Co-transplantation of mesenchymal stem cells with islet grafts

by

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Abstract

Mesenchymal stem cells (MSCs) have immuno-regulatory, anti-inflammatory, and pro-angiogenic properties through the secretion of a myriad of trophic factors, and have been shown to have a beneficial effect on graft function. In this thesis we investigated whether MSCs' have an effect on neonatal porcine islets (NPIs) in vitro and in vivo. For the in vitro studies, NPIs were cultured with or without MSCs for 48 hours and a glucose stimulated insulin secretion (GSIS) assay was administered. NPIs cultured with MSCs had higher cellular insulin content and improved GSIS. For in vivo studies, NPIs cultured with or without MSC were transplanted under the kidney capsule of streptozotocin-induced diabetic B6.129S7-Rag1^{tm1Mom}/J mice and glycemia and weight were measured weekly. An oral glucose tolerance test was administered when mice reached normoglycemia. Co-transplantation of NPIs and MSCs resulted in earlier reversal of diabetes, improved glucose tolerance, higher insulin content and improved vascularization. One experiment conducted with MSCs from a donor with an autoimmune disease resulted in no improved transplant outcomes. Co-transplantation of human MSCs with NPIs is demonstrated to have a beneficial effect on transplant outcomes, likely due to improved early vascularization and islet insulin secretion. Furthermore, donor pathology can impact MSC properties.

Preface

This thesis is an original work by Julie Hayward. Chapter 2 of this thesis has been accepted as J.A. Hayward, C.E. Ellis, K. Seeberger, T. Lee, B. Salama, A. Mulet-Sierra, P. Kuppan, A. Adesida, and G.S. Korbutt, "Co-transplantation of mesenchymal stem cells with neonatal porcine islets improve graft function in diabetic mice" in the journal *Diabetes*. I drafted the article, collected data, contributed to data analysis/interpretation; C.E. Ellis contributed to data analysis and critical revision of the article; K. Seeberger contributed to isolating NPI, data research, and critical revision of the article; T. Lee contributed to image analysis and cell composition quantification; B. Salama contributed to the isolation of islets and transplantation; A. Mulet-Sierra contributed to the data research; P. Kuppan contributed to the characterization of the islet grafts; A. Adesida contributed to the data research and concept/design; G.S. Korbutt was the supervisory author and contributed to concept/design, data analysis/interpretation, and critical revision of the article.

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CHAPTER 1: INTRODUCTION

1.1 DIABETES MELLITUS

Diabetes mellitus is a broad term defined by chronic high blood sugar levels. There are several types, including diabetes mellitus type I (T1DM), diabetes mellitus type II (T2DM), gestational diabetes and maturity onset diabetes of the young (MODY). Diabetes is characterized by polyuria (increased urination), polydipsia (increased thirst), polyphagia (increased hunger), caused by high blood glucose. According to the World Health Organization (WHO), diabetes is diagnosed by a fasting blood glucose of ≥ 7.0 mmol/L and blood glucose of ≥ 11.1 mmol/L two hours post oral glucose tolerance test (1).

The pathogenesis of T2DM is characterized by a progressive decline in insulin sensitivity, followed by decline in insulin secretion. T2DM, formerly known as adult onset diabetes, has been rising at an alarming rate in the pediatric community, with 8 – 45% of newly diagnosed pediatric diabetes cases being T2DM (2). T2DM and insulin resistance is strongly associated with obesity, although the majority of obese individuals do not develop T2DM (3), suggesting there is also a genetic component to the development of T2DM. In fact, twin concordance rates indicate that this is the case (4). Genome wide association studies have found a number of single nucleotide polymorphisms (SNPs) that contribute risk to an individual, including TCF7L2 which carries a 40% higher risk, among many others (4). It is clear that environmental factors, such as lifestyle, as well as a genetic predisposition contribute to disease development. Treatment plans include diet, exercise, and medications to increase insulin sensitivity.

Gestational diabetes is associated with exceeding recommendations for weight gain during pregnancy (5). It is defined by glucose intolerance which starts during pregnancy (6). Gestational diabetes is usually managed by diet and exercise, and usually resolves after birth.

Maturity onset diabetes of the young (MODY) is caused by a rare single gene mutation, often in HNF1A, HNF4A, PDX1, GCK or HNF1B (7). MODY consists of progressive β-cell dysfunction and hyperglycemia (7). Because of the increased incidence of T2DM in the pediatric population, MODY is often mistaken for T2DM because of similar clinical presentations (7). It requires a genetic screen for the mutated gene for diagnosis (7).

1.2 Diabetes Mellitus Type I

T1DM is caused by selective autoimmune destruction of β-cells, the insulin producing cells of the pancreas, resulting in absolute insulin deficiency. Beta-cells are organized within cell aggregates known as the islets of Langerhans. The islets of Langerhans consist of various endocrine cell types; β-, α-, δ-, and γ-cells which secrete insulin, glucagon, somatostatin, and pancreatic polypeptide, respectively. As the autoimmune destruction that occurs in T1DM is selective for β-cells, the disease only impacts insulin secretion.

T1DM accounts for 5-10% of individuals with diabetes (8). The untreated disease is characterized by polyuria, polydipsia, weight loss, polyphagia, and blurred vision (8). Chronic hyperglycemia can lead to susceptibility to infection and ketoacidosis (8). Even with treatment, long-term complications of diabetes include retinopathy, foot ulcers or

amputation, organ dysfunction and damage (8), diabetic neuropathy, renal failure (9), and cardiovascular disease (10).

The autoimmune destruction of β-cells is initiated by a combination of genetic predispositions and environmental factors (11, 12). The pathogenesis of diabetes is strongly associated with haplotypes HLA-DR3-DQ2 and HLA-DR4-DQ8, with 90% of individuals in Scandinavia with T1DM expressing these alleles (11). The appearance of islet autoantibodies, most commonly GAD-65, IA-2 or insulin, are strongly associated with HLA-DR-DQ haplotypes and strong predictors of development of T1DM (11). Genome-wide association studies have identified more than 50 loci associated with genetic risk of developing T1DM, with MHC HLA class II region on chromosome 6p21 conferring about 50% of the susceptibility risk (11).

A number of potential environmental triggers have been identified and studied in the pathogenesis of T1DM (12). According to the WHO, the incidence of T1DM is rising in middle and high income countries (13), and since migrants tend to acquire the risk of the new country (14), this strongly suggests an environmental role in pathogenesis. Major areas of study have been viral infection, intestinal microbiota, hygiene hypothesis, infant diet, toxins, birthweight and infant growth (12). A number of infant diet studies, including DAISY, BABYDIAB, ABIS, and DIPP have shown conflicting evidence with association between diabetes and the timing of exposure to gluten, root vegetables, and eggs (15-18). Viral infection is a promising line of research. A review of human studies suggests a higher presence of enteroviruses and T1DM, although more research is

required with larger sample sizes (19). Rewers et al postulate that T1DM may be a heterogeneous disease, explaining the contradictory evidence for environmental causes (12). It is clear the topic is complex, and disease onset likely results from an intricate combination of genetic and environmental factors.

According to an economic report by the Canadian Diabetes Association, the number of individuals living with diabetes is expected to nearly triple in Canada between 2000 and 2020, from 1.3 million to 3.7 million (20). The economic burden of diabetes in Canada due to both direct and indirect costs was $5.9 billion in 2000 and is expected to rise to $16.9 billion by 2020 (20). Furthermore, each patient incurs personal costs of $1000 to $15,000 per year (20), and over 57% of patients report being unable to adhere to treatment because of high costs (21), which further exacerbates indirect costs due to increased risk of long term complications. Consequently, this disease is not only an individual concern, but also a significant economic burden.

1.2.1 Insulin Therapy

Exogenous insulin therapy is currently the standard treatment for T1DM patients. Frederick Banting and Charles Best were the first to isolate insulin from the pancreas and reverse hyperglycemia in diabetic patients (22). Banting was awarded the Nobel Prize in Physiology or Medicine for this discovery in 1923. Insulin affords the opportunity for diabetic patients to live relatively normal lives and maintain glycemic control. The typical regimen for T1DM patients is to combine short- and long-acting insulin analogues to mimic as closely as possible normal insulin release of the pancreas by using bolus insulin at mealtimes and

basal insulin between meals. The Diabetes Control and Complications Trial (DCCT) revealed that it is optimal to maintain blood glucose between 3.9 and 6.7 mmol/L, and not exceeding 10.0 mmol/L after meals (23). This intensive glucose control reduced the risk of complications like microvascular complications by 76%, neuropathy by 60%, and renal disease by 50% compared to standard therapy (23). However, intensive therapy comes with a higher risk of hypoglycemic events (23).

Insulin is not an entirely ideal therapy, and it requires daily injections and strict blood glucose monitoring. Because exogenous insulin does not perfectly mimic normal pancreatic function, there is poor overall glycemic control and the risk of long-term complications remains. Insulin therapy comes with a risk of hypoglycemic episodes (23), which can often come without warning symptoms, especially for the hypoglycemic unaware (24). Short term symptoms include tremor, anxiety, cold sweats, confusion, feeling faint, and blurred vision (24). Repeated or severe hypoglycemic episodes can be destructive to the brain (24). Neurological effects include amnesia, stroke, convulsions, and cortical atrophy (24). Additionally, hypoglycemic episodes have the potential to result in coma and sudden death (24).

There have been some recent advancements in insulin therapy beyond the traditional insulin injections. Insulin pumps, which have evolved from large heavy machines in the 1970s to a pump that fits in a pocket today, deliver continuous basal rapid acting insulin with programmed insulin boluses at mealtimes (25). Some models go a step further and have continuous glucose monitor (CGM) technology, such as the Medtronic iPro2 (25).

These glucose monitors measure interstitial blood glucose every 3-5 minutes, providing a continuous up to date glucose reading and a more accurate report of glycemia over time (25). These CGMs can be paired with an insulin pump, or be on their own. Insulin pumps are associated with a greater reduction in HbA1C, lower rates of retinopathy and peripheral nerve issues (26), likely because of improved glycemic control due to the use of only rapid acting insulin (25). There has also been some success with the "bionic pancreas", which involves CGM and two separate pumps delivering insulin and glucagon (25). It was found that use of the bionic pancreas decreased average blood glucose levels and decreased incidence of hypoglycemia (27), although further development of this technology is required (25).

1.2.2 Beta-Cell Replacement

β-cell replacement is theoretically superior to traditional insulin therapy, since it eliminates the need for exogenous insulin and restores normoglycemia. Because the body produces insulin endogenously with β-cell replacement, it is able to act in a physiological manner and respond to blood glucose directly. It improves variability of blood glucose and reduces risk of hypoglycemia. β-cell replacement has shown to improve cardiovascular (28) and renal function (29), and to decrease risk of cardiovascular death and overall mortality (30). Furthermore, insulin independence improves the patient's quality of life as the individual no longer requires daily injections, constant blood glucose monitoring, and removes the fear of hypoglycemia.

The two current methods of β-cell replacement are whole pancreas and islet transplantation.

1.2.3 Whole Pancreas Transplantation

Pancreas transplantation is a method of β-cell replacement accomplished by transplanting a whole pancreas into a patient. One-year graft survival rates are 74%, which drops to 51% at five years' post-transplant (31).

A major advantage of whole organ transplant is that the tissue is immediately vascularized (32) since the donor vascular structure of the organ remain intact. Pancreas transplantation, however, involves major surgery and is associated with a high surgical morbidity, including pancreatic thrombosis, pancreatic leak, hemorrhage, abscess, hernia, and major infection (33).

1.2.4 Islet Transplantation

Islet transplantation is an alternative method to β-cell replacement. This procedure is minimally invasive, does not require surgery or general anesthesia (34), and is associated with a low morbidity (32). Complications from islet transplantation are relatively minor, including portal vein thrombosis, which can be resolved with anticoagulants (35).

Paul Lacy, an anatomist and researcher, and Walter Ballinger have often been credited as the founders of islet transplantation, due to their successful work with rat islet transplantation in diabetic streptozotocin induced diabetic rats in 1973 (36). Although the

rats did not reach normoglycemia, they were able to reduce hyperglycemia and resolve diabetic symptoms such as polyuria (36).

Between 1990 and 2000, only 8.2% of islet grafts were still functional at one-year post-transplant (37). In 2000, the landmark Edmonton Protocol reported insulin independence of 7/7 patients at one-year post-transplant (37). The Edmonton Protocol used an advanced technique of islet isolation using collagenase digestion of the pancreas and a glucocorticoid free immunosuppressive regimen (37). The pancreas is enzymatically dissociated and the islet-rich endocrine tissue, which is only 1-2% of the pancreas, is isolated from the exocrine tissue (34). The tissue is separated into top and bottom layers via gradient purification, resulting in the top layers containing significantly more islets than bottom layers (38). The resulting small volume of islets is injected through the hepatic portal vein (37). Shapiro and colleagues were able to resolve hypoglycemic episodes with a suboptimal dosage of islets using the Edmonton Protocol, and completely resolve hyperglycemia and maintain insulin independence in 100% of patients in their breakthrough study (37).

The initial expectation was not realized, however, and a five-year follow up study revealed only 7.5% of patients were able to sustain insulin independence (39). There was at least partial graft survival in 82% of patients who tested positive for C-peptide (39). Furthermore, Ryan and colleagues were unable to see a pattern to predict graft function as transplant outcomes were independent of factors such as donor age, sex, or weight, or number of islets transplanted (39). Progressive loss of graft function, evidenced by increasing HbA_{1C} scores, indicates loss of islets over time (39). According to Ryan and

colleagues, perfect β-cell function is very rarely achieved after transplant, suggesting there may be an early loss of islets (40). There is significant loss of islets in the immediate post-transplant period due to inflammatory events (41), apoptosis (42), and hypoxia (43). Mouse models using syngeneic islet transplants have found up to 60% of the islet graft undergoes apoptosis, of which 30% occurs in the first 3 days after transplantation (42). Activated coagulation factors and insulin release immediately post-transplant is indicative of a non-specific inflammatory response and islet damage following transplantation, peaking at 15 and 30 minutes respectively (41).

Another factor contributing to early islet loss may be inadequate blood supply in the immediate post-transplant period (44). During islet isolation the inter-islet vascular connections are broken, and the islets are avascular during transplantation (44). It takes 2-4 days for the angiogenic process to begin, and completes in about 14 days (45, 46). Mouse models have shown there is markedly decreased oxygen tension in the islet graft up to 9 months after transplantation (47).

There are many complications associated with immunosuppressive therapy such as infection, hypertension, hyperlipidemia, impaired renal function, and a 3-4 fold risk of cancer (48).

1.3 Islet Function and Survival

1.3.1 Normal Islet Function in Non-Diabetics

The average pancreas has between 300,000 and 1.5 million islets, and only about 60% of the islet mass is necessary to maintain glucose control (49). The pancreas is composed of highly heterogeneous tissue; aside from pancreatic islets, there is acinar, ductal, vascular, and nerve cells (50). This environment is likely extremely important for the proliferation, differentiation, and regeneration of islets (50).

Islets are composed of β-cells, α-cells, δ-cells, and γ-cells which secrete insulin, glucagon, somatostatin, and pancreatic polypeptide respectively. In rodents, islets are organized with non β-cells surrounding a β-cell core (50). Human islets are less structured, with β-cells, α-cells, δ-cells, and γ-cells dispersed throughout (50). Islet structure and morphology is very important for normal function (51). It has been noted that dissociation of islets into single cells severely impacts GSIS (51). This implies that cell-to-cell communication and the organized structure within the islet is essential for proper islet function and insulin secretion (51).

The pancreatic islets are an extremely well vascularized tissue (44). They comprise only 1-2% of the pancreatic mass, yet they receive 15-20% of blood flow to the pancreas (44). There is a greater density of blood vessels within the islets compared to the pancreatic exocrine tissue, with features that provide greater partial pressure of oxygen within islets (44). Islet vasculature is formed during embryonic development by VEGF signaling (52).

This process is involved in pancreatic differentiation and influences morphology (44). Blood flow to endocrine cells occurs before islet formation (44).

1.3.2 Revascularization

During the isolation process, the highly specialized islet vascular connections are severed (44). Immediately after transplantation, the islet graft is avascular and it takes several days to several weeks to re-establish blood flow through construction of new capillaries (45, 46), and there is decreased oxygen tension for up to 9 months post-transplantation (47). As a result, the graft is receiving much less than it's normal oxygen requirements. Furthermore, the graft is only supplied with portal venous blood, which contains only 85% oxygen saturation, whereas arterial blood contains 96% oxygen saturation (53). This has deleterious effects on the islets, and inadequate blood supply in the post-transplant period is likely a major contributor to early islet death (44).

In new islet grafts, the endothelial cells make up the lining of new capillaries and blood vessels are recruited from several sources (44). Up to 40% are intra-islet endothelial cells from the donor (44). A smaller portion is derived from the recipient's bone marrow (44). As a result the vasculature structure in the transplanted graft is chimeric, consisting of both donor and recipient endothelial cells (44). VEGF and other angiogenic factors are highly involved in this revascularization process (44).

1.3.3 Inflammatory Response

In the immediate post-transplant period, islets come into direct contact with the recipient's blood (54). The transplanted islets come into contact with platelets and complement proteins, which triggers coagulation cascades and inflammatory events (54). Bennet et al dubbed this process "IBMIR", or "instant blood mediated inflammatory reaction" (54). They first characterized this process in 2000 by observing islets come into contact with ABO-compatible human blood in a closed loop system (54). They observed visible blood clotting within 5 minutes and complement activation, indicated by C5b-9 complexes (54). Additionally, IBMIR causes islet damage evidence by major insulin dumping within 5 minutes after being exposed to whole blood (54). Addition of heparin prevented islet morphology damage, which suggests islets are damaged by platelet activation and coagulation cascades (54). Naziruddin and colleagues measured activated complement proteins and pro-inflammatory cytokines in patients receiving islet transplantations (55). They found elevated C5b-9, IL-6 and IL-8 shortly following transplantation, providing evidence for coagulation and inflammation responses in the patient (55). Cytokines have been shown to be mediators of β-cell damage and pancreatic injury (56). Pro-inflammatory cytokines have an adverse effect on β-cells and can be cytotoxic (57). Co-culture of islets and IL-1β, with or without TNF-α and IFN-γ, results in suppression and loss of β-cells (56). Cytokines cultured with islets have a higher percentage of cells undergoing apoptosis, as assessed by co-expression of insulin and TUNEL (57). Inhibition of cytokines or macrophages improves islet function after transplant, indicating these cells are involved in

transplanted pancreatic injury (53). Exposure of rodent or human islets to IL-β, TNF-α and IFN-γ, for several days has been shown to cause β-cell dysfunction, indicated by a decrease in insulin secretion – glucose oxidation, as well as diminished glucose responsiveness (56, 58-60). It is clear that IBMIR is a major contributor to the loss of islets following transplantation.

1.4 Neonatal porcine islets

Neonatal porcine islets (NPIs) are a viable alternative to cadaveric donor islets (61, 62). Since the number of diabetic candidates far exceed the supply of human donor tissue, it is necessary to look for alternative sources of islets. Pigs are an ideal source of tissue because they breed quickly and have sizable litters (62). Furthermore, porcine insulin was traditionally used for insulin therapy for T1DM patients until synthetic insulins were developed. It is structurally almost identical to human insulin, and differs only in one amino acid (alanine and threonine, in pigs and humans respectively). NPIs in particular have distinct advantages over adult porcine islets. Namely, housing costs are reduced (63) and they are hardier in culture (64-66), unlike adult pig islets which are quite fragile (67). Fetal porcine islets also have the advantage of reduced housing costs, but these islets have poor glucose responsiveness (68) and take 2-3 months in vivo to reverse hyperglycemia (69).

Embryonic stem cells (ESCs) may be another alternative to creating an unlimited supply of donor islets. Rezania and colleagues had success in reversing diabetes in mice with their stage 7 β-like cells, although these cells were not identical to β-cells and had a delayed

response to glucose during perifusion (70). ESCs will require further study on the appropriate transcription factors required in vitro to be able to create β-cells that are identical to native β-cells.

NPIs are naturally resistant to hypoxia (64), pro-inflammatory cytokines (65), streptozotocin (65), and hyperglycemia (66). Emamauelle and colleagues cultured human, mouse and neonatal porcine islets in hypoxic conditions and measured viability and function (64). They determined that NPIs are able to resist apoptosis under hypoxic conditions, unlike human and mouse islets (64). NPIs had impaired glucose responsiveness during hypoxic periods, but recovered fully during reoxygenation, indicating NPIs are very resistant to hypoxia (64).

NPIs are also resistant to hyperglycemia (66). In fact, when NPIs are transplanted into diabetic mice along with a curative mouse islet transplant on the opposing kidney, they fare significantly worse than mice receiving NPI transplants only (66). So not only are NPIs resistant to hyperglycemia, it actually helps them develop a sufficient β-cell mass more quickly (66).

1.5 Mesenchymal Stem Cells

Mesenchymal stem cells (MSCs) are multipotent, adult progenitor cells that have the capacity to differentiate into osteocytes (bone), chondrocytes (cartilage), and adipocytes (fat). Once thought to be only present in the bone marrow, they can be isolated from a

variety of tissues such as Wharton's Jelly, the pancreas, adipose tissue, umbilical cord blood, and likely every adult tissue (71, 72).

According to Clinicaltrial.gov, MSCs are being studied to treat a multitude of diseases, including ulcerative colitis, graft vs host disease (GvHD), muscular dystrophy, multiple sclerosis and T1DM. Treatment for GvHD using mesenchymal stem cells has been promising, as a number of clinical trials has showed the benefit (73). Particularly, in one trial Ringden et al were able to completely resolve GvHD symptoms in 6/8 patients (74). Carlsson and colleagues, in their clinical trial, were able to preserve C-peptide secretion in newly diagnosed T1DM patients compared to controls (75). Another trial reported that administration of allogenic umbilical- and autologous bone-marrow derived MSCs through the pancreatic artery of T1DM patients had lower HbA1C and fasting blood glucose compared to controls (76). Furthermore, they required less insulin after one year (76). Importantly, these trials showed no adverse effects of MSC administration (74-76).

1.5.1 Characterization

The International Society for Cellular Therapy (ISCT) defines that MSCs must meet the following criteria: adherence to plastic, ability to difference into osteocytes, chondrocytes, and adipocytes, and expression of the appropriate markers (77). MSCs must express CD105, CD73 and CD90 (≥ 95%), but not express hematopoietic markers CD45 and CD34 (≤ 2%) (77). They exhibit a spindle-like morphology, similar to fibroblasts, and exist at a frequency of 0.01-0.001% in the bone marrow (78).

MSC isolation from bone marrow depends on the property of adherence to plastic (79). Mononucleated cells are isolated by centrifugation from bone marrow aspirates, plated, and after the first or second passage the hematopoetic cells are washed away, as they are non-adherent (79).

1.5.2 Immunoregulatory Properties

MSCs display immunosuppressive, immunomodulatory, and angiogenic effects. They secrete a multitude of trophic factors that mediate these properties.

MSCs inhibit T cell and B cell proliferation when co-cultured for 3 days (80). Interestingly, MSCs do not favor any T cell type, as they inhibit CD4+ and CD8+ cells with equal suppression (80). Glennie and colleagues investigated the method of arresting T cell proliferation and found that they inhibit T cell division, and this effect is reversible with MSC removal (80). They can significantly impair proliferation of allogenic lymphocytes *in vitro*, and can even delay, although not prevent entirely, rejection of skin grafts when injected intravenously (56). Additionally, T cell suppression is dose dependent, confirming that it is MSC specific effects that cause T cell suppression (81). MSCs also induce production of T regulatory cells, as there is an increased population of FoxP3+ cells when PBMCs are cultured together with MSCs (82).

MSC and B cell co-culture results in suppression of LPS-stimulated B cell differentiation into plasma cells (83). Furthermore, Asari and colleagues found that cell-cell contact is not necessary for this suppression of B cell differentiation, as MSCs and B cells separated by

transwells was sufficient to suppress B cell proliferation (83), but MSC supernatant did not exert any effect (84). This suggests there may be crosstalk between B cells and MSCs, and B cell signals may cause MSCs to secrete trophic factors. Additionally, MSCs downregulate production of immunoglobulins such as IgM (83), and upregulate production of IgM (85). They also promote generation of IL-10 producing B regulatory cells (85).

MSCs have been shown to impair the activity and maturation of dendritic cells (86). In particular, MSCs cultured with dendritic cells results in a higher proportion of dendritic cells expressing CD83, indicating an immature phenotype (86). Similar to B cells, dendritic cell suppression was not cell contact dependent, as separation in transwell chambers produced similar effects (86). Furthermore, MSC cultured with already matured dendritic cells resulted in reduced secretion of IL-12 and IFN- γ, and upregulated secretion of IL-10, indicating a shift toward a more regulatory phenotype (86).

Macrophages co-cultured with MSCs are induced to switch from expressing pro-inflammatory M1 to anti-inflammatory M2 markers (87). M1 macrophages are involved in early phases of tissue damage and promote inflammation, whereas, M2 macrophages are involved in later stages when tissue repair has begun.

1.5.3 Secreted Factors

MSCs secrete hepatocyte growth factor (HGF), interleukin-10 (IL-10), transforming growth factor-β (TGF-β), interleukin-6 (IL-6), matrix metalloproteinase 2 (MMP2), vascular endothelial growth factor (VEGF), annexin A1, and likely others (88, 89).

Annexin A1 may be largely responsible for the improvement in glucose stimulated insulin secretion (GSIS) when MSCs and islets are co-cultured, as GSIS improvement disappears when an Annexin A1 siRNA is used in co-cultures (89). Furthermore, GSIS improvement can be replicated by addition of Annexin A1 to islet cultures instead of MSCs (89).

MSCs induce dendritic cells to produce more IL-10, as well as secrete IL-10 themselves (86). IL-10 contributes to T cell suppression, as IL-10 knockdown abolishes most of this effect in MSC cultures (90).

HGF and TGF-β plays a role in suppression of T cell proliferation, as blocking HGF and TGF-β activity restores proliferation (91).

1.5.4 Factors affecting potency

MSC proliferation, trophic factor secretion, and viability can vary greatly across preparations, and may be determined by donor characteristics. Age of the donor can influence MSC properties, in particular. Choudhery and colleagues compared MSCs isolated from young (2-3 months) and aged (23-24 months) mice and found that MSCs from aged mice had significantly decreased proliferation, VEGF secretion, higher apoptosis, and impaired wound healing ability (92). A comparison of human MSCs from young (1-5 years) vs. old (50-70 years) patients showed a decrease in colony forming unit (CFU) ability and impaired MMP-9 and MMP-2 expression and activity (93). There may also be sex differences between MSC characteristics. Male donors have been shown to have better chondrogenic differentiation (94, 95). Interestingly, chondrogenic differentiation declines

with age in males but not females (96). LPS activated female MSCs produce less pro-inflammatory cytokines and more VEGF compared with male donors, which may explain female's protective advantage during cardiac injury compared to males (95).

There is emerging evidence that besides age and sex, disease status can affect the potency of MSCs. Autoimmune diseases in particular have been associated with altered morphology (56), decreased proliferative capacity (97-99), altered gene expression (100), reduced expression of trophic factors (98, 99), and reduced inhibitory effects on T cells (97, 100) Additionally, Cramer et al found that MSCs from diabetic donors have a decreased proliferative capacity compared with non-diabetic donors (101). Therefore MSCs from persons with diabetes may have impaired function due to the person's autoimmune status and diabetic state.

1.5.5 Co-culture with Islets

Islet culture with MSCs is associated with prevention of β-cell death and improvement of islet function (88). Co-cultured islets exhibit a higher ADP/ATP ratio, which indicates higher metabolic activity and correlated with higher insulin secretion, better GSIS and reduced islet death (88). Furthermore, these effects were also seen with MSC conditioned media, indicating trophic factors in the media are responsible for these effects (88). Rackman et al in fact found that Annexin-A1 knockdown abolishes the improvement in GSIS, indicating Annexin A1 is responsible for this effect (89).

When islets are cultured with pro-inflammatory cytokines, it results in β-cell death and impairment of GSIS (57). Addition of MSCs to these cultures rescues the islets; β-cell death is prevented and GSIS is restored (57). HGF is likely at least partially responsible for the protection against pro-inflammatory cytokines as addition of HGF to the cultures without MSCs was able to rescue GSIS to normal, non-cytokine treated levels (57). These effects were not observed with fibroblasts, a cell type with similar morphological characteristics and also adhere to plastic (57). This suggests the cytoprotective effects are specific to MSCs.

The question of whether cell-to-cell contact between islets and MSCs is necessary in order to have beneficiary effects on islets is currently unclear. Studies thus far have presented conflicting evidence. As described above, even with physical separation of MSCs by transwells, we still observe an inhibition of B cell proliferation (83) and dendritic cell suppression (86). Culture of islets in MSC conditioned media is able to improve GSIS and improve islet survival (88), suggesting trophic factors are responsible for these effects. Sato et al. used transwells to show that separation reduces, but not erases, T cell suppression (103). This suggest there may be more than one mechanism at play influencing T cell activity by MSCs.

1.5.6 Co-transplantation with Islets

With MSCs immunosuppressive and immunomodulatory properties, using them to combat the clinical problems with islets transplantation seems intuitive. There has been a number of experiments using animal models looking at whether co-transplantation of

islets and MSCs have any beneficial effects on the efficacy of transplant and achievement of normoglycemia in vivo.

Bartholomew et al observed a prolongation of skin graft survival with IV administration of baboon MSCs, which was comparable to the survival time of recipients taking immunosuppressive drugs (56). Measurement of lymphocyte activity in the recipient baboons showed significantly reduced proliferative activity in vivo (56). This is a significant implication that islet transplantation recipients could potentially take a reduced amount of immunosuppressive drugs, which would greatly enhance quality of life.

MSC co-transplantation may result in improved graft function. Several studies have found that MSC co-transplantation improves glucose tolerance in rodent models when receiving rodent islets and MSCs (88, 89, 104). Additionally, Park et al found higher total insulin content of the transplanted grafts (88). Borg and colleagues showed in their syngeneic mouse model that co-transplantation improves beta cell survival by significantly less caspase 3 at 14 days (105). Increased insulin content and beta cell survival may be a contributor to the improvement in glucose tolerance.

Co-transplantation studies have been shown to reduce the number of islets necessary to achieve normoglycemia (103-106). Figliuzzi et al, using rat islets and MSCs transplanted in rat recipients, 100% were able to reach normoglycemia with just 2000 islets when MSCs were present, while the islets alone group all remained hyperglycemic (106). 3000 islets were necessary to reverse hyperglycemia without MSCs (106). Ito et al found similar

results transplanting rat islets and MSCs into NOD-SCID mice. Transplantation of 500 islets resulted in 8/10 and 3/10 diabetes reversal with and without MSCs, respectively (104). Diabetes reversal with a smaller islet mass is very clinically relevant, as two donor pancreases are necessary for transplantation.

Co-transplantation has been shown to help islets retain normal structure during transplantation. Rackman et al demonstrated that co-transplantation of MSCs and islets in rodent models helped islets retain a normal islet structure (103). Immunohistochemistry for insulin showed that islets transplanted alone were less organized and more amorphous in structure (103). In fact, the total average aggregate size in the grafts were significantly higher in the islets alone group, indicating they merged together to form a large islet mass, whereas regular islet morphology was maintained when MSCs were co-transplanted (103). This is highly relevant since revascularization of smaller aggregates is more efficient (103). Furthermore, maintenance of islet morphology is important for normal function and insulin secretion, since it is highly dependent on interactions between β-, α-, and δ-cells (51). The maintenance of islet morphology likely contributes to improved outcomes of these groups (103).

Several studies have found that co-transplantation improves revascularization of transplanted grafts. MSCs secrete VEGF, which is known to promote vascularization and angiogenesis (88). Mice deficient in both VEGF alleles do not survive embryogenesis due to irregular blood vessels with few endothelial cells (107), and conversely overexpression of VEGF during development results in embryonic death due to excessive vascularization

(108). Co-transplantation has been shown to significantly increase the number of capillaries formed within islet grafts compared to islets alone (88, 104, 106, 109), although one study found no differences in CD31 (105).

Rackman and colleagues showed that improvement of islet function may be due to trophic factor secretion (89). While addition of MSCs to islet cultures improves GSIS; this effect can be replicated with the addition of Annexin-A1 only (89), indicating improvement in glucose tolerance may be largely due to Annexin-A1 (89). Furthermore, siRNA Annexin-A1 knockdown abolishes GSIS improvement (89).

1.6 Hypothesis, Objectives and outline of thesis

My hypothesis is that co-transplantation of mesenchymal stem cells with neonatal porcine islet grafts will improve the therapeutic efficacy of islet transplantation. While co-transplantation models of islets and MSCs have been investigated, and found to be beneficial, in previous studies, thus far these studies have been conducted in rodent models using rodent islets and MSCs. So far, no study has looked at the effect of transplanting NPIs with human MSCs, which is clinically relevant.

In this study, we wanted to assess the effect of transplanting NPIs and human MSCs in a streptozotocin induced diabetic B6.129S7-Rag1^{tm1Mom}/J mouse model. Our objectives were and to examine the metabolic differences in mice transplanted with and without MSCs, and explore the reasons for the differences.

NPIs were cultured with or without MSCs for 48 hours and transplanted into streptozotocin induced diabetic B6.129S7-Rag1^{tm1Mom}/J mice. Blood glucose and weight were monitored weekly until all mice reached normoglycemia, then mice were given an oral glucose tolerance test. Islet grafts were assessed for insulin, vascularization, and total cellular insulin content. To examine graft differences earlier in the post-transplant period, grafts were taken at 2, 3, and 4 weeks to assess histological differences in insulin and CD31.

1.7 References

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CHAPTER 2: COTRANSPLANTATION OF MESENCHYMAL STEM CELLS WITH NEONATAL PORCINE ISLETS IMPROVE GRAFT FUNCTION IN DIABETIC MICE[1]

2.1 INTRODUCTION

Despite the initial optimism following the successes of the Edmonton Protocol in 2000 (1), there still exist many hurdles preventing islet transplantation from replacing insulin as the gold standard treatment for patients with diabetes. However, despite early insulin independence, long-term graft attrition gradually revert recipients to exogenous insulin dependency (2). Loss of islet graft function is partly due to a significant loss of β-cell mass in the first hours to days after infusion, mediated by a non-specific inflammatory response characterized by pro-inflammatory cytokines (3). Cytokines are damaging to islet structure and function; co-culture of islets and cytokines results in β-cell death and impairment of glucose stimulated insulin secretion (GSIS) (4). A second contributing factor to islet death is the hypoxic conditions immediately post-transplant. Pancreatic islets have a dense native capillary network, and β-cells receive 10-15 times more blood flow than the surrounding exocrine tissue (5), approximately 15-20% of the pancreatic blood supply despite comprising only 1-2% of the pancreas volume (6). During isolation and culture this vasculature is destroyed, leaving the islets avascular prior to transplantation (5). Moreover, it takes several days to re-establish islet vascularization thereby exposing islets to hypoxia (5).

[1] A version of this chapter has been published in the journal *Diabetes*

The challenges of hypoxic and inflammatory-mediated islet destruction could be ameliorated by co-transplantation with mesenchymal stem cells (MSCs). MSCs are multipotent, progenitor cells that can be isolated from a variety of tissues, including bone marrow, adipose tissue, Wharton's jelly, umbilical cord blood, pancreas, and likely every adult tissue (7, 8). MSCs have been reported to have anti-inflammatory, pro-angiogenic and immunoregulatory effects (9, 10) through the secretion of trophic factors such as hepatocyte growth factor, transforming growth factor-β, interleukin-6, vascular endothelial growth factor (VEGF) (11) and annexin A1 (12). They may be capable of controlling inflammation within and surrounding the islet graft in the post transplantation period, as well as stimulating rapid graft vascularization. MSCs have already been demonstrated to modulate the diabetic milieu in humans. Carlsson et al. (13) reported preserved C-peptide secretion in patients with newly diagnosed T1DM given intravenous autologous bone marrow-derived MSCs. A second trial reported that patients with T1DM given a combination of allogenic umbilical- and autologous bone marrow-derived MSCs through the pancreatic artery had lower HbA_{1C} and fasting glycemia compared to patients that did not receive MSCs, and required less exogenous insulin one year after MSC delivery (14). Importantly, no severe adverse events were reports in either trial (13, 14).

An additional challenge to islet transplantation becoming a widespread therapy is that cadaveric donor islets are in limited supply and human islet quality can range greatly between donors. As previously reported, neonatal porcine islets (NPIs) may be a safe and effective alternative to human donor islets (15, 16). These islets are in virtually unlimited supply, easy to isolate, can proliferate after transplantation, and are resistant to hypoxia

(17), pro-inflammatory cytokines (18), and hyperglycemia (19). While the effect of both rodent MSCs and islets have been examined in mouse transplant models (20-25), further studies are needed to examine the effects of clinically relevant human MSCs and β-cell sources such as NPIs on islet engraftment and functional outcome.

It is known that the MSC human donor characteristics can have an effect on the therapeutic potential of MSCs (26). For example, MSCs from older donors have altered morphology and reduced proliferative ability, secretion of trophic factors, angiogenic potential, viability, and wound healing ability compared to younger human donors (27-30). There are also sex related differences in trophic factor secretion (31). There is little data on the effects of disease status of donors on the characteristics of MSCs, but some studies indicate donors with autoimmune diseases have abnormal MSCs (26). Donors with certain autoimmune diseases have been shown to have MSCs with altered morphology (32), decreased proliferative capacity (32-34), altered gene expression (35), decreased expression of trophic factors (35, 36), and reduced inhibitory effect on T cells (32, 35). With the promise of novel therapies for autoimmune diseases such as T1DM using MSCs, it is critical to learn more about the effect of using autologous MSCs in the treatment of autoimmune diseases.

In this clinically relevant study we investigate the function of transplanted NPIs in the presence of human MSCs. We report that the co-transplantation of MSCs results in faster normalization, improved glucose tolerance, and improved early angiogenesis in our diabetic mouse model. We also report that MSCs from a donor with an autoimmune disease produced dramatically different outcomes when co-transplanted with NPIs.

2.2 Materials and Methods

2.2.1 NPI isolation and preparation of human MSCs

Porcine pancreases were obtained from 1 to 3 day Duroc neonatal piglets from the University of Alberta Swine Research Centre (1.5-2.0 kg body weight), and NPIs were isolated and cultured for 5-7 days as described previously (16). A total of 6 independent NPI isolations were used for the transplant studies.

To prepare bone marrow derived MSCs, human bone marrow was extracted from seven patients during orthopedic surgery (Division of Orthopedic Surgery, University of Alberta) following informed consent. For expansion, cells were plated in Modified Essential Medium alpha (MEMα; Cellgro) supplemented with 2.5 ng/mL basic fibroblast growth factor (bFGF, Millipore), 10% FBS (Gibco), 1 mM sodium pyruvate (Gibco), 10 mM HEPES (Gibco), 100 U penicillin/1000 U streptomycin (Biowhitaker) at a density of 166,000 cell 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). Cells were counted and re-seeded into supplemented MEMα culture medium at a density of 5,000-10,000 cells/cm^2 and underwent 5 passages prior to transplantation. As we previously reported (37), MSCs isolated using this protocol express by FACS analysis the classic MSC surface antigens CD29, CD44, CD73, CD90, and CD105.

2.2.2 Graft preparation and pre-transplant graft characterization

Bone marrow MSCs were enzymatically detached from culture plates, counted, and 2 x 10^6 cells were added to a 100 mm low adherence culture dish (Corning) with 6,000 NPIs in a total volume of 10 mL culture media. Controls included 6,000 islets cultured alone. Cells were culture for 48 hours in DMEM low glucose (5.6 mM glucose; Gibco) with 1% FBS, 20 ng/mL EGF, 20 ng/mL bFGF, 10 mM HEPES, 100 U/mL penicillin, 0.1 mg/mL streptomycin, and 71.5 μM β-mercaptoethanol.

To determine whether MSC co-culture had an effect on the cellular composition or ß-cell proliferation of the NPI grafts, immunostaining was performed using previously published methods (15, 16). Primary antibodies included; insulin (1:1000, DAKO), glucagon (1:5000, Sigma-Aldrich) proliferating cell nuclear antigen (PCNA,1:300, DAKO) and appropriate species specific secondary antibodies Alexa Fluor 488 or 594 (1:200, Molecular Probes, Eugene). Insulin, glucagon, and double insulin and PCNA stained cells were quantified using Image J; http://rsbweb.nih.gov/ij/). Prior to transplant, total cellular insulin content of the grafts was determined and a static incubation assay was used to assess GSIS (previously published methods; (15, 16). RNA was also extracted from grafts (RNeasy Mini Kit: QIAGEN). cDNA was synthesized using the High-Capacity Reverse Transcription Kit (Thermofisher) and Relative Quantification was performed using TaqMan Gene Expression Assays (Thermofisher) utilizing SDS software on the ABI Prism 7900HT. Validated primer sets were: PDX1 (Ss03373351_m1), insulin (Ss03386682_u1), glucagon (Ss03384069_u1), pancreatic polypeptide (Ss03375477_m1),

somatostatin (Ss03391856_m1), and glyceraldehyde-3-phosphate dehydrogenase (Ss03375629_u1) a housekeeping gene. Analysis was by Relative Quantification (RQ) software (ABI7900HT) utilizing the delta-delta Ct method and plotted as RQ. Controls were; no template RT control, no template qPCR control and human MSC cDNA to verify probe specificity to porcine cDNA.

2.2.3 Transplantation and follow-up

Male, inbred, B6.129S7-Rag1^{tm1Mom}/J mice (Jackson Laboratories) were used as recipients. Animals were maintained under virus-antibody-free conditions in climatized rooms with free access to sterile tap water and pelleted food. Mice were rendered diabetic by intravenous injection of 185 mg/kg streptozotocin (freshly dissolved in acetate buffer; Sigma) 2-4 days before transplantation. Blood samples were obtained from the tail vein for glucose measurement (OneTouch UltraMini glucose meter). Grafts consisting of 3000 NPI alone or 3000 NPI + 10^6 MSCs were transplanted under the left kidney capsule Rag mice (16). Grafts were aspirated into polyethylene tubing (PE-90) and pelleted by centrifugation then gently placed under the kidney capsule with the aid of a micromanipulator syringe. To examine whether NPI-MSC cell-to-cell contact is essential, in 2 separate cohorts of transplants, mice were implanted under the kidney capsule with 3000 NPI and immediately after implantation mice were injected intravenously (IV) via the tail vein with 10^6 MSCs obtained from 2 independent bone marrow donors. Controls included mice transplanted with 3000 NPI under the kidney capsule but with no IV MSC injection.

All mice were monitored for non-fasting blood glucose levels at 3 days post-transplant, and once a week thereafter. When the blood glucose level was ≤11.1 mM for two consecutive weeks, mice were deemed normalized. After normalization, an oral glucose tolerance test (OGTT) was performed on transplanted mice (16). After a 12 hour fast, D-glucose (3 mg/g) was administered as a 50% solution intragastrically into non-anesthetized mice. Blood samples were obtained from the tail vein at 0, 15, 30, 60, and 120 min. All mice subsequently underwent a survival nephrectomy of the graft-bearing kidney, which was taken for morphological analysis or assessment of cellular insulin content (16). Nephrectomized animals were subsequently monitored to confirm a return of hyperglycemia.

2.2.4 Characterization of harvested grafts

The graft bearing kidneys were prepared for immunohistological analysis by fixation in 4% w/v paraformaldehyde (BDH Laboratory Supplies), then embedded in paraffin and 5 µm sections were prepared. Paraffin sections were processed and immunostained. After rehydration, antigen retrieval for tissue samples was performed with Tris EDTA Buffer (pH 9.00). The samples were then blocked with 20% normal goat serum (NGS, Jackson for ImmunoResearch Laboratories Inc) for 1 hour. Tissues were stained with a guinea pig anti-insulin antibody diluted at 1:1000 (Dako) and a rabbit anti-CD31 antibody diluted at 1:50 (Abcam) in 5% NGS. Secondary antibodies used were AlexaFluor 594 goat anti-rabbit and AlexaFluor 488 goat anti-guinea pig (Molecular Probes, Eugene) diluted at 1/200 in 5% NGS. Slides were cover slipped with Prolong Gold Anti-fade (Invitrogen) to preserve

fluorescence. Negative controls included sections of the same tissues incubated without primary antibodies and only the secondary antibody whereas positive controls included sections of neonatal porcine pancreas for both insulin and CD31 staining. Separate negative and positive controls were employed for each independent staining procedure and subsequent imaging. Slides were visualized with an Axioscope II microscope equipped with an AxioCam MRC and analyzed with Axiovision 4.6 software (Carl Zeiss).

To assess the effects MSC may have on the degree of graft vascularization in the early transplant period, a cohort of mice were implanted with either NPI alone or NPI + MSCs and then the grafts were harvested at 3 weeks post-transplant and immune-stained for insulin and CD31-positive cells. In these grafts the CD31 positive area and the DAPI positive cell number were measured using the Histogram feature of ImageJ and hand counting, respectively. The ratio of CD31 positive vasculature was then calculated by dividing the amount of CD31 positive pixels by the number of DAPI positive cells in the graft.

The grafts were also measured for total cellular insulin content (16). Extracted kidneys were homogenized and sonicated at 4°C in 10 mL of 2 mM acetic acid (containing 0.25% BSA). After 2 hours at 4°C, tissue homogenates were resonicated, centrifuged (10,000 g, 25 minutes), and supernatants were collected. Pellets were then further extracted by sonication in an additional 5 mL of acetic acid. The second supernatant was collected after centrifugation, combined with the first supernatant, total volume was measured, and samples were assayed for insulin content (MesoScale Mouse/Rat Insulin Kit).

2.2.5 Statistical analysis

Data are expressed as mean of *n* independent observations unless otherwise specified, with an individual biological replicates shown as points. Statistical significance of differences was calculated by either Student's t-test with the Holm-Sidak method for correction for multiple comparisons where appropriate if the sample approximated a normal distribution, or Mann Whitney U-test if a normal distribution could not be assumed. Median time to normoglycemia was compared using the Mantel-Cox log-rank test.

2.3 Results

2.3.1 Characterization of NPI grafts pre-transplant

Quantification of the proportion of beta- and alpha-cells in the grafts revealed no difference when NPIs were cultured alone or with MSCs (Table 2.1). Moreover, there was also no differences in the number of proliferating insulin/PCNA double positive ß-cells. In contrast, co-culture of NPIs with MSCs resulted in significantly more cellular insulin content compared to NPIs cultured alone (34.60 ± 0.75 µg/pancreas vs. 27.43 ± 3.22 µg/pancreas, respectively; $p<0.05$) (Table 2.1).

TABLE 2.1: COMPARISON OF NPI GRAFTS PRE-CULTURED FOR 48 HOURS WITH OR WITHOUT MSCS

Condition	Insulin Content (µg/pancreas)	Cell Composition (% of total)		
		β	α	PCNA+ β+
NPI	27.43 ± 3.22	15.43 ± 0.44	12.38 ± 0.35	5.20 ± 1.80
NPI+MSC	34.60 ± 0.75*	13.02 ± 0.44	11.60 ± 0.33	5.10 ± 2.20

Data are expressed as mean ± SEM of 4 independent experiments. NPIs were pre-cultured for 48 hours in the absence (NPI) or presence of 10^6 human bone marrow derived MSCs (NPI + MSC). *p<0.05 (Student's t-test).

During a GSIS assay, there was a significant difference (p<0.01) in the amount of insulin secreted between the two culture groups at low glucose (2.8 mM), high glucose (20.0 mM), and the stimulation index (SI) (Table 2.2).

TABLE 2.2: GLUCOSE STIMULATED INSULIN SECRETION OF NPI GRAFTS PRE-CULTURED FOR 48 HOURS WITH AND WITHOUT MSCS

	% Cellular Insulin		
	2.8 mM	20.0 mM	SI
NPI	0.60 ± 0.04	0.93 ± 0.12	1.50 ± 0.12
NPI + MSC	0.83 ± 0.03*	1.65 ± 0.06*	2.01 ± 0.04*

Data are expressed as mean ± SEM of 4 independent experiments. In each experiment, NPIs were pre-cultured for 48 hours in the absence (NPI) or presence of 10^6 human bone marrow derived MSCs (NPI + MSC). SI, stimulation indices, were calculated by dividing the amount of insulin released at high glucose (20.0 mM) by that released by low glucose (2.8 mM). *p<0.01.

Gene expression analysis of the NPI and NPI + MSC co-cultures revealed co-cultures had significantly higher levels ($p<0.01$) of pancreatic polypeptide (1.6 fold increase). There was no difference in insulin, glucagon, somatostatin and PDX1 gene expression (NPI + MSC vs. NPI respectively; (Fig. 2.1)).

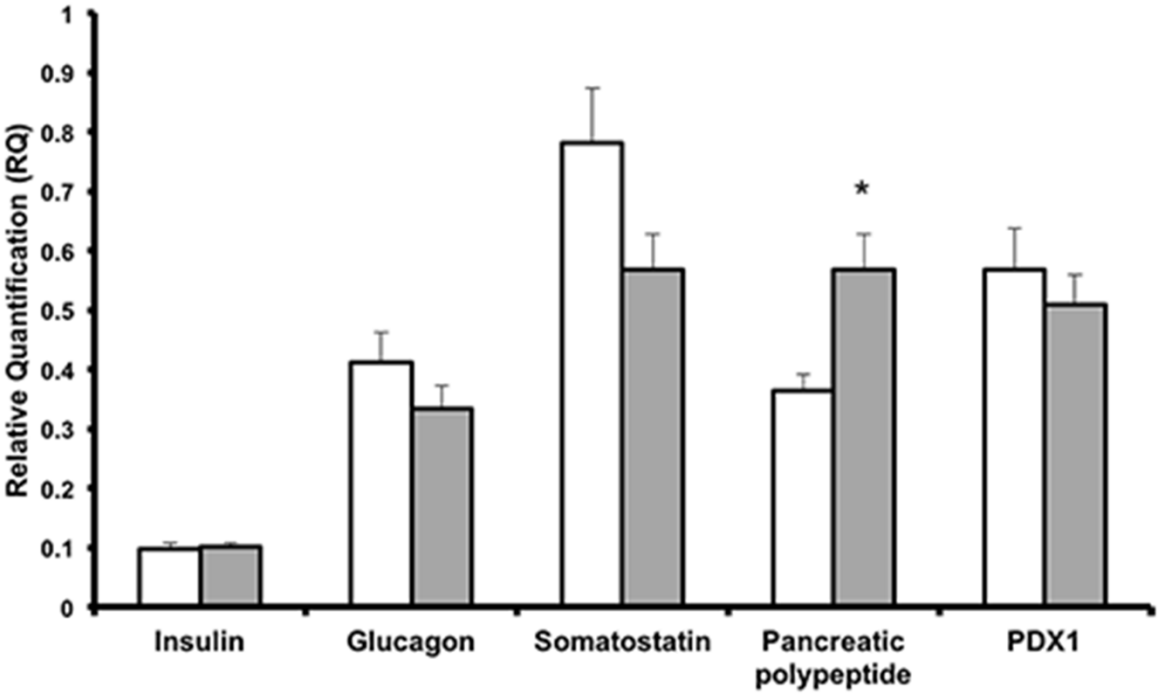

FIGURE 2.1. RELATIVE QUANTITATIVE RT-PCR COMPARISON OF NPIS PRE-CULTURED FOR 48 HOURS WITH AND WITHOUT MSCS. There were no significant differences in the insulin, glucagon, somatostatin and PDX1 transcripts within NPIs (white bars) and NPIs co-cultured with MSCs (grey bars). There were however significantly more pancreatic polypeptide transcripts found in the NPIs co-cultured with MSCs compared to NPIs alone (*$p<0.05$). Expression was normalized to GAPDH (endogenous control) and pig islet mRNA as a calibrator. Relative gene expression is plotted against Relative Quantification (RQ) values. Data are expressed as mean ± SEM

2.3.2 Transplantation of NPI and MSC

Metabolic follow-up of glycemia and weight was measured on the transplanted mice weekly, and an oral glucose tolerance test was administered once recipients reached and maintained normoglycemia. MSC and NPI co-transplanted recipients (*n=14*) exhibited significantly lower glycemia at weeks 18 to 20, and 22 post-transplant compared to those with islets alone (*n=14*) (Fig. 2.2A). Average weight decreased immediately after transplant, and subsequently increased following weeks 3 post-transplant. Weight was comparable between co-transplanted and islet alone recipients (Fig. 2.2B). Mice with co-transplants began to reach normoglycemia significantly earlier than islet only recipients with a median time to normoglycemia of 19.5 weeks compared to 25 weeks ($p<0.001$ Fig. 2.2C). Furthermore, at 20 weeks 64% of mice in the co-transplant group had obtained normoglycemia compared to 0% with islets alone; co-transplanted mice demonstrate a more rapid return to glycemia. At the end of the follow-up period 100% of the mice in both groups obtained normoglycemia. Following nephrectomy of the graft bearing kidney, all mice returned to hyperglycemia within 24 hours, confirming that resolution of hyperglycemia was due to the graft and not regeneration of β-cells.

Mice transplanted with islets alone were less glucose tolerant compared to co-transplantation recipients (Fig. 2.2D). During an oral glucose tolerance test, mice with NPI and MSC grafts had significantly lower glycemia compared to islet alone grafts at 15 (15.6 vs.19.7 mM; $p<0.05$, respectively) minutes post glucose challenge. Furthermore, the

glucose response curve was significantly lower in mice with NPI and MSC grafts compared to NPI alone grafts (area under the curve; 1278 ± 74 vs. 1072 ± 50 mM·min; $p<0.05$).

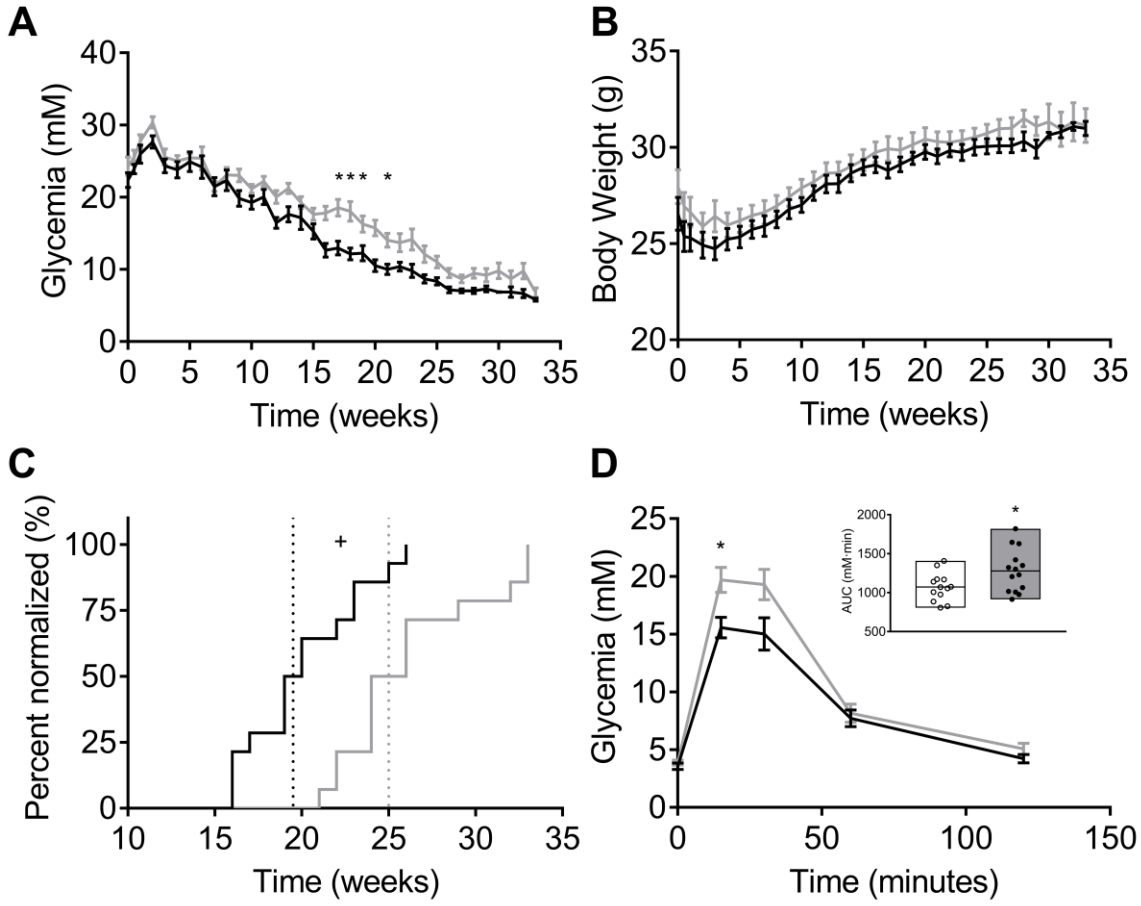

FIGURE 2.2. WEEKLY METABOLIC FOLLOW-UP OF DIABETIC B6/RAG-/- MICE transplanted with 3000 NPI (n = 14; grey line) or 3000 NPI + 106 MSC (n = 15; black line) under the kidney capsule. (A) Blood glucose levels assessed weekly from 0 to 35 weeks post-transplant. (B) Body weight assessed weekly from 0 to 35 weeks post-transplant. (C) Percentage (%) of mice achieving normoglycemia (glycemia < 11.1 mM for 2 consecutive weeks) from week 10 to week 35. (D) Blood glucose values during OGTTs in transplanted mice transplanted with NPI alone (n =14) or NPI + MSC (n=15). Insert represents respective area under the curve for OGTT (D inset). Vertical lines in C demonstrate median time to normalization. *$p < 0.05$ (Student's t-tests with Holm-Sidak corrections for multiple comparisons where appropriate); +$p < 0.001$ (Mantel-Cox log-rank test).

When we examined whether cell-to-cell contact is required for MSCs to exert an effect on NPIs, those mice transplanted with NPIs alone under the kidney capsule and given IV administration of MSCs exhibited no significant difference in weekly glycemia, time to normalization, glucose tolerance, or graft cellular insulin content compared to mice not given IV MSCs (Fig. 2.3A-D).

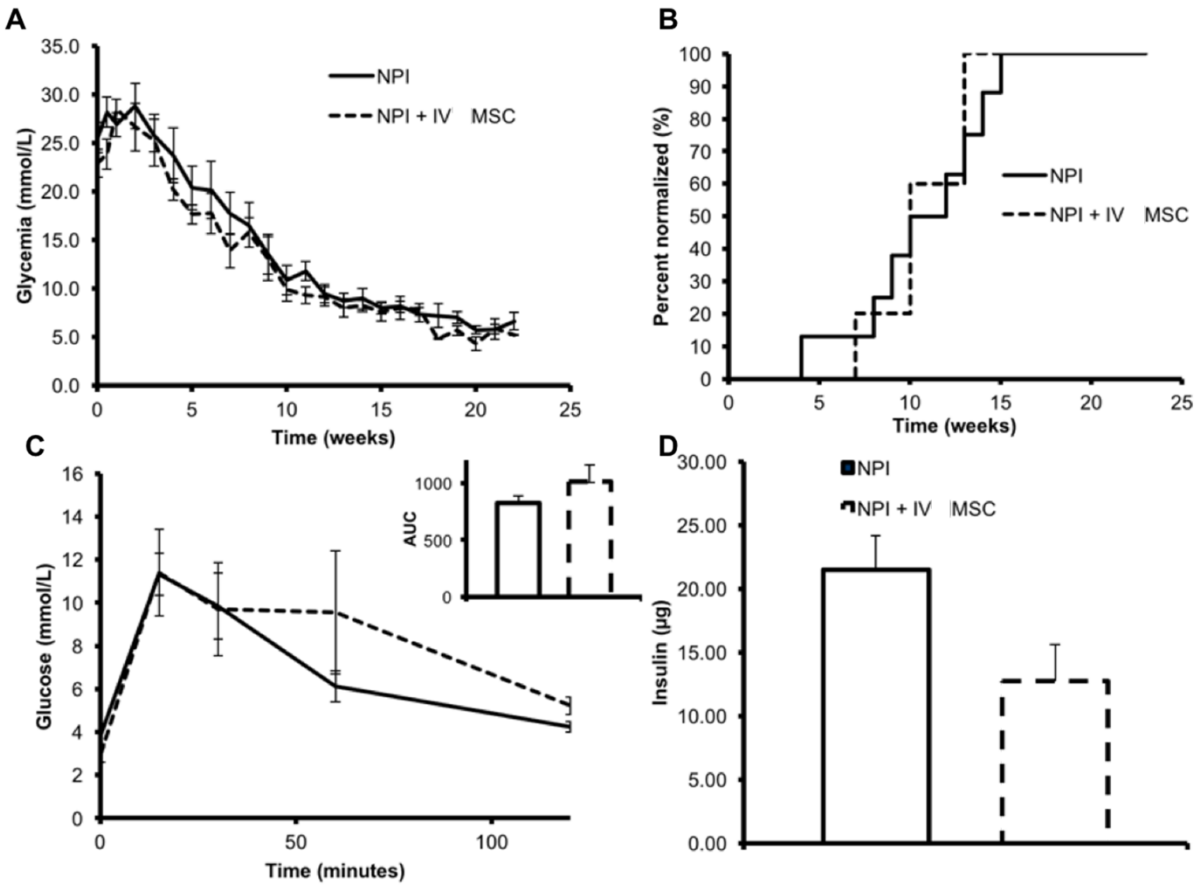

FIGURE 2.3. MICE TRANSPLANTED WITH NPIS UNDER THE KIDNEY CAPSULE WITH ADMINISTRATION OF INTRAVENOUS (IV) MSCS. Weekly metabolic follow-up of diabetic B6/Rag$^{-/-}$ mice transplanted with 3000 NPI under the kidney capsule (solid line) or without 3000 NPI + 10^6 IV MSC (dashed lines). A) Blood glucose levels assessed weekly from 0 to 25 weeks post-transplant. (B) Percentage (%) of mice achieving normoglycemia (glycemia < 11.1 mM for 2 consecutive weeks). (C) Blood glucose values during OGTTs in transplanted mice transplanted with NPI alone (solid line bar, n =8) or NPI + MSC (dashed line bar, n=7). Insert represents respective area under the curve for OGTT (D inset). (D) Total cellular insulin content of

grafts containing NPI alone (solid line bar) or NPI + MSC grafts (dashed line bar) at 25 weeks after transplantation. Data are expressed as mean ± SEM.

2.3.3 Morphological characterization and insulin content of grafts

Immunohistochemical examination of the grafts at 35 weeks post-transplant revealed highly vascularized tissue, consisting predominantly of insulin-positive cells (Fig. 2.4). Insulin staining at 3 weeks post-transplant was minimal (Fig. 2.4A, C) compared to 35 weeks post-transplant (Fig. 2.4E, G), although no differences between the two groups at either time point was observed.

Additionally, immune-histological analysis of the grafts for TUNEL showed no evidence of apoptosis in either NPI or NPI + MSC at week 2 or 3 post-transplant (data not shown).

To assess graft vascularization, grafts were obtained at 3 or 35 weeks post-transplant and immunostained for CD31 positive vasculature (Fig. 2.4). We hypothesized that the more rapid normalization in the co-transplant mice could be a result of earlier revascularization due to MSC secretion of trophic factors. In a separate cohort of mice transplanted with or without MSCs, grafts were collected at 3 weeks post-transplant to assess vascularization. Compared to the islet alone graft (Fig. 2.4B), the NPI + MSC graft exhibited markedly increased CD31 positive vasculature (Fig. 2.4D) at week 3. An abundance of CD31 positive vasculature were observed throughout the co-transplant graft, while CD31 positive vasculature were comparatively sparse in the islet only graft. At week 35 grafts were observed to have similar amounts of CD31 positive vasculature in the two groups (Fig. 2.4F,

H). Moreover, quantification of comprehensive images at week 3 post-transplant demonstrated significantly more CD31 positive vasculature in co-transplant grafts versus NPI alone grafts (Fig. 2.4I) (117.4 ± 24.7 vs. 35.6 ± 6.3 area/cell, $p<0.05$).

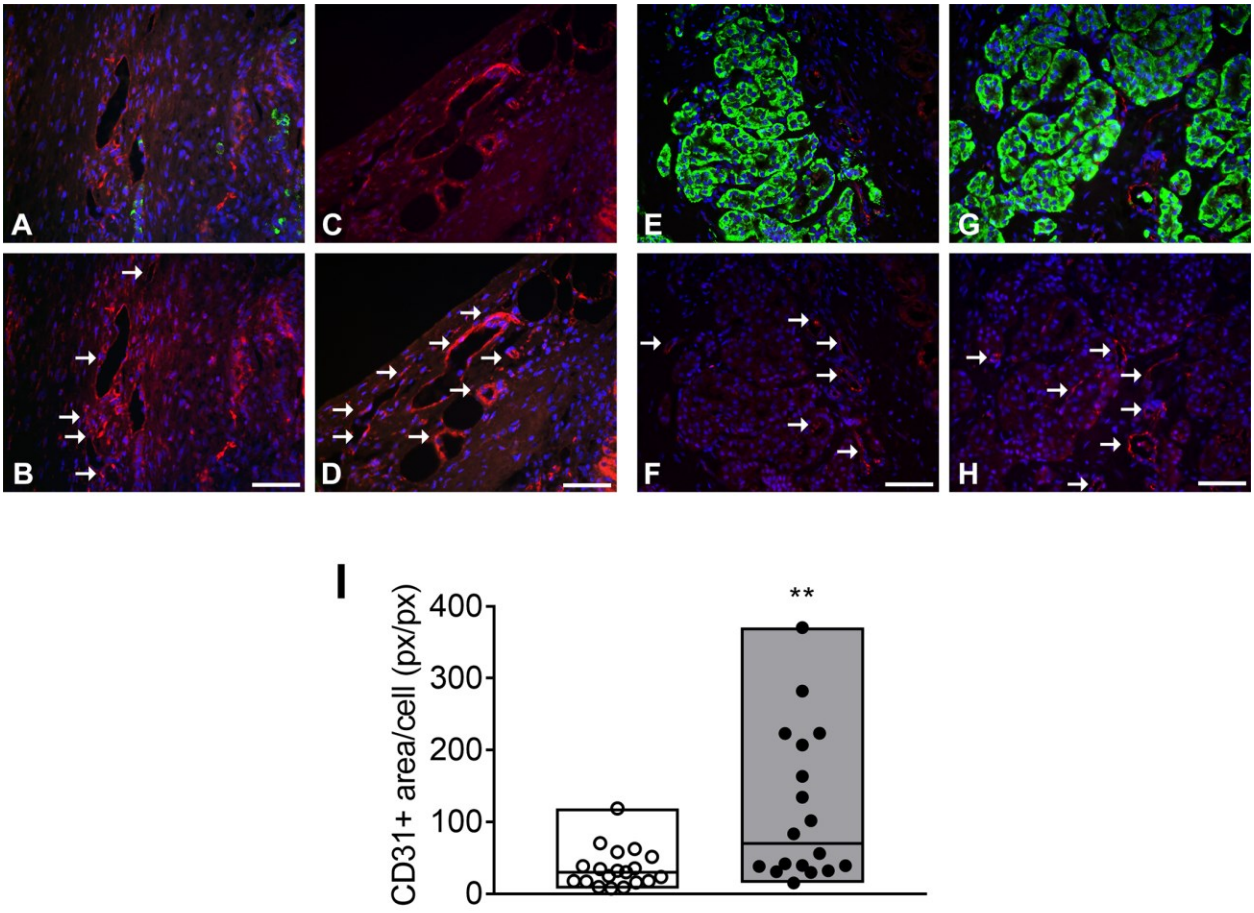

FIGURE 2.4. REPRESENTATIVE IMMUNOFLUORESCENCE IMAGES OF GRAFTS containing NPI alone at 3 weeks (A, B) and 35 weeks post-transplant (E, F) or NPI + MSC grafts at 3 weeks (C, D) and 35 weeks post-transplant (G, H). Grafts were stained for insulin (green) and CD31 (red). Scale bars = 25 μm. (I) Quantification of CD31 positive vasculature in NPI alone grafts (white bars and open circles) and NPI + MSC grafts (grey bars and black circles) at 3 weeks post transplantation. **$p < 0.01$ (Student's t-test).

Grafts were removed after mice reached normoglycemia and a subset of the grafts were homogenized to measure total cellular insulin content (Fig. 2.5). Recipients in the co-

transplanted group had nearly 1.5 times more insulin than islet only recipients (17.5 µg ± 2.37 vs. 12.0 µg ± 0.77; $p<0.05$).

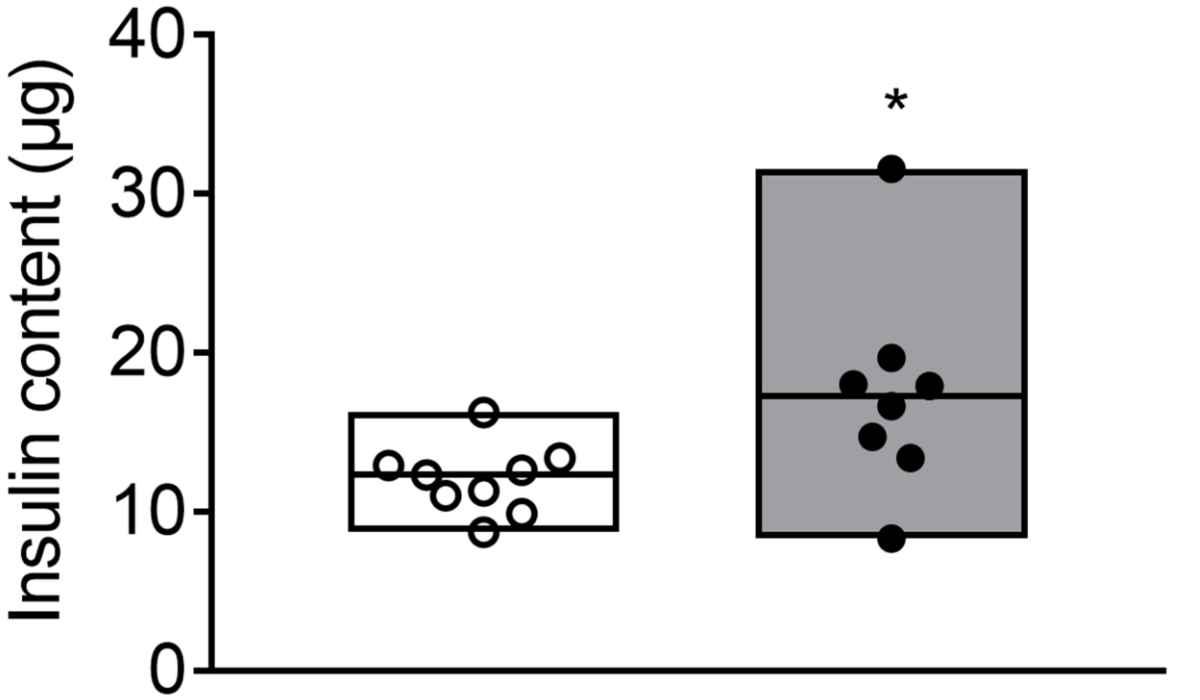

FIGURE 2.5. TOTAL CELLULAR INSULIN CONTENT OF GRAFTS containing NPI alone (n=7; white bar and open circles) or NPI + MSC grafts (n=10; grey bar and black circles) at 35 weeks after transplantation. Line is at median. *$p < 0.05$ (Mann-Whitney U-test).

2.3.4 EFFECT OF MSC DONOR PATHOLOGY

One paired experiment with a single NPI preparation and MSC donor was found to have contrasting results from the other experiments (Fig. 2.6). A review of the MSC donor information revealed that the donor had an autoimmune disease (psoriatic arthritis). In this paired experiment, co-transplantation recipients (*n=6*) exhibited no difference in glycemia than islet only recipients (*n=4*) in weeks 1-32 post-transplant. No difference in

weight was observed between groups (Fig. 2.6B), although weights did increase steadily at week 5 post-transplant, after an initial decline. Interestingly, mice with islets alone began to reach normoglycemia significantly earlier than mice with co-transplants median time to normoglycemia of 25.5 weeks compared to 32 weeks (p=0.05). (Fig. 2.6C), Glucose tolerance (Fig. 2.6D) and total graft insulin content (9.3 μg ± 1.73 and 10.3 μg ± 0.83 for NPI and NPI:MSC respectively; data not shown) did not differ between co-transplant and islets only recipients.

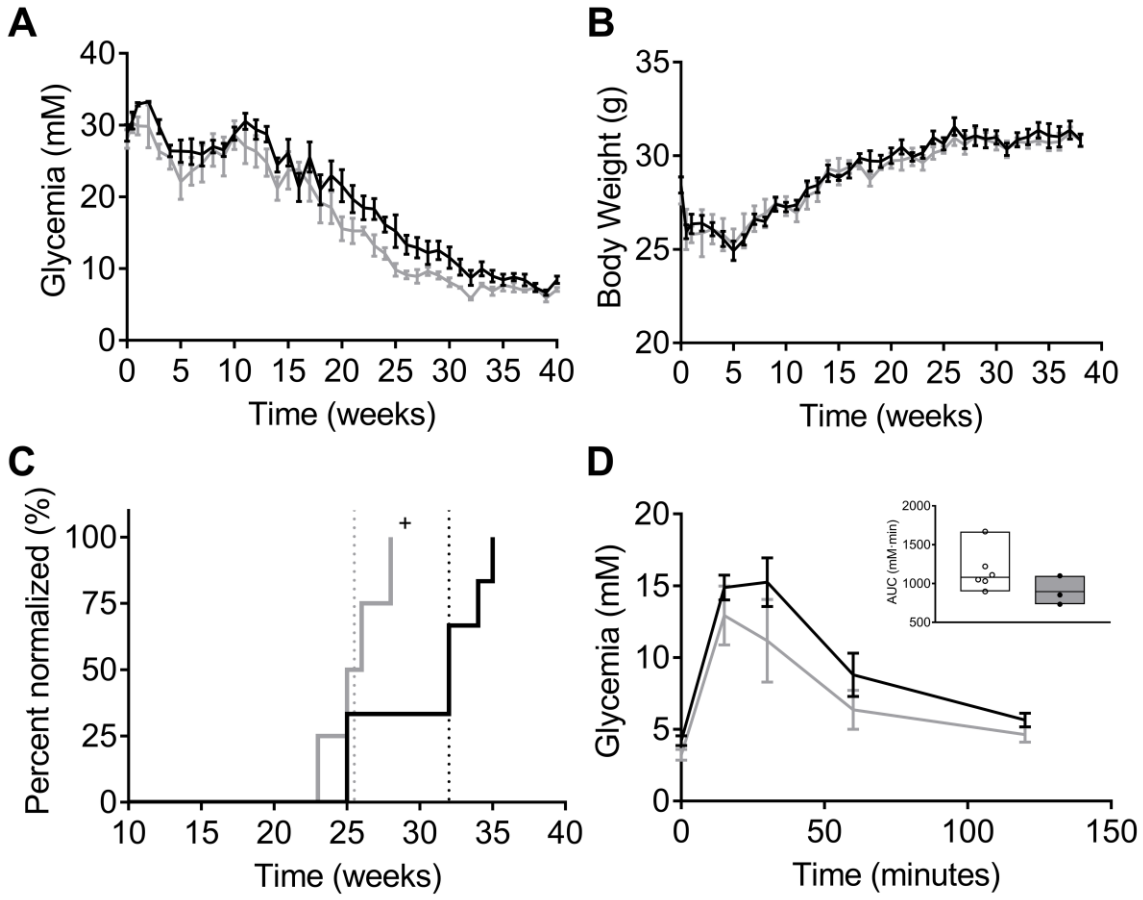

FIGURE 2.6. WEEKLY METABOLIC FOLLOW-UP OF DIABETIC B6/RAG-/- mice transplanted with 3000 NPI (n=4; solid line) or 3000 NPI + 106 Psoriatic Arthritis donor MSCs (n=6; dashed line) under the kidney capsule. (A) Blood glucose assessed weekly from 0 to 40 weeks post-

transplant. (B) Weight assessed weekly from 0 to 40 weeks post-transplant. (C) Percentage (%) of mice achieving normoglycemia (glycemia < 11.1 mM) for 2 consecutive weeks) from week 10 to 40. (D) Blood glucose values during OGTTs in transplanted mice transplanted with NPI alone (n =4) or NPI + MSC (n=6). Insert represents respective area under the curve for OGTT (D inset). Vertical lines in C demonstrates median time to normalization. +p = 0.05 (Mantel-Cox log-rank test).

2.4 Discussion

In this clinically relevant study we demonstrate that co-transplantation of human bone marrow derived MSCs with NPIs under the kidney capsule of streptozotocin induced diabetic mice resulted in better functional outcomes compared with NPIs alone. Mice receiving co-transplants achieved normoglycemia significantly sooner, exhibited lower glycemias, were shown to be more glucose tolerant, and significantly more graft cellular insulin content was recovered post-transplant. Prior to transplantation NPI and MSC co-cultures contained significantly more total cellular insulin content and moreover exhibited greater GSIS compared to NPIs cultured alone.

Several studies have shown that co-transplantation of rodent MSCs and islets result in lower blood glycemia (20-23, 38). This is in accordance with our results, as we found significantly lower glycemia for several weeks midway through the transplant period (Fig. 2.1A). Our results also agree with evidence that co-transplantation reduces the time to achieve normalization compared to islets alone (22, 23). Similarly, Ito et al. (21) reported superior glucose tolerance in mice receiving rodent islet and MSC co-transplants, in concordance with our findings. Since the proportion of time a patient spends in a hyperglycemic state is directly related to the risks of developing long term complications and comorbidities (39), this is of great clinical significance. The mechanisms by which

MSCs improve glycemic control are not yet known, but some evidence supports the secretion of trophic factors as a potential explanation. In particular, annexin A1 appears to play a significant role in enhancing GSIS and annexin knockdown severely impairs MSC function (12). Moreover, our data support this observation, since NPIs co-cultured with MSCs exhibited significantly increased GSIS as well as total cellular insulin content. Furthermore, MSCs have been found to upregulate certain genes involved in insulin secretion and synthesis in β-cells (40). However, when NPIs were co-cultured with MSCs we observed no significant differences in the insulin, glucagon, somatostatin and PDX1 transcripts however significantly more pancreatic polypeptide transcripts were found in the NPIs co-cultured with MSCs compared to NPIs alone.

Rapid development of a vascular system is important for islet function after transplant (5), and earlier angiogenesis could contribute to improved NPI function, as evidenced by the increase in CD31 positive vasculature in the co-transplanted grafts. MSCs secrete VEGF, a trophic factor that induces angiogenesis (41). Many studies involving transplanted MSCs have found an increase in capillaries (20, 21), increased endothelial cell positive areas (38), or VEGF production (42) in graft areas, although one has shown no increase in vascularization (23). Additionally, mice deficient in pancreatic VEGF expression exhibited impaired glucose tolerance (41), indicating that VEGF may potentially improve glucose tolerance. Fabryoya et al. (42) used rodent MSCs to enhance vascularization of an implanted scaffold, intended to create adequate vascularization in previously unsuitable sites for transplantation such as subcutaneous and the greater omentum (42). MSCs were unable, however, to provide adequate vascularization for the intramuscular site (40). The

lack of any effect when MSCs are administered IV (Fig. 2.3), indicates that MSC action may be site dependent, and cell-to-cell contact may be necessary. Further investigation is required to identify the specific mechanisms by which MSCs affect the graft microenvironmnent.

Co-transplant recipients also exhibit superior glucose tolerance (Fig. 2.2D) and higher total pre-culture and post-transplant graft cellular insulin content (Table 2.2 and Fig. 2.5). We have previously reported that human MSCs prevent β-cell apoptosis in human islets *in vitro* (37), and *in vivo* studies have suggested that rodent MSCs can prevent rodent β-cell apoptosis (23). A lack of immunostaining for TUNEL on early time point grafts indicate no apoptosis in grafts from either group at 3 weeks post-transplant (data not shown). This suggests MSCs do not prevent β-cell apoptosis in NPIs. This does not contradict previous findings, however; it is known that NPIs are more resistant to hypoxia than human islets (17) and have the ability to proliferate after transplant (16). It is not unexpected that NPIs are less sensitive to hypoxic conditions in the initial post-transplant period.

MSC donor characteristics, such as age and sex, have an impact on the therapeutic potential of MSCs (26). Less is known about how the specific disease status can affect characteristics of MSCs (26). However, autoimmune diseases as a whole have been associated with some dysfunction (26), including altered morphology (32), decreased proliferative capacity (32-34), altered gene expression (35), decreased expression of trophic factors (33, 35), and reduced inhibitory effect on T cells (32, 35). In one of our paired experiments, we discovered that the MSC donor had psoriatic arthritis (PA), an autoimmune disease in

which joints are targeted for immune attack and inflammation. The experiment using the PA MSCs from this donor was conducted using the same protocol as the experiments discussed above. In this experiment, mice in the co-transplantation group had no differences in glycemia (Fig. 2.6A), significantly increased time to normalization (Fig. 2.6C), no difference in glucose tolerance (Fig. 2.6D), and no difference in insulin content (data not shown). The positive effects that were exemplified in the previous experiments disappeared, and in fact PA MSCs had a detrimental effect on the ability of NPIs to reverse diabetes. This suggests that these MSCs did have functional impairment, such as a reduction in VEGF expression or reduced anti-inflammatory effects. Some studies show that MSCs from diabetic donors may have decreased proliferative capacity and differentiation potential (26, 28). Furthermore, MSCs from rodent and human diabetic type 2 donors have been shown to have reduced proliferative capacity, higher levels of senescence and apoptosis, and decreased differentiation potential (28, 43). This is likely caused by the diabetes microenvironment of high glucose and increased concentrations of advanced glycation end products and reactive oxygen species (28). Therefore, T1DM donors may have impaired MSCs for two reasons; autoimmune disease status and diabetic microenvironment. It is critical to learn more the functional capacity of MSCs from donors with T1DM, as using autologous MSCs from these patients may not be as effective.

In this study we have demonstrated that MSCs have a beneficial effect on the efficacy of NPI transplantation and in vitro function; they encourage islets to be curative faster and increase the glucose tolerance of islets after reversal of diabetes, potentially due to earlier development of a vascular system and enhanced glycemic control.

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CHAPTER 3: DISCUSSION

3.1 GENERAL DISCUSSION

There were 1.3 million individuals with T1DM, costing the government $5.9 billion in Canada in 2000, and those numbers are expected to triple by 2020 (1). Insulin therapy, the current gold standard treatment for individuals for T1DM, is a burden on patients due to cost, constant monitoring, and daily injections. Furthermore, exogenous insulin therapy does not provide perfect glucose control and fluctuations are unavoidable (2). Hypoglycemic events are the most dangerous complication of insulin therapy (2). The list of long-term complications of T1DM is long, and includes retinopathy, limb amputation (3), renal failure, and neuropathy (4).

β-cell replacement is ideal as it provides endogenous insulin, which reduces glycemic variability. Whole pancreas transplant comes with a high chance of morbidity and mortality due the necessity of major surgery (5). Islet transplantation is more ideal, as it does not even require general anesthesia (6) and complications are minor (7). Significant advance was made on the field of islet transplantation in 2000 when 7/7 patients sustained insulin independence after one-year post-transplantation (8). A five-year follow-up indicated, however that insulin independence is not sustained (9).

There may be several reasons for loss of graft function. There are early inflammatory events, known as IBMIR, that cause massive islet damage and coagulation (10). Transplanted islets also lack sufficient blood supply, as their pancreatic connections were severed during isolation and they remain hypoxic until they can be revascularized in the recipient, which

takes several days to several months (11). Another issue is the consistency of donor islets, and islet quality or transplant outcomes cannot be predicted by donor characteristics (12). Taken together, these factors contribute to early loss of islets, which may be up to 60% (13).

NPIs are a viable alternative to cadaveric donor islets; pigs are an ideal source of donor tissue as they breed rapidly and have large litters, creating a virtually unlimited supply (14). Additionally, NPIs are naturally resistant to hypoxia (15), pro-inflammatory cytokines (16), and hyperglycemia (17).

MSCs are ideal in solving the above issues in islet transplantation. They are anti-inflammatory, pro-angiogenic, and beneficial to islets in culture. Co-transplantation studies using rodent islets and MSCs have been shown to improve islet graft function. Several studies have shown that the addition of MSCs can reduce the number of islets necessary to cause reversal of diabetes (18-21). MSCs may also help islets retain a normal morphology in vivo (20). Importantly, several studies have found that MSC co-transplantation improves vascularization (18, 22, 23). Mice receiving co-transplants tend to have improved glucose tolerance (19, 20, 22), which may due to MSC's secretion of Annexin-A1 (24).

In this study, we assessed the effects of co-transplanting NPIs and human bone marrow derived MSCs in a streptozotocin induced diabetic B6.129S7-Rag1^{tm1Mom}/J mouse model. We found that co-culture of NPIs and MSCs results in higher insulin content and improved GSIS (Table 2.2), but no difference in total number of β-cells (Table 2.1) and no difference in insulin transcripts (Fig. 2.1). Co-transplantation recipients had better blood glycemia

(Fig. 2.2A), glucose tolerance (Fig. 2.2D), and normalized significantly earlier (Fig. 2.2C) than NPI alone recipients. Addition of MSCs by IV injection did not improve metabolic outcomes (Fig. 2.3), demonstrating that cell-cell contact may be necessary.

We demonstrated that co-transplantation grafts had better vascularization at an early time point (Fig. 2.4I), which may account for earlier normalization. Additionally, we found that co-transplant grafts had higher total insulin content (Fig. 2.5), which could contribute to better glucose tolerance.

Interestingly, we found that co-transplantation with MSCs from a donor with an autoimmune disease (psoriatic arthritis), negated these beneficial effects. In this particular study, co-transplant mice did not have improved glycemia (Fig. 2.6A), glucose tolerance (Fig. 2.6D), and actually took significantly more time to normalize (Fig. 2.6C). This may be clinically relevant as it calls into question whether it is optimal to use autologous MSCs to treat individuals with T1DM.

3.2 Conclusions

In this study, we found that bone marrow derived MSCs have beneficial effects on NPIs, in vitro and in vivo. C0-culture of NPI and MSCs results in higher total insulin and better GSIS. Co-transplantation results in better glucose tolerance and earlier normalization. Co-transplant grafts at 3 weeks have better vascularization, which may account for improved transplant outcomes.

There are still many areas of research ahead for this project. We have found evidence that autoimmune disease in the MSCs donor impairs MSC activity and negates the improvement in co-transplantation outcomes. This is however, a single MSC donor with one particular autoimmune disease. Further research is required into other autoimmune diseases, particularly T1DM, to determine whether these MSCs are impaired as well. In Carlsson and colleagues' clinical trial administering MSCs to newly diagnosed T1DM patients, they used autologous bone marrow derived MSCs as the source (25). There is some evidence that autologous MSCs may be superior to allogeneic MSCs to treat ischemic cardiomyopathy, although both sources showed improved outcomes (26). Based on our findings, however, autologous MSCs might be impaired specifically in individual's with autoimmune diseases, so treating T1DM with autologous MSCs may require special consideration. In fact, one study has shown that MSCs from diabetic mice has decreased proliferative capacity and diminished differentiation potential, suggesting they may be impaired (27). Additionally, MSCs from T2DM human donors have been shown to have a reduced proliferative capacity, higher levels of senescence and apoptosis, and decreased differentiation potential, likely caused by the diabetes microenvironment of high glucose and reactive oxygen species (28). Given that autologous MSCs may be impaired due to autoimmune disease status and the diabetes microenvironment, further research is crucial, since it will determine whether autologous or allogeneic donors are used to treat T1DM patients.

Another issue is the safety of infusing MSCs into the vascular system. Animal models show that IV administered MSCs tend to accumulate in the lungs, and distribution after lung

entrapment cannot be demonstrated (29). However, there is a number of clinical trials that have demonstrated the safety of IV MSC administration in treating diabetes, Crohn's disease and myocardial infarction and reported no adverse events (25, 30, 31). Our study, however, has found no efficacy when infusing MSCs intravenously (Fig. 2.3), so in this context direct cell-cell contact between islets and MSCs may be necessary to promote better glucose tolerance and vascularization.

The question still remains about the method of transplanting MSCs with islets in clinical islet transplantation. Currently, islets are infused into the hepatic portal vein during transplant, an ideal site for minimal invasiveness and easily resolved complications. However, islets are exposed to IBMIR (32) and hypoxic conditions (33), and about 60% of the graft is lost following transplantation (34). MSCs could potentially improve the hepatic portal site, as we have demonstrated they increase vascularization in our model (Fig. 2.4I). We have demonstrated that IV administration of MSCs did not lead to improved transplant outcomes, so clinically MSCs would likely need to be co-injected into the portal vein with islets, rather than IV administration.

Another promising area of research is the source of MSCs. Adipose-derived MSCs are more plentiful in the tissue by approximately 500 fold (35) and secrete comparable levels of IL-6, IL-8, and VEGF, and higher levels of bFGF (36) compared to bone marrow-derived MSCs. Since adipose-derived MSCs are more plentiful and easily isolated, they are an excellent candidate source of MSCs for co-transplantation. Further research is required to examine whether they have similar beneficial effects on islets in vitro and in vivo.

Further research is required before MSCs are adapted into the clinical for islet transplantation. The MSC source needs to be determined; bone marrow or adipose tissue, and autologous or allogeneic. Adipose-derived MSCs may be more easily isolated and expanded for clinical use compared to bone-marrow-derived MSCs, so adipose-derived MSCs need to be studied for their effect on islets. Autologous MSCs may have superior effects clinically on diseases such as ischemic cardiomyopathy (26), and Carlsson et al have turned to autologous MSCs for their clinical trial on T1DM patients (25). These MSCs may not be ideal, however, in the case of T1DM, an autoimmune disease, and comparisons of autologous versus allogenic MSCs in the context of T1DM need to be examined before translation into the clinic.

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