorable than whole pancreas transplantation because the surgical procedure is less invasive [5,6]. However, the use of chronic immunosuppression and the loss of graft function less than 5 years after transplantation are the significant drawbacks to clinical islet transplantation [2,7]. Islet transplantation has not been approved as an acceptable standard therapy. The transplant surgery is prescribed to T1DM individuals who are no longer responsive to insulin therapy and/or patients with T1DM who require renal replacement [2,5,6]. Reducing or eliminating the risks of islet transplantation is crucial to justify the procedure as a standard treatment of T1DM.

One of the means to resolve the issue of islet graft failure is to utilize cell-based therapies that protect transplanted tissue from functional impairment or cell death [8-10]. This strategy offers the opportunity to develop graft specific therapy, and to utilize tissues derived from patients. Direct attachment of helper cells to the islet graft can be achieved without disrupting islet viability or function [8-10]. In contrast, drug therapy can cause non-specific or adverse effects throughout the body. To localize the effect of drug therapy, chemical modification of islet cells must take place, which is not a favorable approach [11]. In recent years, mesenchymal stem cells (MSCs) have gained a large amount of attention as a potential stem cell therapy for autoimmunity, transplantation and

inflammation [12-14]. These cells are derived from connective tissue with the ability to differentiate into bone, fat and cartilage [12-14]. Although research initially focused on generating tissues from MSCs by differentiation, many are exploiting another aspect of these cells, the capacity to induce regeneration and immunoregulation [12-14]. After transplantation into animals with myocardial infarction, sepsis or graft versus host disease, MSCs migrated to the site of inflammation and promoted cell repair or prevented tissue rejection by decreasing the activity of alloantigen activated immune cells [12-14]. In T1DM, loss of islet β cells occurs after autoimmune mediated destruction. Due to the immuno-regulatory properties of MSCs, researchers are investigating clinical approaches to delay the progression of T1DM, to mitigate late complications of diabetes, and to potentially cure diabetes with MSC transplantation [15]. In islet transplantation, inflammation and immune rejection are key mechanisms of islet graft impairment and loss [16,17]. Thus, the role of MSC therapy in islet transplantation is currently being investigated [15].

In Chapter 2, this study focused on the effects of mesenchymal stem cells (MSCs) on islets *in vitro*. MSCs are believed to exert beneficial effects on islets. In cell culture, MSCs from human bone marrow can improve the survival and function of rodent and human islets by secreting growth factors [10,18]. MSCs also enhance islet transplant outcomes by inducing graft revascularization and preserving islet cell morphology [19-23]. However, the ability of human MSCs to protect human islets after transplantation is not well known. Inflammation in the

post transplant period is recognized as a major factor in islet graft dysfunction [16,17]. Elevated pro-inflammatory cytokines, not related to allogeneic islet rejection, are reported in the islet graft [11,16,17]. To mimic the effect of MSCs on islets after transplantation, we tested the ability of MSCs to protect islets from the pro-inflammatory cytokines interferon γ, tumour necrosis factor α, interleukin 1β *in vitro*.

We demonstrated that MSCs have immunomodulatory properties that can protect islets [24]. While both bone marrow and pancreatic derived MSCs are protective, the effect of bone marrow MSCs on human islets is more robust than pancreatic MSCs. We also confirmed that this effect was MSC dependent, as fibroblasts from human skin did not have any beneficial effects on human islets. Glucose stimulated insulin secretion and total insulin content were preserved in islets co-cultured with MSCs after pro-inflammatory cytokine exposure. A decrease in islet β cell apoptosis was also observed in the presence of MSCs. The protective effect was correlated with elevated levels of hepatocyte growth factor (HGF) and matrix metalloproteinase 2 (MMP2). Consequently, we evaluated the role of HGF in our co-cultures. HGF alone preserved glucose responsiveness but the overall protection was decreased in comparison to MSCs.

In Chapter 3, we examined the function of other soluble factors that may play a role in the protection of islets from pro-inflammatory cytokines and to further understand the mode of action in MSC islet co-culture. The study in Chapter 2 demonstrated that HGF alone did not replicate the cytoprotective effect

of MSCs. A combination of factors may be necessary to achieve the level of protection of islets observed with MSCs. In addition, islet and MSC contact may be important. After pro-inflammatory cytokine exposure, the physical interaction between islets and MSCs was increased suggesting that cell contact played a role in the anti-inflammatory effect. To determine the role of soluble factors and cell contact in MSC mediated protection of islets, we tested the function of human islets by comparing direct and indirect contact co-cultures.

Direct cell contact enables greater protection than indirect contact. In direct contact, insulin content recovery and islet function from cytokine treated islets was significantly better than islets treated with cytokines only. On the other hand, the indirect cell contact experiment showed that islets cultured in MSC conditioned medium did not restore islet function or improve insulin content recovery. But MSCs cultured in a separate compartment from human islets preserved islet function. The presence of MSCs is essential for protection, which led us to conclude that stimulation of MSCs with pro-inflammatory cytokines is important to achieve this protection. The amount of secreted factors from untreated MSCs are likely not sufficient to protect islets. However, loss of insulin content did occur after cytokine exposure when MSCs and islets were cultured in separate compartments that prevented cell contact but allowed diffusion of soluble factors. Cell contact, therefore, was integral to protect islet insulin content. As direct contact co-culture protects islet function and islet insulin content, soluble factors together with cell contact have synergistic effects to enhance the immunomodulatory properties of MSCs on islets.

Limitations in the Cell to Cell Contact Study and Future Directions

The implication of this study is that replacement of MSCs with growth factors alone does not completely replicate the effects of cell therapy. Not only does this suggest that MSCs must be present at the transplant site, but also MSCs need to be in direct contact with islets to provide the greatest protection. An additional benefit of localized delivery is that MSCs can release growth factors directly at the graft site over a prolonged period. Drug based therapy may depend on multiple doses at higher than therapeutic levels to ensure that sufficient concentrations of the drug can affect the transplant graft. These results led us to pursue localized delivery of islets and MSCs into a diabetic mouse model. One limitation of our *in vitro* model is that the survival and function of these islet and MSC aggregates has not been tested in conditions that mimic the instant blood mediated inflammatory reaction (IBMIR) of clinical islet transplantation. IBMIR is an intense inflammatory and coagulatory reaction that occurs after infusion of islets into the liver [11,16]. A significant amount of islet loss occurs due to IBMIR in rodent transplant models [11,16]. And, medical imaging of clinical islet transplant recipients revealed 25% islet loss is related to activation of IBMIR. Investigation of IBMIR will also need to be assessed for transplantation of islets with MSCs [11,16]. Duprez *et al*. utilized an *in vitro* model of coagulation with human blood to show that islets coated with MSCs have decrease activation of the coagulatory pathway [25]. While our co-culture is similar to the model presented by Duprez *et al*., the bMSCs do not coat human

islets. Instead, the bMSCs form aggregates that adhere to islets. Thus, future experiments can utilize this *in vitro* model of IBMIR to better predict graft survival and graft function in a hepatic portal vein model.

Based on the ability of MSCs to protect islets in cell culture, in Chapter 4, MSC therapy for islet transplantation was evaluated in a mouse model of diabetes, the transplantation of human islets with human mesenchymal stem cells. We speculated the bone marrow derived MSCs would release factors that protect islets from inflammation and promote islet revascularization.

In our model, the kidney capsule was the site for islet transplantation because the kidney is a common site that yields consistent success in rodents [1,17,21,22]. In addition, the graft can be retrieved without euthanizing the animal. Our results demonstrate that co-delivery of islets and MSCs under the renal capsule has a poor outcome. Rates of diabetes reversal are decreased and average blood glucose levels are higher in the co-transplant group versus the islet transplant only group. At this transplant site, compaction of islets [26] and loss of islet architecture [21] are several factors that contributed to loss of graft function. We initiated a pilot study that indicates systemic delivery of MSCs can improve islet transplantation. Since revascularization occurs over two weeks, the beneficial effect of the initial MSC infusion is likely related to decreased inflammation at the transplant site. A secondary infusion of MSCs three weeks after the islet transplant also reduced hyperglycemia, which indicates that the injection of MSCs is beneficial to islet graft function.

Limitations in Islet:MSC Transplantation Model and Future Directions

Systemic infusion of MSCs is a promising approach to improve islet transplantation with human donor tissue; but the mechanism of action has not been determined. Several modes of action may explain these beneficial effects with MSCs including the production of soluble factors to attenuate inflammation in the transplant microenvironment, migration of MSCs to the islet graft to initiate vascularization, or transfer of sub-cellular units, such as mitochondria, to facilitate cell repair [12-14]. In diabetic mice, systemic administration of MSCs resulted in cell migration to the pancreases and kidneys to mediate tissue regeneration [27-31]. However, engraftment of MSCs can be low because islet grafts are not vascularized until several weeks after transplant. To determine the extent of cell migration to the islet graft, MSCs that express green fluorescent protein can be infused. The presence of MSCs at the graft will be detected by measuring the expression of human DNA or green fluorescent protein (GFP). However, the search for human DNA or GFP expression is not able to detect mitochondrial transfer. To distinguish between engraftment of cells and sub-cellular compartments, the effect of MSCs after intravenous injection can be compared to MSCs delivered in an immunoisolation, biocompatible device. Alginate microcapsules [34] or Theracyte^{TM} chambers [35] are some of these devices that can support MSC or stromal cell survival. Soluble factors produced by MSCs in these devices can continue to diffuse systemically [34,35]. But cells and their sub-cellular compartments cannot migrate out of these devices. Improvements in

graft function with these immunoisolation devices are due to factors produced by MSCs.

Because subpopulations of MSCs may also have different regenerative properties, another variable is to determine the ideal MSC for transplantation. One group has demonstrated that MSCs with high expression of aldehyde dehydrogenase (ALDH) can improve rodent islet graft function and engraftment [36]. For transplantation, a population of MSCs enriched in ALDH expression can be investigated for improvement in graft function. For patients, the use of autologous or allogeneic MSCs is another area for investigation. While patient derived tissues are not immunogenic, MSCs from patients with long standing chronic illness may not be suitable. Fiorina *et al.* compared MSCs from healthy mice to those with autoimmune diabetes [37]. The MSCs from healthy mice were more potent than the MSCs from the diabetic mice in their ability to prevent the occurrence of autoimmune diabetes [37]. Thus, patient derived MSCs may not be the optimal source of cells for transplant. Moreover, allogeneic MSCs should not stimulate a robust immune rejection because they are immunoregulatory.

Delivery of islets and MSCs to the same transplant site is preferable to systemic injection of MSCs because the number of cytoprotective cells present at the transplant site is known and consistent. Islet and MSC co-transplant grafts are large in volume. Consequently, sites that can avoid the complication of islet compaction including the omentum [38] and hepatic portal vein [19,23] are necessary to test the effect of MSCs. For clinical islet transplantation, the hepatic portal vein is the main transplant site. The clinical relevance of intraportal islet

and MSC infusion is an important area for future investigation. Issues regarding safety and function of MSCs in the liver are also necessary to evaluate. For instance, the genetic stability of MSCs in the liver is not known [39]. Accumulation of chromosomal aberrations has been reported in cell culture of MSCs, which increases the risk for tumourigenesis [39]. Removal of cancerous cells within the liver is dangerous and difficult. The function of MSCs in the liver is also unclear. Mouse MSCs promote revascularization [19] but human MSCs may be more variable in function, depending on donor characteristics and culture methods. Interaction of MSCs with immune cells in the liver may promote an inflammatory phenotype from MSCs. In addition to concerns about MSC graft function and risks for neoplastic transformation, the liver remains a poor site for islet graft survival and function. Several transplant centres have investigated alternative sites including bone marrow [40] and muscle [41].

An attractive alternative is to bioengineer a microenvironment that best preserves islet function using a biocompatible device. The device can be transplanted into a location that requires minimally invasive surgery. The graft can also be easily removed. For clinical transplantation, large animal studies will be necessary to advance MSC therapies for islet transplantation. Large animal models of islet transplantation are already available; Berman *et al*. have described the transplantation of monkey islets and MSCs into non-human primates [23].

5.2 GENERAL CONCLUSIONS

The objective of this thesis was to investigate the ability of mesenchymal stem cells to improve islet survival and function in cell culture as well as after transplantation. We have demonstrated bone marrow and pancreatic derived MSCs can secrete factors that improve islet function but bone marrow MSCs are more potent in their cytoprotective properties. The source of pancreatic derived MSCs is limited to cadaveric pancreases because partial resection of the pancreas from living donor is unsafe and medically unnecessary. Because bone marrow can be extracted from living patients without fatal consequences, this cell source remains more favorable to pancreatic derived MSCs. Thus, we used MSCs from bone marrow for all our subsequent experiments.

In our co-cultures, we also observed that physical interaction between MSCs and islets occurred. Cell contact increased in the presence of cytokines. To determine whether the addition of secreted factors alone could replace the use of MSCs for islet cell therapy, the protection of islets by MSCs in direct and indirect contact co-cultures was examined. bMSC-conditioned medium did not protect islets. However, MSCs did protect encapsulated islet function, which led us to conclude activation of MSCs increased the secretion of cytoprotective factors that ameliorated islet function. The greatest protection from pro-inflammatory cytokines in islet and MSC co-culture was observed when the two tissues were in direct cell contact. Thus a combination of an adequate amount of secreted factors and cell contact is important for MSC protection of islets.

To address the clinical applicability of this co-culture approach, islets and MSCs were delivered into a preclinical model of islet transplantation. Transplantation of islets and MSCs into the renal capsule, however, produced unexpected results. Islet transplant outcome was superior without MSCs. The large amount of tissue with co-transplantation resulted in islet graft compaction, decreased islet revascularization, and distorted islet graft morphology. To circumvent the compaction of islets in the kidney capsule, an alternative strategy for co-transplantation was explored in which MSCs were delivery systemically. Intravenous administration of MSCs after islet transplantation improved reversal of diabetes and helped to lower blood glucose levels. These are the first reported results of improved function with donor human tissues.

Future studies will need to identify the mode of action on the islet graft after systemic injection of MSCs. After systemic delivery, the number of MSCs that migrates to the graft may be low. Moreover, localized delivery of MSCs remains more desirable for clinical transplantation. An alternative site for consideration is the hepatic portal vein. To address the safety concerns of transplanting stem cells, another strategy may be to implant a biocompatible device at a peripheral site. Overall, these studies demonstrate that MSC therapy is a promising solution to the poor long term outcomes of clinical islet transplantation.

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APPENDIX A

EFFECT OF HUMAN BONE MARROW DERIVED MESENCHYMAL STEM CELLS ON NEONATAL PORCINE ISLETS

A1.1 INTRODUCTION

Islet cell transplantation is a potential cure for type 1 diabetes (T1DM) because most recipients can achieve insulin independence with good glycemic control and do not experience the hypoglycemic incidences common to insulin therapy [1]. In addition, the surgery is less invasive and less risky than the whole pancreas transplantation [1]. Because of the favourable metabolic outcomes, islet transplantation has become an attractive alternative to daily insulin therapy. But, the desirable outcomes do not last long term. Loss of graft function occurs within 5 years post-transplantation in the majority of islet recipients. Therefore, islet transplantation is for patients who have severe type 1 diabetes and/or with end stage renal disease [1]. Also, the limited availability of human donor cadaveric tissue continues to be a significant obstacle for widespread islet cell therapy. Seeking an abundant or readily available islet supply, one solution is to harvest islets from an animal source. Porcine insulin, for example, has a similar molecular structure and it functions to regulate blood glucose in the same range as human islets [2]. Insulin extracted from the porcine pancreas has been utilized for many years as insulin therapy for TIDM [2]. In addition, clinical trials of pig islet

transplantation for the treatment of T1DM have been initiated in the past few years [3]. These clinical trials indicate that pig islets are a viable source of tissue for xenogeneic islet transplantation [3].

Porcine islets have been isolated from animals at the stages of adult, neonatal and fetal [2]. Adult pigs are considered to be a poor islet source because the success of islet isolation is inconsistent [2]. On the other hand, neonatal pig islets (NPI) can be isolated with a simple and reproducible method [4]. Moreover, transplantation of NPI into small and large animals has been successful [4,5]. Reversal of diabetes and insulin independence can be attained [4]. Thus, NPI are considered to be a potential source of islets for clinical islet transplantation. However, one major limitation of utilizing neonatal pig islets is the delay in reversing diabetes due to the lengthy maturation period to become glucose responsive tissue [2,4]. NPI require eight to ten weeks to reach full functional maturity after transplantation into diabetic immunodeficient mice [4]. The implication is that NPI recipient would require insulin injection to maintain the strict glycemic control while waiting for porcine islets to mature. However, when blood glucose concentrations are well controlled, the functional maturation of neonatal porcine islets after transplantation is delayed-to a greater extent [6]. These results indicate the complexity of delivering NPI for β-cell replacement therapy. As a result, NPI are not fully ready as a suitable islet source for clinical transplantation. For NPI transplantation to be a substitute of islet transplantation, solutions are necessary in accelerating the functional maturation of NPI prior to transplant or in reducing the lag time in NPI maturation after transplantation.

Several approaches to expedite porcine islet maturation have been explored [7-11]. For instance, the culture of NPI with natural or chemical compounds as well as the co-culture of NPI with feeder/helper cells can promote islet survival and differentiation [7-9]. *In vitro*, the maturation of NPI occurred in the presence of fetal bovine serum, insulin like growth factor 1, nicotinamide, sodium butyrate, other growth factors and chemical compounds [7]. However, these treatments did not improve islet function after transplantation [8]. Another option for investigation may be to co-culture with support cells; one such example is culturing NPI with testicular sertoli cells to accelerate maturation in vitro and in vivo [10,11]. NPI conditioned with sertoli cells prior to transplantation had demonstrated a shortened maturation period after NPI were transplanted [10,11]. However, a clinically viable source of sertoli cells has not been reported. We, therefore, suggest that a suitable surrogate should have similar regenerative properties as sertoli cells; such a cell may be the mesenchymal stromal/stem cell (MSC) derived from human bone marrow.

Mesenchymal stem/stromal cells are multipotent cells that are known to increase angiogenesis, improve tissue survival, and decrease tissue inflammation after islet transplantation [12,13]. These cells can be isolated from connective tissues including human bone marrow, adipose, and umbilical cord blood. MSCs also have potent immunoregulatory properties [12,13]. They have been used for the successful treatment for arthritis and multiple sclerosis in animals [13]. In humans, MSCs have been used for steroid refractory graft versus host disease

[13]. Clinical trials on treatment of sepsis are also underway [13]. MSCs are primarily thought to exert many of the reported beneficial effects by secretion of soluble factors [12,13]. On the other hand, concern over the possibility of MSCs differentiating into bone or cartilage or tumorogenic tissues cannot be ignored. Nevertheless, treatment with MSCs is believed to be a safe cell therapy for clinical applications because numerous phase I clinical trials have not reported ectopic tissue formation with mesenchymal stem cells [14].

Based on the regenerative properties of MSCs, several groups including our own have explored the beneficial effects of MSCs on the Islets of Langerhans in cell culture as well as for islet transplantation [15-18]. MSCs derived from human bone marrow or umbilical cord blood in co-culture with rodent islets improved islet survival and glucose sensitive insulin secretion in culture [15-17]. After transplantation, rodents treated with islets and MSCs reversed diabetes at a higher frequency than islet only controls [19-22]. The MSCs helped to improve islet engraftment by inducing angiogenesis and maintaining islet organization [19-21]. In addition to improving adult islet function, human umbilical cord blood MSCs induced the maturation of neonatal rat islets [23], whereas, neonatal rat islet controls became fragmented in cell culture [23]. Taken together, our research objectives were to determine the effects of bone marrow derived MSCs in co-culture with NPI and to study the effects of MSC on NPI function after transplantation. NPI were either aggregated with MSCs or cultured with MSC aggregates. To evaluate the co-culture model, the primary outcomes were islet function, which can be determined by glucose stimulated insulin secretion, and

total cellular insulin content. The optimal NPI:MSC co-culture based on islet function or cellular insulin content was utilized for transplantation into immunodeficient mice rendered diabetic with streptozotocin. For transplantation, weekly glycemia, percentage of euglycemic animals and graft composition were determined.

A2.1 MATERIALS AND METHODS

A2.2 Isolation and Culture of Neonatal Porcine Islets (NPI)

Islets were isolated from the pancreases from one to four days old Duroc piglets. The pancreases were removed, minced and digested with a collagenase XI blend (Sigma, Oakville, Canada). The pancreatic digests were then filtered with a 500 μm mesh and the filtrate was cultured for five to nine days in modified Hams F10 medium as previously described [4]. Complete media changes were performed every two to three days.

A2.3 Preparation of Human Bone Marrow Mesenchymal Stem Cells (bMSCs)

Bone marrow was extracted from three patients of the age 24, 46 and 69 years old at the Division of Orthopedic Surgery, University of Alberta, with signed informed consent. To isolate mesenchymal stem cells, bone marrow was cultured in Modified Essential Medium alpha (MEMα, Cellgro Manassas, VA) supplemented with 2.5 ng/mL basic fibroblast growth factor (bFGF, Millipore, Billerica, MA), 10% fetal bovine serum (FBS, Gibco), 1 mM sodium pyruvate

(Gibco), 10 mM HEPES (Gibco), 100 U penicillin/1000 U streptomycin (Biowhittaker, Walkersville, MD) at a density of 166,000 cells per cm^2 [15]. Non-adherent cells were removed by changing the medium every 2-3 days. Once confluent, the cell monolayer was washed with versene and was detached with 0.05% trypsin-EDTA (Invitrogen, Carlsbad CA). Cells were counted and re-seeded into MEMα culture medium at a density of 5000-10000 cells/cm^2. All cell cultures were maintained at 37^{o}C, 5% CO_2 in a humidified incubator.

A2.4 Alginate Co-encapsulation of NPI and bMSC

To study the effect of human bone marrow derived mesenchymal stem cells (bMSCs) on neonatal porcine islets, we devised a co-culture system that could support the growth of both tissues. The culture medium consisted of Hams F10, a medium utilized in NPI culture, supplemented with 10% FBS, a component essential for bMSC growth and favorable for NPI maturation. To prevent the formation of large cell clusters, NPI and bMSC were co-encapsulated in alginate microcapsules.

Prior to co-encapsulation, the NPI and bMSCs were aggregated or bMSCs alone were aggregated. Bone marrow derived MSCs were prepared by enzymatic detachment with trypsin from culture plates. After counting, the 2.0 x 10^6 cells were added to a 100 mm low adherence culture dish (Corning) with 4000 neonatal porcine islet equivalents in a total volume of 10 mL. For bMSC aggregate formation, the same numbers of MSCs were added to another low adherence culture dish. The culture medium for aggregation consisted of DMEM low

glucose (5.6 mM glucose, Gibco) with 1% FBS, 20 ng/mL EGF, 20 ng/mL bFGF, 10 mM HEPES, 100 U penicillin/1000 U streptomycin, and 71.5 μM β-mercaptoethanol. After 24 hours, these cultures were washed in calcium free HBSS to prevent alginate cross-linking and the tissues were resuspended in equivalent volumes (0.2 mL) of HBSS with 1.5% w/v alginate (MVG, Pronova Biomedical, Oslo, Norway) for encapsulation. Alginate microcapsules were formed by extrusion of this tissue alginate mixture through an electrostatic spray device into a calcium chloride solution (120 mmol/L $CaCl_2$, 10 mmol/L HEPES, 0.01% Tween 20), yielding particles of 300-600 μm in diameter. The encapsulated tissues were cultured in modified Hams F10 with 10% FBS for extra 3 days. The treatment groups consisted of two methods for co-encapsulation: aggregation of MSCs with NPI prior to encapsulation and aggregations of MSCs prior to encapsulation with NPI. The control groups included non-encapsulated and encapsulated neonatal porcine islets cultured alone.

A2.5 Characterization of Alginate Encapsulated NPI:bMSC Co-cultures

To determine glucose responsiveness, treatment and control groups were assessed using a static incubation assay [15]. Tissues were collected and washed twice by gravity sedimentation over 30 minutes. Preparations were then divided into representative aliquots and incubated at 37^{o}C for 2 hours in 1.5 mL Roswell Park Memorial Institute 1640 medium (RPMI) supplemented with 2.0 mM L-glutamine, 0.5% w/v BSA with 2.8 mM and 20.0 mM glucose respectively. Culture supernatant was collected and stored at temperature of minus 20^{o}C for

measurement of insulin content at a later time by rodent insulin immunoassay (Meso Scale Discovery, Gaithersburg, MD). Representative aliquots were also taken to assess cellular insulin contents using previously described protocols [15]. For the encapsulated NPI, the alginate microcapsule was dissolved with a dissociation medium (135 mM NaCl, 2.7 mM KCl, 1 mM Na_2HPO_4, 0.44 mM KH_2PO_4, 0.8 mM $MgSO_4 \cdot 7H_20$, 2.8 mM D-glucose, 10 mM HEPES, 1 mM EGTA, 4.2 mM $NaHCO_3$, 0.5% w/v fraction V BSA, 100 U penicillin/1000 U streptomycin, pH 7.4) prior to dividing the samples for cellular insulin content determination. To assess cellular insulin content, islet samples were re-suspended in 2 mM acetic acid containing 0.25% w/v BSA. The samples were sonicated and centrifuged (10 minutes, 800 g, 4^oC) to remove cellular debris. A 50 μL sample of the supernatant was vacuum dried and was reconstituted in 250 μL phosphate buffer with 0.5% w/v BSA (pH 7.4). Total cellular insulin content was measured using a mouse/rat insulin immunoassay (Meso Scale Discovery). Cross reactivity of porcine insulin with the rodent insulin immunoassay was confirmed at 140% (in lab measurement with a porcine insulin standard).

A2.6 Localized Transplant of NPI and Human Mesenchymal Stem Cell Aggregates

To determine the effect of MSCs on NPI maturation *in vivo*, non-encapsulated NPI and bMSCs co-culture were transplanted into immunodeficient B6 Rag -/- mice (C57Bl/6 $Rag1^{tm1\text{-}mom/J}$, from the Jackson Laboratory, Bar Harbor, Maine). The recipient mice, 8-12 weeks old, were rendered diabetic with a single intraperitoneal dose of streptozotocin (180 mg/kg, Sigma) dissolved with

citrate buffer (pH 4.5). To verify the diabetic status of the mice, blood was collected from the tail vein and blood glucose concentrations were determined using an OneTouch glucometer (LifeScan, Milpitas, CA). Mice were considered diabetic after two consecutive blood glucose readings greater than 20.0 mM. Prior to transplantation, NPI and bMSCs co-cultured were aggregated for 24 to 48 hours in supplemented DMEM medium (DMEM low glucose (5.6 mM glucose, Gibco) with 1% FBS, 20 ng/mL epidermal growth factor (EGF, R&D Systems, Minneapolis, MN), 20 ng/mL bFGF, 10 mM HEPES (Gibco), 100 U penicillin/ 1000 U streptomycin (Biowhittaker, Walkersville, MD), and 71.5 μM β-mercaptoethanol (Sigma-Aldrich)). The composite NPI:bMSC tissues were collected and transferred into PE50 polyethylene tubing (Becton Dickenson, MD). The tissues were collected into a pellet by centrifugation and implanted into a space that was created under the left renal capsule.

A2.7 Post-Transplantation Assessment

Each week, blood was collected from the tail vein and blood glucose levels were determined using an OneTouch glucometer. The main outcome was the achievement of euglycemia defined by two successive readings less than 11.0 mM glucose. For mice that achieved the primary outcome, graft function was assessed by an oral glucose tolerance test (OGTT). No food intake was allowed for these mice for 6 to 12 hours followed by an oral dose of dextrose (50% solution, 3mg/g body weight). Blood glucose readings were measured at 0, 15, 30, 60 and 120 minutes intervals after the oral glucose gavage. Graft function

was confirmed by a survival nephrectomy, in which the graft bearing kidney was removed and blood glucose values were recorded for an additional 2 to 5 days. The secondary outcome was graft composition, which was assessed by graft insulin content and graft histology.

A2.8 Insulin Content of NPI Grafts

To prepare specimens for insulin content determination, grafts were collected from each group at survival nephrectomy. The engrafted kidney (left) was removed and non-grafted sections were cut away from the kidney. The grafts were immediately frozen in liquid nitrogen and stored at minus 80^{o}C until it is ready for processing the cellular insulin content. Graft tissues were homogenized, followed by sonication at 4^{o}C in 10 mL of 2 mM acetic acid containing 0.25% w/v BSA. After a 2 hour incubation at 4^{o}C, the tissue was re-sonicated, centrifuged at 8000 xg for 20 minutes. The supernatant was collected. The tissue pellet was re-extracted with an additional 5 mL of 2 mM acetic acid containing 0.25% w/v BSA. Insulin content was determined with an immunoassay (Meso Scale Discovery, Gaithersburg, MD).

A2.9 Histology and Immuno-staining

To prepare transplanted grafts for histology, grafts were collected from each group at survival nephrectomy. The engrafted kidney (left) was removed and fixed with z-fix. Graft bearing kidneys were embedded in paraffin. Sections with 5 μm in thickness were cut with a microtome and placed on positively

charge glass histobond slides. For Masson's trichrome sections, slides were rehydrated and placed in Bouin's solution (60 C, 30 minutes) and cooled to room temperature (30 minutes). The slides were washed in warm water. Filtered trichrome stain was dripped onto the slides (20 minutes, room temperature). The slides were placed into 0.5% acetic acid (2 minutes), followed by 100% ethanol and coverslipped.

For immunohistochemstry, after rehydrating samples, the slides were quenched with hydrogen peroxide and washed in distilled water followed by PBS. Samples were blocked with 20% v/v goat serum (Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA) for 15 minutes at room temperature before staining. Primary antibodies for use were polyclonal guinea pig anti-insulin (1:1000 dilution, Dako, Mississauga, ON, Canada), mouse anti-glucagon antibodies (1:5000 dilution, Dako), and. Secondary antibodies were biotinylated goat anti-guinea pig and biotinylated goat anti-mouse. Avidin biotin complexes (Vector Laboratories) were bound for 30 minutes at room temperature before the addition of diaminobenzidine (Vector Laboratories). When a brown precipitate was formed on control slides, the reaction was stopped with distilled water and counterstained with hematoxylin and eosin (Sigma).

For immuno-fluoresence, primary antibodies used were polyclonal guinea pig anti-insulin (1:1000 dilution, Dako, Mississauga, ON, Canada), mouse anti-glucagon antibodies (1:5000 dilution, Dako), and monoclonal rabbit anti-cytokeratin 7 (1:50 dilution, Dako). Alexa fluor conjugated secondary antibodies were utilized – 1:200 Alexa Fluor 488 anti-guinea pig (Molecular Probes, Eugene,

OR), 1:200 Alexa Fluor 594 anti-mouse (Molecular Probes), and 1:200 Alexa Fluor 594 anti-rabbit (Molecular Probes). All antibodies were diluted in phosphate buffered saline. Slides were sealed with Prolong Gold Antifade and DAPI nuclear staining (Molecular Probes) to preserve fluorescence. Slides were visualized with an Axioscope II microscope equipped with an AxioCam MRC and analyzed with Axiovision 4.6 software (Carl Zeiss, Gottingen, Germany) as well as an AxioCam Colibri.

A2.10 Statistical Analysis

Results are reported as mean ± SEM. Data were analyzed by a Kruskal Wallis multiple comparisons test at a level of significance of 0.05. A Mann Whitney U test with Bonferroni corrections was performed for data considered significantly different. All statistically analyses were performed with STATA 11 (StataCorp LP, College Station, TX).

A3.1 RESULTS

A3.1.1 Effect of bMSCs on Neonatal Porcine Islets

Total cellular insulin content was measured from treatment and control groups an indicator of overall β cell mass. Insulin content in the treatment groups is reported as a value of insulin content from non-encapsulated NPI, which served as the baseline control. The percentage insulin recovery from NPI (60.1±6.1%)

was decreased after encapsulation (EC-NPI). However, aggregation of NPI and bMSC prior to co-encapsulation exhibited improved insulin recovery at 97.3±10.8% ($p<0.05$ compared to EC-NPI group). Co-encapsulation of NPI with aggregated bMSCs did not significantly improve insulin recovery compared to EC-NPI at 71.3±7.1% ($p>0.05$).

To measure islet function, glucose stimulated insulin secretion (GSIS) from neonatal pig islets was assessed by a static incubation assay at 2.8 mM and 20.0 mM glucose. The ratio of insulin secretion at 20.0 mM glucose compared to 2.8 mM glucose was reported as a stimulation index (SI). With all treatment groups, no significant difference in islet function based on SI and insulin release was observed. The results indicate the alginate microcapsule was not a barrier to insulin secretion (Appendix Table 1). In addition, stimulation indices in all groups were less than 1.5. These values for stimulation indices demonstrate no increase in islet functional maturation. Insulin release (% of insulin content) at 2.8 mM glucose was between 2.5 and 4.5% while insulin release at 20.0 mM glucose was between 3 and 5% for both control and MSC co-cultured NPI.

A3.1.2 Islet Morphology and Physical interaction of NPI and bMSCs

In co-culture with NPI, the bMSCs appeared to self aggregate and form spheroid cell clusters. Few bMSCs directly interacted with the NPI. The aggregates of bMSC were much smaller in size than most of the NPI tissue. The structural integrity of the neonatal porcine islets appeared to be best maintained with alginate microencapsulation. In addition, the morphology of the islets was

also not disrupted after co-culture with bMSCs. As insulin content recovery is better in the co-encapsulated NPI and bMSC than the EC-NPI alone, the tissue were examined for β-cell proliferation or beta progenitor cell differentiation. To determine cell proliferation, the expression of the proliferating cell nuclear antigen (PCNA) in insulin positive cells was determined. Co-expression of insulin with the proliferation marker PCNA was not markedly different between treatment and control groups. Furthermore, insulin expression was not greater in NPI:bMSC co-culture than controls. Cytokeratin 7 (CK7) positive ductal cells are putative β-cell progenitors. To assess the effect of bMSCs on the CK7 population, co-expression for insulin and CK7 was determined. Minimal co-expression of insulin and CK7 was observed in all culture conditions.

A3.1.3 Outcome of Transplant in Mice

Non-fasting blood glucose concentrations were determined from mice transplanted with NPI alone or NPI with MSC aggregates. After seven weeks, average glycemia in the co-transplant group were significantly lower (17.6±0.7) than the control (28.7±1.5). By the eleventh week, mice in the co-transplant group had average glycemia of 10.8±1.1 compared to average glycemia in the islet control (20.3±1.4). In addition, mice in the co-transplant group exhibited less polyuria based on visual inspection. Weight gain of the co-transplanted was superior to the control mice in the post transplant period ($p<0.05$). Overall average weights were greater in mice transplanted with NPI and MSCs from week five to ten. At week eleven, two out of four mice achieved normoglycemia in the

co-transplant group but no mice transplanted with NPI alone (n=5 mice) were able to reverse diabetes. At fourteen weeks, all mice in the co-transplant group were normoglycemic compared to one mouse in the NPI alone group.

When the graft function was assessed by an oral glucose tolerance test, the co-transplant group demonstrated better glucose tolerance compared to the NPI only group (Appendix figure 3C). Grafts from the co-transplant and NPI only groups were evaluated for morphology and cell composition. In the islet only group, numerous insulin positive cells were present but they did not form discrete cell clusters. In comparison, the co-transplant group demonstrates distince aggregates of insulin positive cells. Moreover, Masson's trichrome staining shows the formation of collagen matrix in the graft. The NPI alone grafts have abundant collagen deposition but the collagen forms thick bundles. In the co-transplant graft, the collagen organizes into narrow fibrils that surround discrete structures which resembles islet aggregates. CK7 expression is also present in the co-transplant group only (Appendix figure 4).

A4.1 DISCUSSION

Neonatal porcine islets (NPI) are being considered as a possible alternative source of insulin producing tissues for islet transplantation [1-3,24]. But, the delay in functional maturation continues to be a limitation for clinical islet transplantation [4]. In culture, growth factors can exert beneficial effects on NPI including an increase in β cell mass [9,10]. However, the long term benefits of

growth factors on NPI maturation or proliferation prior to transplantation are not significant [9]. Growth factors alone may not be sufficient to complete maturation. On the other hand, our lab has previously shown that the addition of a support matrix with growth factors could accelerate maturation [25]. Neonatal porcine cell clusters cultured in alginate microcapsules with autologous serum achieved normoglycemia significantly earlier than non-encapsulated neonatal porcine controls [25]. One drawback of encapsulation is that the alginate microcapsule becomes a barrier to the formation of new blood vessels into the NPI graft [26]. As diffusion is the primary means that encapsulated islet exchange nutrients, remove waste and regulate glucose metabolism, glycemic control with encapsulated islets may not mimic physiological function. Thus transplantation of neonatal islets without a barrier is preferable. One possible solution is to transplant accessory cells which protect tissue function, support cell survival and induce angiogenesis. Co-transplantation of NPI with sertoli cells has improved not only graft survival but also decreased the lag time to euglycemia compared to islets alone [10,11]. But, sertoli cells are isolated from testicular tissue, which is not readily available; if the testicular tissue is derived from a human source, sertoli cells are not likely to be a source of clinically relevant tissue for transplantation.

Human bone marrow derived mesenchymal stem cells as an alternative accessory cell source with various regenerative and immunomodulatory properties [12,13]. MSCs can be easily isolated from bone marrow, adipose and cord blood. They can facilitate cell or tissue engraftment and mitigate the rejection of donor

grafts [12,13]. In several studies, MSCs have been reported to improve islet cell survival and function [15-18]. Co-transplantation of islets with MSCs resulted in increased islet revascularization, decreased marginal islet mass, and improved islet transplant success [19-22] Our objective is to determine the ability of human bone marrow derived mesenchymal stem cells (bMSCs) to improve NPI maturation in cell culture or after transplantation. For cell culture, the effect of aggregating NPI and bMSC was compared to co-culturing NPI with bMSC aggregates. As aggregating NPI with bMSC resulted in better insulin content, this co-culture method was tested in diabetic mice. NPI and bMSC were aggregated and transplanted into immunodeficient diabetic mice in comparison to mice transplanted with NPI only; the co-transplant mice had a greater improvement in blood glucose levels.

A4.1.1 Co-culture of NPI and bMSCs In Vitro

Bone marrow mesenchymal stem cells secrete bioactive factors that can improve cell survival and cell function. We tested the effects of co-culturing bMSC with NPI by assessing the glucose sensitive insulin release and insulin content after four days of culture; no changes in GSIS were detected in co-cultures compared to islets alone. Insulin content, however, was greatest from NPI co-cultured with bMSCs, suggesting that the bMSCs are promoting beta cell proliferation or differentiation. To assess cell proliferation, expression of proliferating cell nuclear antigen (PCNA) was determined. Co-localization of insulin and PCNA expression was observed but no difference in between the

treatment groups were observed. Thus, pre-existing β-cells did not increase proliferate in co-culture with bMSCs.

Ductal cells in porcine tissue are reported to represent a putative progenitor population of β-cells. For islet cell differentiation, the expression of ductal cell marker, CK7, and insulin was assessed. Immunostaining shows that cells did not co-express insulin and CK7. Thus, islet progenitor cells within the ductal epithelium did not mature into β-cells in the presence of MSCs. As no markers of cell differentiation and no changes in cell proliferation were detected in the co-culture or control groups, the positive effect of bMSCs in the treatment group may be related to prevention of islet loss during encapsulation. MSCs can maintain islet morphology [21]. Culture of NPI with bone marrow MSCs did not improve functional maturation but did preserve islet morphology and islet cell mass for transplantation.

A4.1.2 Co-transplantation of NPI and bMSC in Diabetic Mice

The beneficial potential of bone marrow MSCs was explored in a transplant model. Because the maturation of NPI grafts occurs over weeks rather than days, we wanted to assess the long term effects of MSCs on NPI maturation could be detected after transplantation. MSCs also produce various growth factors and cytoprotective agents to promote angiogenesis, beta cell survival and reduce inflammation. We observed that NPI co-cultured with bMSC had lower blood glucose concentration 5 weeks post-transplantation. Although, only one mouse achieve normoglycemia at 10 weeks, NPI and MSC co-transplanted mice

were healthier because average weight gain was significantly better than NPI alone transplanted mice. By 14 weeks, all mice in the co-transplant group had achieved normoglycemia but only one mouse in the NPI alone group was euglycemic. Moreover, islet graft function was superior in the co-transplant group compared to the NPI alone based on an oral glucose tolerance test. Organization of the graft into islet like structures occurs in the co-transplant group. Discrete insulin positive structures and the ductal cell compartments are present only in the NPI + MSC co-transplant group. Rackham *et al.* reported that mouse islets co-transplanted with mouse MSCs performed better than islets transplanted alone [21]. They demonstrated that improvements in islet organization and angiogenesis were responsible for improved islet function [21]. Because MSCs can improve adult islet survival, MSCs may also exert a stromal effect to support islet maturation. Stromal support from biocompatible matrices can improve maturation. Alginate microencapsulated islets have superior transplant outcomes because the microcapsule provides a three dimensional support structure [25]. Our preliminary data from these studies demonstrate that bMSCs may prevent islet fragmentation in cell culture and could supported NPI formation after transplant,.

A5.1 CONCLUSION

The renal capsule provides an environment that can support NPI maturation. The addition of MSC helps to maintain islet structure. In contrast to

human islets, NPI are more resistant to hypoxia [27]. Thus, compaction of NPI in kidney capsule with bMSCs is not detrimental to NPI survival and function. For future experiments, changes in NPI graft composition can be examined. Because MSCs secrete factors that modulate vascularization and cell survival, increases in blood vessel density, and decreases in progenitors, such as CK7 ductal cells may be observed. Overall, co-transplantation is a promising method to expedite neonatal islet maturation. This co-transplant model may also provide insight into neonatal islet development.

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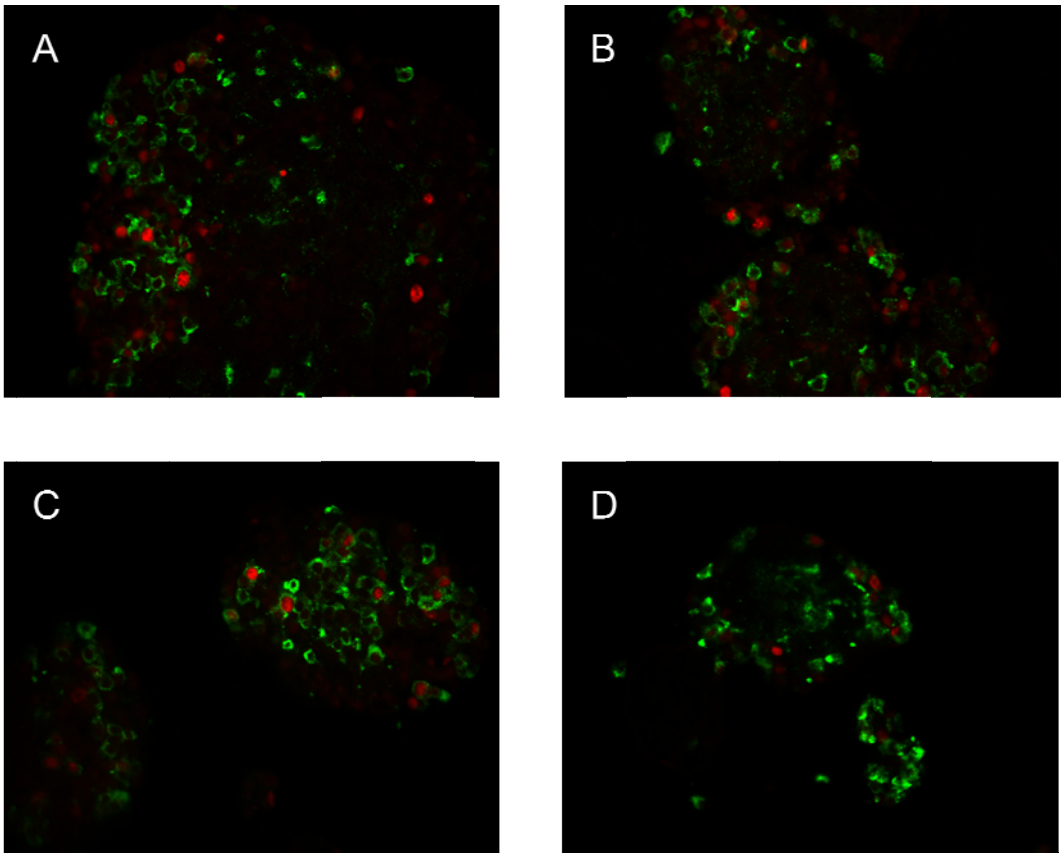
Appendix Table 1: Assessment of co-encapsulated neonatal porcine islets and human bone marrow derived mesenchymal stem cells for total cellular insulin content and insulin secretory capacity

Culture Conditions	% Recovery	Insulin Release (% insulin content)		Stimulation Index
	Insulin Content	2.8 mM Glucose	20.0 mM Glucose	
NPI	100.0	2.7±0.7	3.5±0.7	1.5±0.2
EC-NPI	60.1±6.1	3.9±1.2	4.4±1.3	1.3±0.1
EC-NPI + bMSC	97.3±10.8*	2.7±0.8	3.1±0.9	1.2±0.1
EC-NPI + bMSC aggregates	71.3±7.1	4.2±1.7	4.6±1.9	1.3±0.1

Results are reported as % recovery of total cellular insulin content (compare to non-encapsulated neonatal porcine islets). Islet function is assessed by a static glucose stimulated insulin secretion assay. The stimulation index (SI) is calculated as a ratio of insulin release at high glucose versus low glucose. % insulin release is reported as insulin measured at 2.8mM glucose or 20.0mM glucose divided by insulin content for corresponding islets. Values are expressed as mean ± SEM (n=5).

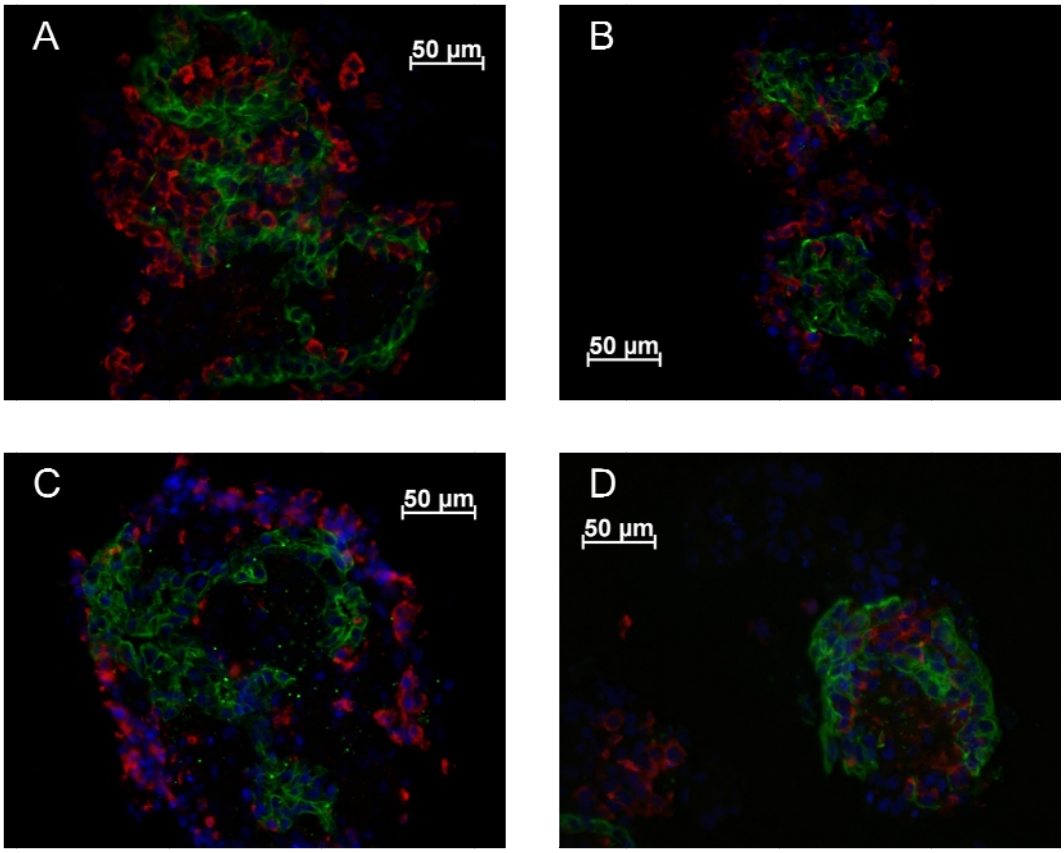
* $p < 0.05$ for EC-NPI vs. EC-NPI + bMSC
NPI – neonatal porcine islets
bMSC – bone marrow derived mesenchymal stem cell
EC – alginate microencapsulated.

Appendix Figure 1: Assessment of co-encapsulated neonatal porcine islets and human bone marrow derived mesenchymal stem cells for β cell proliferation



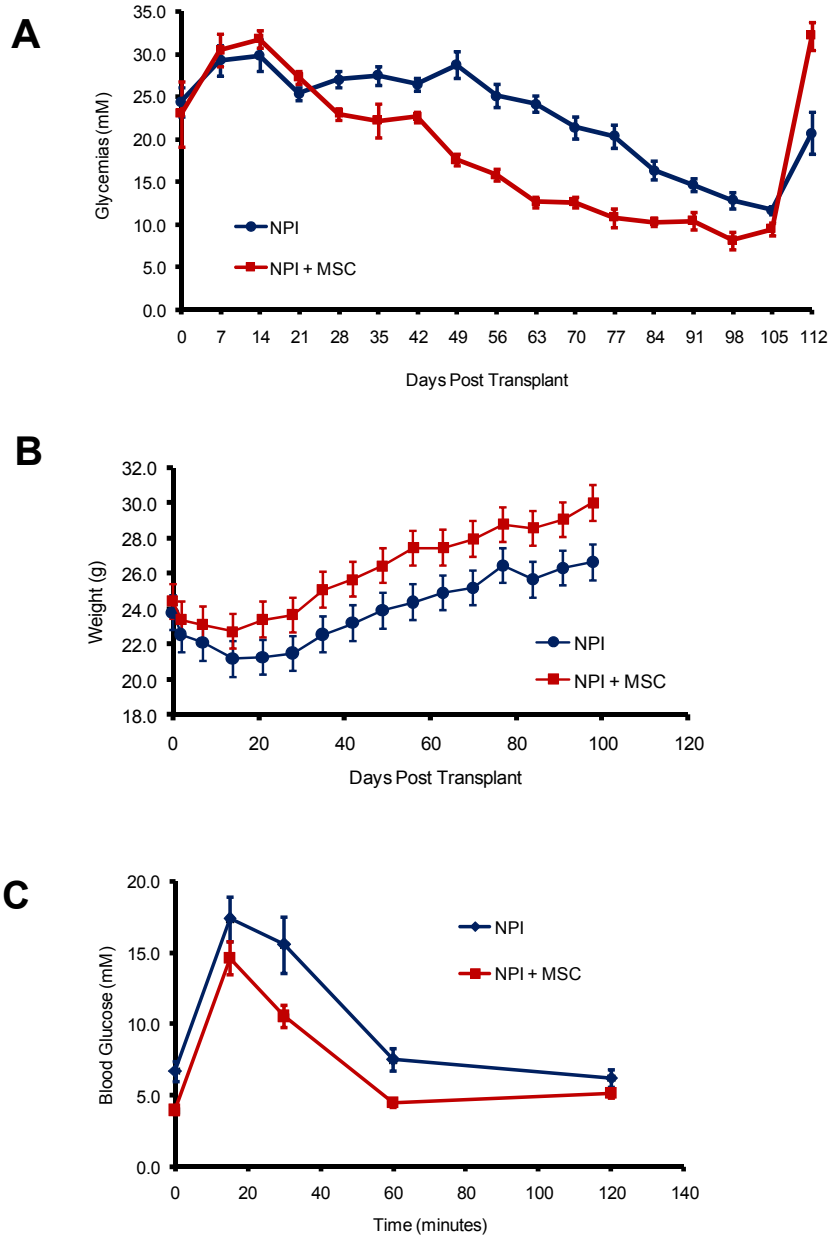
Insulin positive cells are marked green and proliferating cell nuclear antigen expression is marked red. A) Non-encapsulated NPI, B) Encapsulated NPI, C) Encapsulation of aggregated NPI + bMSC, D) Encapsulation of NPI with bMSC aggregates.

Appendix Figure 2: Assessment of co-encapsulated neonatal porcine islets and human bone marrow derived mesenchymal stem cells for CK7 ductal cell differentiation into insulin positive cells.



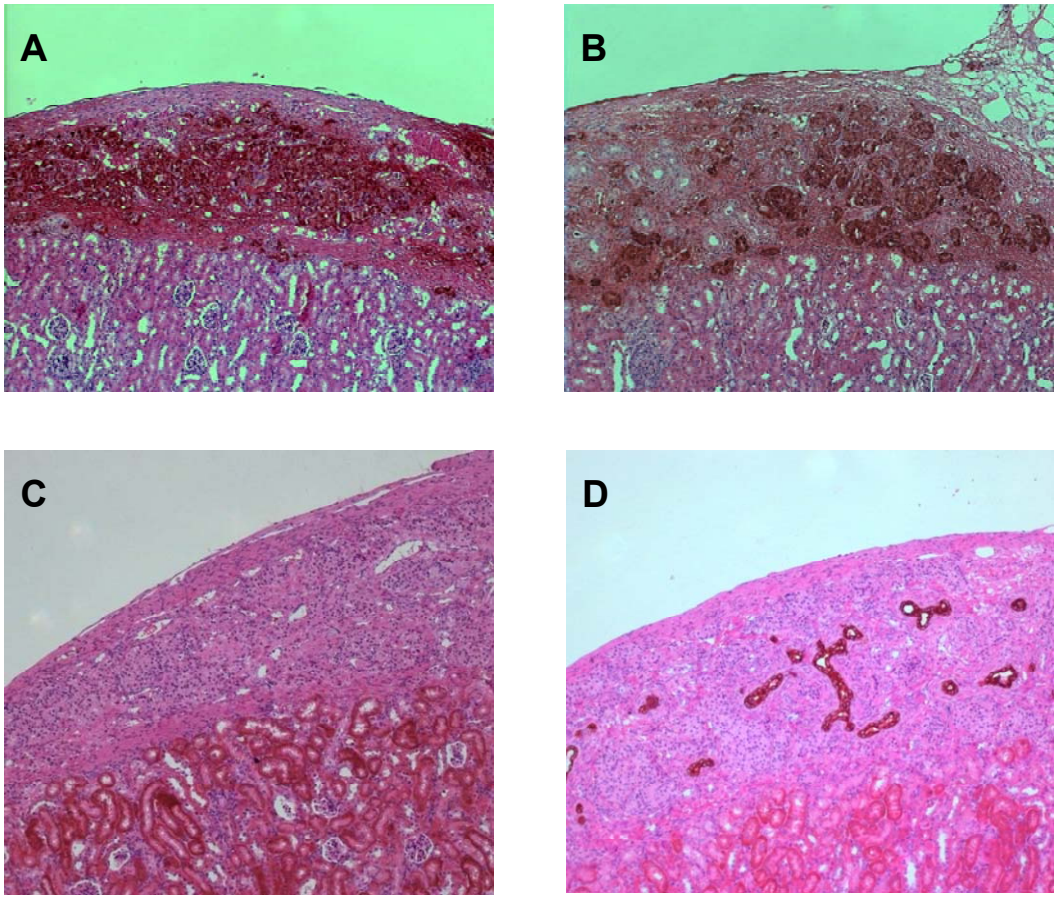
Insulin positive cells are red, cytokeratin 7 positive cells are green and cellular nuclei are blue (DAPI stain). A) Non-encapsulated NPI, B) Encapsulated NPI, C) Encapsulation of aggregated NPI + bMSC, D) Encapsulation of NPI with bMSC aggregates.

Appendix Figure 3: Co-transplantation of Neonatal Porcine Islets and Human MSCs Into the Renal Capsule Improves Islet Transplant Outcomes.



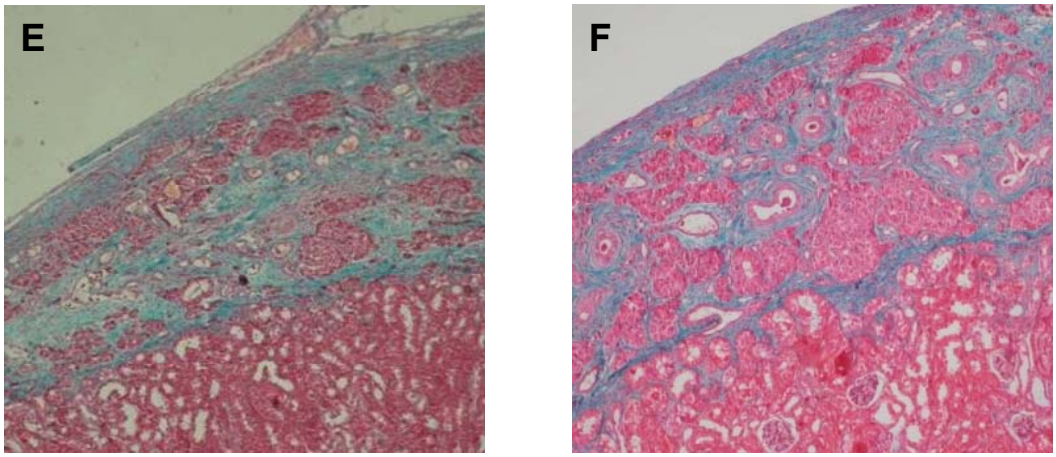
A) The average glycemia are reported as mean ± SEM (n=4-5). A survival nephrectomy was performed on week 15. B) The mice weights are reported as mean ± SEM (n=4-5). C) The oral glucose tolerance values are mean ± SEM (n=4-5).

Appendix Figure 4: Histological Evaluation of Neonatal Porcine Islet and Neonatal Porcine Islet + MSC Grafts Under the Kidney Capsule of Diabetic Mice.



Overall morphology was determined after staining grafts for insulin (A and B) and CK7 (C and D) and counter staining for hematoxylin and eosin. (A and C) NPI transplant graft; (B and D) NPI + MSC transplant graft. Insulin staining appears more organized with distinct aggregates of insulin positive cell clusters in the NPI + MSC co-transplant compared to the NPI alone. CK7 expression is absent in the NPI alone; however, in the NPI + MSC transplant graft, CK7 expression is maintained. Magnification is x100.

Appendix Figure 4: Histological Evaluation of Neonatal Porcine Islet and Neonatal Porcine Islet + MSC Grafts Under the Kidney Capsule of Diabetic Mice.



Sections were also stained for extracellular matrix using a Masson's trichrome stain (E and F). (A, C, and E) NPI transplant graft; (B, D and F) NPI + MSC transplant graft. Masson's trichrome staining demonstrates collagen deposition in blue-green. The collagen fibrils outline distinct clusters of cells in the NPI + MSC graft but thick collagen is present in the NPI alone. Magnification is x100.