Improving Islet Function and Engraftment by Co-culturing Islets with Mesenchymal Stem Cells and the Formation of Pseudoislets

by

ANISSA FRANCES MARIE GAMBLE

A thesis submitted in partial fulfillment of the requirements for the degree of

Master of Science

in

Experimental Surgery

Department of Surgery

University of Alberta

ABSTRACT

Islet transplantation has proven to be efficacious in preventing severe hypoglycemia and restoring insulin independence in selected patients with type 1 diabetes. The well-established procedure has gained popularity due to notable refinements over the past two decades. However, islet transplantation is not a permanent solution for glycemic control as multiple infusions are often required to achieve and maintain insulin independence, and chronic lifelong immunosuppression is required. Substantial islet loss occurs prior to and post-transplant due to multiple factors including nutrient depletion, the instant blood-mediated inflammatory reaction, and potent host auto- and alloimmune responses. To circumvent islet loss, this thesis evaluates the effect of co-culturing islets with mesenchymal stem cells and alternatively reformation of islets into pseudoislets as two potential discrete strategies to improve long term islet function and survival.

We first evaluated the effect of co-culturing murine and human islets for 48 hours with human adipose-derived mesenchymal stem cells. *In vitro*, murine islets co-cultured with adipose-derived mesenchymal cells demonstrated superior islet yield, vitality, survival and function relative to islets cultured alone. In an immunodeficient mouse model, we demonstrated a marginal islet mass co-transplanted with adipose-derived mesenchymal stem cells without prior co-culture failed to restore normoglycemia as efficiently as islets alone. However, islets co-cultured with adipose-derived mesenchymal stem cells for 48 hours and subsequently co-transplanted into mice had improved glycemic profiles relative to islet cultured and transplanted alone. We demonstrated

preserved mouse islet function and recovery during a co-culture and co-transplantation with adipose-derived mesenchymal stem cells relative to islets alone.

In the second part of this thesis, we evaluated the function and efficacy of the bioengineered islets called pseudoislets. Utilizing the Aggerwell® system developed by Mark Ungrin and colleagues from the University of Calgary, human islets were dissociated and re-aggregated into uniform sizes. We sought to determine the optimal size and dose for islet function and engraftment. We found that reaggregated pseudoislets demonstrated improved *in vitro* insulin secretion and hypoxia tolerance. When tested *in vivo* in a chemically diabetic murine model, pseudoislets reversed diabetes at a similar rate and demonstrated improved glucose clearance compared to native islets. We found that dissociated and re-aggregated islets had comparable outcomes when compared to native islets. This intriguing finding provides room for considerable future investigation.

PREFACE

This thesis entitled "*Improving Islet Function and Engraftment by Co-culturing Islets with Mesenchymal Stem Cells and the Formation of Pseudoislets*" is a compendium of four sections: An overview of islet transplantation, the effect of co-culturing islets with adipose-derived mesenchymal stem cells, the potential for creation of pseudoislets and concluding remarks. The body of work is aimed at describing potential avenues to improve islet survival and engraftment by utilizing mesenchymal stem cells and bioengineered cells. The thesis contains a combination of published work and work under peer-review for considered publication.

Chapter 1 affords a brief introduction of islet transplantation, and how this therapy may be applied for select patients with type 1 diabetes mellitus complicated by refractory recurrent hypoglycemia. This chapter has been published (Gamble A. first author) in the peer-reviewed journal ***Islets,*** (Gamble A, Pepper AR, Bruni AB and Shapiro AMJ, "*The Journey of Islet Cell Transplantation and Future Development*." Islets 2018, vol 10, issue 2: e1428511). As first author, my role was researching, designing, creating figures and writing the primary manuscript. ARP was the primary reviewer and provided critical analysis and editing. AB provided strategic designing and information. AMJS performed critical review and had a large role in writing the manuscript as the senior corresponding author.

Chapter 2 provides insight of the effect of co-culturing murine and human islets with human adipose-derived mesenchymal stem cells. The work presented is close to completion with the intention of imminent submission to the journal **Diabetes** (Gamble A, first author, with Pawlick RL, Pepper AR, Bruni A, A Adesida, P Senior, G Korbutt

and Shapiro AMJ as co-authors). I performed all major experimental steps in this study, including murine pancreas distensions, islet isolation procedures, all *in vitro* assays, animal monitoring and assessments, data analysis and manuscript preparation. RLP assisted pancreas distensions, perifusions, performed transplants, aided with data analysis, and contributed to article writing and editing. ARP aided with pancreas distensions, transplants, data analysis and contributed to article writing and editing. AA laboratory provided cryopreserved MSCs. PS and GK provided input for experimental design, techniques and article revisions. AMJS gave insight for experimental design, analysis and performed final edits to the manuscript as senior corresponding author.

Chapter 3 examines bio-engineered disaggregated and then reaggregated islet cells called "pseudoislets," in an effort to improve islet function and engraftment. This is not my original work, but I contributed in a significant way to the experimental design and execution, where I conducted most experiments, animal monitoring and aided in data analysis and manuscript preparation including generation of figures. I am the second author of this manuscript which is accepted for print in the journal ***Diabetologia*** (Yu Y, Gamble A, Pawlick R, Pepper AR, Salama B, Toms D, Razian G, Ellis C, Bruni A, Gala-Lopez B, Lu L, Vovko H, Chiu C, Abdo S, Korbutt G, AMJ Shapiro and Ungrin M). YY is the principle investigator of the project and it is important to note this research is a part of his Doctor of Philosophy thesis. YY contributions were experimental design, pseudoislets culture, *in vitro* analysis and the formation of the article. RLP contributed to experimental design, *in vitro* analysis, transplants, and a large portion of article writing and editing. ARP performed transplants, contributed to experimental design and aided article formation and editing. AB contributed to experimental design and BG performed

transplants. All other authors performed tasks from the University of Calgary or contributed to preliminary data from the University of Alberta. Both AMJS and MU designed the study and performed final edits to the manuscript as senior corresponding authors.

Chapter 4 serves to summarize and conclude the research presented in this thesis and its relevance to the islet transplant research field. Segments of this chapter are published as a book chapter in Elsevier Science & Technology Books second edition ***Encyclopedia of Endocrinology Diseases*** entitled "*Transplantation: Pancreatic and Islet Cells*," (Gamble A, Bruni A, and Shapiro AMJ). Editors in chief are H.Ilpo and M.Luciano published October 2018. Within this section, AG contributed to research, structure, writing, editing and figure creation. AB aided with article writing and editing. As corresponding author, AMJS performed final edits and contributed to article formation.

DEDICATION

I dedicate this thesis to my family and friends who have unconditionally supported my journey throughout life. I want to acknowledge the struggle and adversity of every person living with type 1 diabetes, and the horizon for everyone to one day become insulin independent.

ACKNOWLEDGEMENTS

I would first like to thank Dr. A.M.J Shapiro for the incredible opportunity to pursue my graduate studies under his supervision. It is people like Dr. A.M.J Shapiro who altruistically dedicate all of their focus and efforts to someday eradicate the necessity for patients with type 1 diabetes to undergo daily insulin injections and constant blood glucose monitoring.

My lab members: Dr. Antonio Bruni, Dr. Andrew Pepper, Ms. Rena Pawlick, Dr. Boris Gala-Lopez, Dr. Braulio Garza and Dr. Mariusz Bral. Without their endless help and patience, this thesis would not be possible. Dr. Bruni, thank you for your daily mentorship and guidance, it goes without notice. Dr. Pepper, your expertise, and patience is inspiring and greatly appreciated. Ms. Pawlick, thank for teaching me essential laboratory skills that I intend to imply in my future endeavors. Dr. Gala-Lopez, Dr. Garza and Dr. Bral, thank you for your expertise and wise life knowledge. A special thank you to Dr. Gang Xu for his contagious passion for science during my short time with them in Dr. A.M.J Shapiro's lab.

A special thanks to my advisory committee: Dr. Peter Senior and Dr. Gregory Korbutt. Their generosity of time, support and guidance have contributed substantially to my success during graduate studies. Their passion for diabetes is contagious and motivates me to pursue future research. In addition, thank you to Dr. Tom Churchill and Dr. Fred Berry for being a part of my defense committee.

Lastly, thank you to my family: Anitra, Michael, Alyssa, and Csaba. I am forever grateful to be blessed with a loving and supportive family.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

AUC	Area under curve
β-cell	Beta-cell
BMI	Body mass index
CFA-PI	Centrifugal-forced-aggregation Pseudoislet
CITR	Collaborative Islet Transplant Registry
CMRL	Connaught Medical Research Laboratories
DL	Device-less
HbA1C	Glycosylated hemoglobin
HBSS	Hank's Buffered Saline Solution
IBMIR	Instant-blood mediated inflammatory reaction
IE	Islet equivalents
IFN-γ	Interferon-gamma
IL	Interleukin
IL-1β	Interleukin-1-beta
i.p	Intraperitoneal
IPSCs	Induced Pluripotent Stem Cells
IPGTT	Intraperitoneal glucose tolerance test
IT	Islet transplantation
KC	Kidney capsule
MSC	Mesenchymal Stem Cell
ROS	Reactive oxygen species
SEM	Standard error of mean
sGSIS	Static glucose stimulated insulin secretion
SI	Stimulation index
STZ	Streptozotocin
T1DM	Type 1 diabetes mellitus
TNF-α	Tumor necrosis factor-alpha
Treg	Regulatory T-cell
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling

Chapter 1

INTRODUCTION

The Journey of Islet Cell Transplantation and Future Development

Chapter 1: The journey of islet cell transplantation and future development.

ISLETS
2018, VOL. 10, NO. 2, 80–94
https://doi.org/10.1080/19382014.2018.1428511

Taylor & Francis
Taylor & Francis Group

REVIEW

Check for updates

The journey of islet cell transplantation and future development

Anissa Gamble [a,c], Andrew R. Pepper[a,b,c], Antonio Bruni [a,c], and A. M. James Shapiro [a,b,c]

[a]Alberta Diabetes Institute, University of Alberta, Edmonton, AB, Canada; [b]Clinical Islet Transplant Program, University of Alberta, Edmonton, AB, Canada; [c]Members of the Canadian National Transplant Research Project (CNTRP), Canada

ABSTRACT

Intraportal islet transplantation has proven to be efficacious in preventing severe hypoglycemia and restoring insulin independence in selected patients with type 1 diabetes. Multiple islet infusions are often required to achieve and maintain insulin independence. Many challenges remain in clinical islet transplantation, including substantial islet cell loss early and late after islet infusion. Contributions to graft loss include the instant blood-mediated inflammatory reaction, potent host auto- and alloimmune responses, and beta cell toxicity from immunosuppressive agents. Protective strategies are being tested to circumvent several of these events including exploration of alternative transplantation sites, stem cell-derived insulin producing cell therapies, co-transplantation with mesenchymal stem cells or exploration of novel immune protective agents. Herein, we provide a brief introduction and history of islet cell transplantation, limitations associated with this procedure and methods to alleviate islet cell loss as a means to improve engraftment outcomes.

ARTICLE HISTORY
Received 8 January 2018
Accepted 12 January 2018

KEYWORDS
immunosuppression; islet engraftment; islet transplantation; mesenchymal stem cells; pluripotent; type 1 diabetes; β cells

Islet cell transplantation overview

Introduction and brief history

Globally, diabetes affects over 382 million people, with roughly 10% presenting with type 1 diabetes mellitus (T1DM) and is expected to rise to 592 million by 2035.[1] An annual 3% growth rate affords an escalating financial burden where the International Diabetes Federation estimates in Canada alone diabetes-related health care costs was $14 billion in 2015. These are expected to climb to a staggering $16 billion per annum by 2020.[2] Although the etiology of T1DM is incompletely elucidated, it is characterized as a multifactorial autoimmune disease resulting from specific immune-mediated destruction of pancreatic beta (β) cells within the islets of Langerhans. Classic symptoms include polyuria, polydipsia, and polyphagia with confirmation of diagnosis marked by hyperglycemia, low or indetectible serum C-peptide levels, elevated glycosylated hemoglobin (HbA_{1c}), and one or more positive autoantibody markers.[3] Those with T1DM must administer frequent exogenous insulin therapy to maintain normoglycemia. Continuous glucose monitoring systems (CGM) and insulin pumps may further help mitigate glycemic fluctuation. Recently, the FDA approved a closed-loop technology that infuses glucose regulatory hormones (insulin and glucagon) in response to glycemic fluctuations. While tighter glycemic control with medical intervention has been clearly shown to reduce secondary complications, it substantially increases risk of severe hypoglycemic reactions. T1DM is associated with a shortened life expectancy by 13 years.[4]

In consequence, the research community has focused on new avenues to arrest T1DM at the time of diagnosis. Intensive "new-onset" pilot trials conducted by TrialNet, a group of researchers aimed at identifying the prognosis and prevention of T1DM, have demonstrated means to sustained honeymoon periods and delayed diabetes onset.[5] In Brazil, Voltarelli and colleagues are currently conducting clinical trials aimed to reset the immune system in new-onset diabetes through administration of peripheral blood autologous bone marrow-derived hematopoietic stem cells coupled with immunodepleting conditioning (NCT00315133).[6-8] This approach led to impressive reversal in the diabetic state in 21 children and adolescents with new-onset T1DM, but was also associated with substantial side-effects. To date, no protocol has yet to eradicate exogenous insulin therapy entirely without substantial recipient risk. The growing

CONTACT A. M. James Shapiro amjs@islet.ca Canada Research Chair in Transplantation Surgery and Regenerative Medicine, 2000 College Plaza, 8215 112th St, Edmonton, AB, Canada, T6G 2C8.

A version of this section was published as a Review in *Islets 2018* Feb; e1428511.
(Gamble, A., et al. (2018). "The journey of islet cell transplantation and future development." Islets 10(2): 80-94.)

ORIGINAL ARTICLE

The Journey of Islet Cell Transplantation and Future Development

Anissa Gamble [1, 3], Andrew R. Pepper[1, 2,3], Antonio Bruni[1, 3], and A.M. James Shapiro[1,2, 3*]

[1]Alberta Diabetes Institute, University of Alberta, Edmonton, AB, Canada

[2]Clinical Islet Transplant Program, University of Alberta, Edmonton, AB, Canada

[3]Members of the Canadian National Transplant Research Project (CNTRP)

*Corresponding Author: A.M.J. Shapiro, amjs@islet.ca

Dr. A.M. James Shapiro

Canada Research Chair in Transplantation Surgery and Regenerative Medicine

2000 College Plaza, 8215 112th St Edmonton AB Canada T6G 2C8

+1-780-407-7330

1.1 Chapter Overview

Intraportal islet transplantation has proven to be efficacious in preventing severe hypoglycemia and restoring insulin independence in selected patients with type 1 diabetes. Multiple islet infusions are often required to achieve and maintain insulin independence. Many challenges remain in clinical islet transplantation, including substantial islet cell loss early and late after islet infusion. Contributions to graft loss include the instant blood-mediated inflammatory reaction, potent host auto- and alloimmune responses, and beta cell toxicity from immunosuppressive agents. Protective strategies are being tested to circumvent several of these events including exploration of alternative transplantation sites, stem cell-derived insulin producing cell therapies, co-transplantation with mesenchymal stem cells or exploration of novel immune protective agents. Herein, we provide a brief introduction and history of islet cell transplantation, limitations associated with this procedure and methods to alleviate islet cell loss as a means to improve engraftment outcomes.

1.2 Islet Cell Transplantation Overview

1.2.1 Introduction and Brief History

Globally, diabetes affects over 382 million people, with roughly 10% presenting with type 1 diabetes mellitus (T1DM) and is expected to rise to 592 million by 2035. [1] An annual 3% growth rate affords an escalating financial burden where the International Diabetes Federation estimates in Canada alone diabetes-related health care costs was $14 billion in 2015. These are expected to climb to a staggering $16 billion per annum by 2020. [2] Although the etiology of T1DM is incompletely elucidated, it is characterized as a multifactorial autoimmune disease resulting from specific immune-mediated destruction of pancreatic beta (β) cells within the islets of Langerhans. Classic symptoms include polyuria, polydipsia, and polyphagia with confirmation of diagnosis marked by hyperglycemia, low or indetectible serum C-peptide levels, elevated glycosylated hemoglobin (HbA_{1c}), and one or more positive autoantibody markers. [3] Those with T1DM must administer frequent exogenous insulin therapy to maintain normoglycemia. Continuous glucose monitoring systems (CGM) and insulin pumps may further help mitigate glycemic fluctuation. Recently, the FDA approved a closed-loop technology that infuses glucose regulatory hormones (insulin and glucagon) in response to glycemic fluctuations. While tighter glycemic control with medical intervention has been clearly shown to reduce secondary complications, it substantially increases risk of severe hypoglycemic reactions. T1DM is associated with a shortened life expectancy by 13 years. [4]

In consequence, the research community has focused on new avenues to arrest T1DM at the time of diagnosis. Intensive “new-onset” pilot trials conducted by TrialNet, a group of researchers aimed at identifying the prognosis and prevention of T1DM, have demonstrated means to sustained honeymoon periods and delayed diabetes onset. [5] In Brazil, Voltarelli and colleagues are currently conducting clinical trials aimed to reset the immune system in new-onset diabetes through

administration of peripheral blood autologous bone marrow-derived hematopoietic stem cells coupled with immunodepleting conditioning (NCT00315133). [6-8] This approach led to impressive reversal in the diabetic state in 21 children and adolescents with new-onset T1DM, but was also associated with substantial side-effects. To date, no protocol has yet to eradicate exogenous insulin therapy entirely without substantial recipient risk. The growing prevalence of T1DM is concerning, and alternatives to insulin injections are needed desperately.

Beta cell replacement therapy through islet transplantation (IT) provides a potential alternative to exogenous insulin. The history of IT extends 23 years before the discovery of insulin, when Watson-Williams and Harsant in 1893 in Bristol UK attempted to treat a 13 year old boy dying from acute ketoacidosis with transplantation of pieces of sheep's pancreas. [9, 10] Although the patient had minor glycemic improvements, he ultimately died 3 days after this futile first attempt at xenotransplantion. The concept of isolating islets was not revisited till 1972, when Paul E. Lacy restored glycemic control with intraportal vein infusion of islets into chemically-induced diabetic rats. [11] In 1980, David Sutherland and John Najarian, two innovative surgeons working in Minnesota, demonstrated successful intraportal islet transplantation in 10 patients with surgical induced diabetes, where the patients' own islets (autografts) were infused back after islet isolation; ultimately 3 of these patients achieved insulin independence for 1, 9 and 38 months, respectively. [12] The development of the Ricordi® Chamber and the semi-automated method for islet isolation was developed by Camillo Ricordi while working in Paul Lacy's laboratory in St. Louis. [13] This semi-automated method remains state-of-the-art today, and is available commercially (BioRep, Miami, FL, USA). In 1990 David Scharp, also working with Camillo Ricordi and Paul Lacy in St. Louis, reported the first case of transient insulin independence after islet allotransplantation, in the context of recipient immunosuppression. [14] Despite substantial advances, fewer than 8% of the 267 islet transplant attempts between 1980 and 1999 resulted in insulin independence for longer than one year. [15] In 2000, the *Edmonton protocol* developed by Shapiro *et al*. made IT a feasible

clinical procedure. The *Edmonton protocol* was ground-breaking as it utilized a corticosteroid-free immunosuppressive protocol by combining two potent immunosuppressants: sirolimus and tacrolimus, together with an anti-CD25 antibody to protect against rejection and recurrent autoimmunity. This protocol augmented the islet mass with two or more fresh islet preparations, infusing a total islet dose that was substantially higher than had been used previously in clinical islet trials (>13,000 islet equivalents (IE) kg^{-1} recipient body weight). [16] All seven-consecutive treated T1DM subjects remained insulin independent for >1 year with sustained C-peptide production after portal vein infusion. [16] A subsequent 5-year follow of the Edmonton protocol demonstrated that most subjects lost complete insulin independence by year 3-5, with only 10% remaining insulin free by 5 years. However 80% maintained strong C-peptide secretion, which was sufficient to correct the HbA1C <7%, and most importantly protected recipients from severe hypoglycemic events. [17] The success of the Edmonton protocol rejuvenated global interest in clinical IT and at least 30 new islet centres initiated activity. The Collaborative Islet Transplant Registry (CITR) in 2001 allowed progress to be tracked closely. The most recent CITR report registered 1,584 IT infusions in 819 patients between 1999 to 2013, and currently, 27 active registered centers are active. [18] IT has improved substantially over the past 17 years with multiple further refinements including more optimal islet preparation, culture, safer transplant techniques and more effective anti-inflammatory and immunomodulatory interventions. Likely cellular replacement therapies will become mainstay treatment, more practical and cost effective, for larger numbers of T1DM patients.

1.2.2 Islet Cell Transplantation Procedure – Isolation, Purification and Infusion

IT requires sequential steps including donor pancreas procurement, islet isolation, purification, culture and infusion. Attention to detail throughout all steps in this process are required to maximize islet integrity and survival. Organ donation from a multiorgan donor

(neurological determination of death, or more recently also deceased cardiac death donors), after donor family consent. Donor characteristics, including age, body mass index and absence of diabetes in the donor (HbA1C <6.5%) may affect islet yield. [19] While obese donors previously provided the best islet mass, improvements in collagenase enzymes and purification protocols have improved the success of islet isolation from the younger, thinner donors too. After the pancreas is flushed and cooled with preservation solution (University of Wisconsin (UW) or Histidine Ketoglutatate (HTK) solutions via intravascular flush, the pancreas is surgically removed and packaged for transport to the isolation center. It is essential that the pancreatic capsule remains intact and uninjured if the pancreas is to be distended with collagenase satisfactorily once the pancreas reaches the isolation laboratory. Once in the clean room facilities (clinical Good Manufacturing Practice (cGMP) approved), the duodenum, spleen and fatty tissues are dissected away from the pancreas, the pancreas transected at the neck or mid-body, and the pancreatic duct cannulated in both proximal and distal directions. The pancreatic duct is then perfused with cold then warmed collagenase solutions under pressure for 10 minutes to load the pancreatic acinar-islet interface with digestive enzyme. The pancreas is then chopped into multiple pieces (typically 9 or 10 large fragments), and transferred to the Ricordi Chamber where warm collagenase enzyme and serine protease solutions are recirculated while the chamber is shaken to facilitate separation of islets from their exocrine stromal matrix. The Ricordi Chamber serves to both mechanically and chemically digest islets. Once islets are liberated into the solution, the digestion is halted by cooling to 4ºC and the enzyme is further quenched with the addition of collagenase binding proteins (human albumin solution). The islet digest is then purified on a COBE 2991 cell processor using a continuous density gradient of BioChrom Ficoll solution to separate islets from the exocrine tissue, the islets being less dense on centrifugation. Islets are then cultured for 24-72 hours at 20ºC or 37ºC (centre dependent) in media supplemented with insulin, transferrin and selenium. Before transplantation, the purified islet preparation must undergo detailed quality

control testing to assess islet viability, purity, insulin content, cell number and insulin secretory response. Islets must have adequate purity (> 50%), dose (>5000 IEQ kg^{-1}), a settled tissue volume <7cc, and be sterile on Gram stain. [20, 21] The islet culture step may minimize the number of dying islets and acinar cells that are infused into the recipient, but these dying cells also create a toxic milieu for the remaining islets during culture. [19, 22] Maximizing the infused islet mass, matched to the ABO-blood type of the recipient, is important as it decreases the need for multiple donor islet infusions. The fewer the donors required reduces the risk of HLA-recipient sensitization. Although considerable research efforts have been made in the field, the optimal protocol has still to be standardized

Currently intraportal islet infusion remains the gold standard site for implantation. To date, this is the only site that has reliably led to high rates of insulin independence in patients with T1DM. The portal vein may be safely accessed by a minimally invasive percutaneous transhepatic access route. The advantage of this approach is that patients do not require surgery or general anesthesia. The early disadvantage was that some patients developed intraperitoneal bleeding from the liver surface after the portal catheter was withdrawn. Refinement in this technique with occlusion and obliteration of the catheter track using soluble hemostatic paste agents (Avitene paste or D-STAT) have virtually eliminated this complication in the larger centre experience. Administration of therapeutic heparin at 70 units per kg recipient weight delivered intraportally with the islets, and a heparin infusion initiated at 3-5 units/kg/hour then adjusted to main a PTT between 60-80 seconds has further eliminated the risk of branch vein portal venous thrombosis, another recognized complication of this procedure. Maintaining a purer islet preparation of typically 2-3ccs of islet tissue coupled with systemic heparinization has been an important component in mitigating this thrombotic risk. [20] Although intrahepatic infusion is associated now with minimal complications, the intravascular site fails to provide an optimal environment for islet survival, and it has been estimated that >60% of the infused islet mass is destroyed within

minutes to hours by innate immune responses. [23] Means to optimize islet survival throughout all steps in the islet preparation process is seen as key to the success of this approach (**Fig 1.1**).

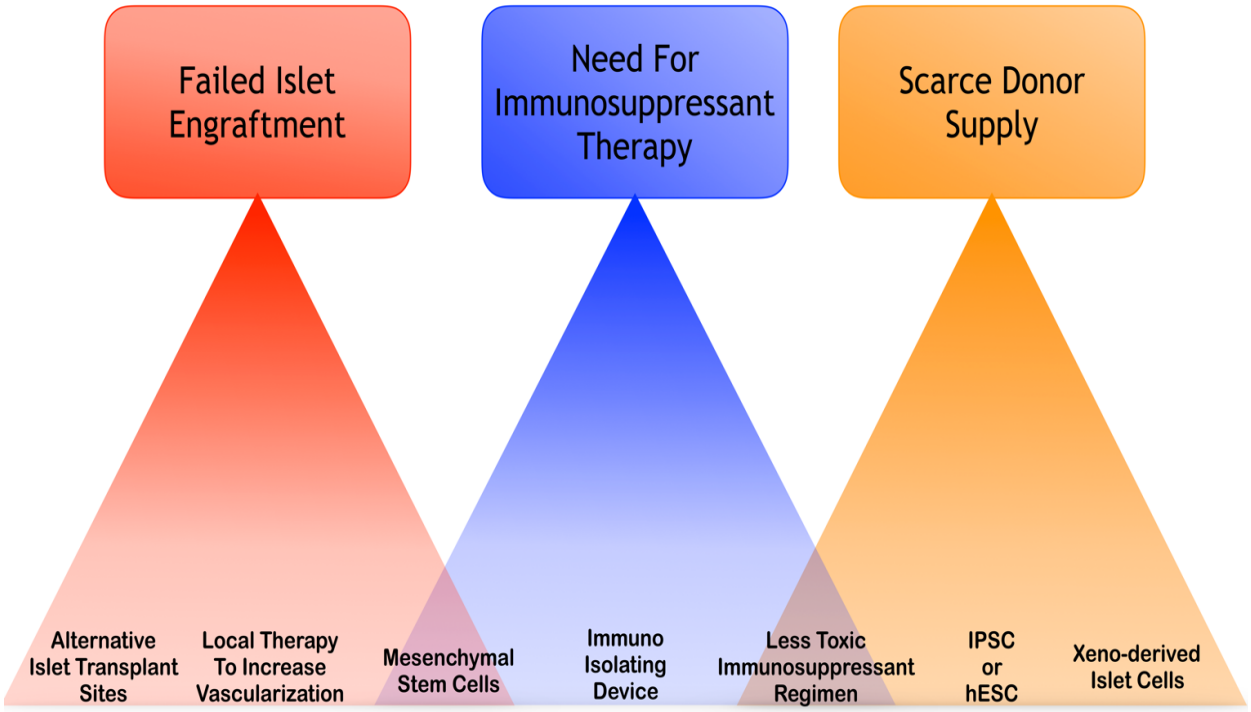

Figure 1.1: Drawbacks of islet transplantation

Islet transplantation is a temporary alleviation from exogenous insulin injections. Drawbacks include, but not limited to failed engraftment, the need for a lifelong immunosuppressant therapy, and scarce donor supply. Displayed in colors are possible avenues to help alleviate these drawbacks.

1.3 Current Limitations and Possible Alleviations in Clinical Islet Transplantation

1.3.1 Instant Blood-Mediated Inflammatory Reaction (IBMIR)

The innate destruction of transplanted islet tissue occurs through an intense reaction called the instant blood-mediated inflammatory reaction (IBMIR). The process is triggered by exposed tissue factor on the islet surface, which attracts platelets that bind and undergo their release reaction, and a cascade of clot adherence and intense inflammatory cellular recruitment follows. [24, 25] Potential means to reduce inflammatory islet stress and protect islets can be achieved through addition of anti-inflammatory agents during islet culture and systemically to the recipient, administration of anticoagulants, or islet coating with a variety of protective macromolecules. [26] The infusion of anticoagulation agents such as dextran sulfate or heparin has been shown to improve islet survival by downregulating the IBMIR response in the experimental setting, but remains to be validated in clinical studies. [27-29]

1.3.2 Alternative Transplantation Sites

As islets are infused intraportally, they embolize and become trapped within the portal sinusoidal capillaries. This may render islets ischemic, and apoptotic or necrotic islet death may ensue. [19, 30] The inability to locate, visualize or biopsy human islets within the intrahepatic site creates a challenge, and has hampered progress as the scale and relative nature of the various insults affecting islet survival cannot be quantified easily. Several investigators have searched for more favorable extrahepatic sites that might obviate IBMIR and provide more ready access for biopsy, imaging and retrieval. Such sites have included the renal subcapsular space, [31] striated muscle, [32] pancreas, [33] omentum, [34] eye chamber, [35] and testis [36,] Although these alternative sites can reverse hyperglycemia in small animal models, thus far only the omentum has recently

allowed a small number of subjects to become insulin independent for short periods of time. Thus far, all attempts to develop a clinically applicable site that is proven to be superior to the intraportal site have remained elusive. The renal subcapsular site is in favored and most efficient for islet implantation in rodents over the intraportal site, but this does not translate in species larger than mice and rats. [37] The subcutaneous space remains an attractive consideration as alternative embryonic stem cell products are being developed for the clinic, mainly because this site is easily retrievable. However, the limited vascularization and low oxygen tension of this site poses challenges. Efforts to improve islet survival within the subcutaneous space may be achieved by the use of a prevascularized technique, which harnesses the natural foreign body reaction, achieved by pre-implanting a catheter. [38] Our laboratory developed a "deviceless" method that implants islets into a prevascularized subcutaneous site created by the temporary placement of a 5 or 6F hollow nylon medical-grade catheter used in angiography. [39] The deviceless method was found to be highly effective in reversing diabetes in full dose and marginal mass islet transplants with human or mouse islets, and with human derived-pancreatic endoderm cells. [39, 40] We have yet to test this approach in larger animals or in patients, so the utility of this approach remains unknown in clinical translation.

A Canadian based biotechnology company, Sernova Corp., developed a permanent plastic mesh-based device with removable plugs called the Cell Pouch™. After Health Canada approval, the device was loaded with human islets and implanted into 3 patients treated at the University of Alberta. While the device was able to reverse diabetes successfully in mice, none of the patients demonstrated any islet function, and had only *de minimus* islet survival upon device explantation. [41,42] Considerable further research is required to refine these and other approaches if they are to be useful in the clinic. Another promising alternative IT site is the omentum based on the expansive surface area, rich blood supply, portal drainage and potential for minimal access surgery. By

folding the omentum upon itself, additional surface for oxygenation and metabolic exchange may be accomplished. [43] The University of Miami is currently completing a Phase I/II clinical trial with this approach, transplanting human allogenic islets coated in autologous plasma and placed using laparoscopic instruments onto the wrapped omentum (NCT02213003). Recently, a 43-year-old diabetic woman was rendered normoglycemia with this approach, and its clinical investigation is ongoing. [44] The intramuscular site is a vascular enriched site that has comparable blood flow to the native pancreas. [45] In Sweden, a 7-year-old girl receiving intramuscular autologous islets after a total pancreatectomy was reported; this subject had detectible C-peptide but failed to gain insulin independence. [46]

1.3.3 Encapsulation Technologies

The potential to shield transplant islets or stem cells from immune attack through micro or macroencapsulation approaches is a concept that has been explored extensively over the past seven decades. Encapsulation utilizes selectively permeable membranes that permit passive diffusion of glucose, insulin, oxygen, carbon dioxide and other nutrient exchange while preventing direct cell-cell contact with immune cells. Factors to consider in evaluating such devices include the site of transplantation, the device configuration, the materials used, and their ability to promote neovascularization and biocompatibility. [47]

Macroencapsulation involves encapsulating multiple islets within a device >1mm diameter and is usually placed in an extravascular space. [48] The use of macroencapsulation dates back to the 1950's when Algire, Prehn, and Weaver transplanted thyroid tissue within a device made of lucite rings, membrane filters, and lucite-acetone seal. [49, 50] Several studies demonstrate islet cell survival within macroencapsulation devices in mice, but translation to larger animals or humans is often limited by fibroblastic overgrowth around the device. [51] In the 1990's a double-membrane sealed device called the Theracyte™ device was developed by Baxter Healthcare and showed initial

promise, but failed to maintain euglycemia. [50, 52, 53] In 2013, Ludwig *et al.* used an oxygenated macro-chamber (Beta-O_2) to implant human islets without immunosuppression beneath the abdominal wall skin of patients. Human islets were stabilized in an alginate matrix. [54] Preliminary published data confirmed that patients had detectible human C-peptide in the complete absence of immunosuppression, but none were insulin independent. [55] ViaCyte Inc. created a macroencapsulation device termed Encaptra ™, which also has an outer plastic weave support matrix and an inner thin immune barrier layer to protect implanted cells. In 2014, ViaCyte Inc. launched a Phase I/II combination trial of human embryonic stem cells derived product (PEC-01, derived from Cyt49 cells, implanted within Encaptra ™) (VC-01™, NCT02239354). In 2017 ViaCyte further initiated a second trial using a perforated macroencapsulation device containing PEC-01 cells, in which it is anticipated that cell survival will be improved by more optimal neovascularization, but recipients in that trial will require full systemic immunosuppression (PEC-direct™ (VC-02™, NCT03163511). Islet Sheet Medical developed a flat sheet device made of ultra-thin biocompatible polymer which showed early promise in small animal models, but failed to be replicated in larger animal studies. [48] The concept of macroencapsulation has been around for several decades, but is still plagued by cell survival challenges as cells are cut off from physiologic gaseous and nutrient exchange. Ongoing studies will help to define the utility of such approaches with the hope that transplants could be conducted without need for chronic immunosuppression. Importantly, these devices have not been tested thoroughly in human patients with autoimmune diabetes, and it remains unknown how effective they may be in preventing recurrent autoimmunity.

The alternative approach of microencapsulation involves coating of individual islets or islet clusters in an immunoprotective envelope. In 1964, Chang *et al.* described microencapsulation [56] and in 1980 Lim and Sun demonstrated islet survival with alginate-polylysine-polyethyleneimine microcapsules. [57] Alternative microencapsulation materials have been explored, including polyethylene glycol, poly methyl methacrylates, alginate, agarose, or chitosan. [48,58]

In one study, Vegas *et al.* maintained normoglycemic for 174 days and suppressed perivascular overgrowth in mice. [59] Human islets have heterogeneous diameters ranging from <50-500μm, and manufacturing a microencapsulation system that accommodates this range has proven to be a challenge. [60] Conformal coating has generated much interest, but even this approach frequently leads to islets that breech the microencapsulation barrier, and when transplanted leave exposed donor antigens accessible to the recipient immune system. Shielding of direct immune cell-to-cell contact may be an over-simplistic approach as it overlooks the cytokine and damage associated membrane products (DAMPs) small molecule cross-talk between contained damaged and dying donor cells that can be sensed by the recipient immune system. Clinical trials of encapsulated pig islets by Diabecell® generated much interest, but the final results of those trials are disappointing as insulin requirements remain unchanged from baseline and pig C-peptide was undetectable. [61,62] Although microencapsulation holds potential promise, materials and nutrient exchange remain suboptimal to sustain islet survival. Ongoing studies will determine if some of these barriers may be overcome with more refined biomaterials and technologies.

1.3.4 Islet Graft Revascularization

The islets of Langerhans constitute ~2% of the total pancreatic mass but receive up to 20% of the pancreatic blood flow. [63] Revascularization is imperative for islet survival after transplantation. Islets have a dense network of sinusoidal capillaries that drain into peripheral venules. [64] The process of islet isolation strips off these capillary networks, and islets must therefore neovacularize if they are to survive. Angiogenesis begins between the first and day post-transplant, and expands for the first 14 days, as new arteriolar vessels grow in from recipient origin. [65] Vascular remodeling may then continue for up to 3 months. [65]

Islets and vascular endothelial cells express high levels of vascular endothelial growth factor (VEGF) that serve to recruit neovascularization. [64,66] Supplementation of VEGF in the

islet graft may have both positive and negative impact, as VEGF also recruits and amplifies inflammation that can also be destructive to islet survival. Cheng *et al.* utilize an adenovirus containing cDNA from human VEGF isoforms and transplants transfected islets into diabetic nude mice and found improved islet revascularization with normoglycemia. [67] VEGF also stimulates release of interleukin, increasing blood flow to ischemic tissue. [68] Hepatocyte growth factor (HGF), fibroblast growth factor (FGF), epidermal growth factor (EGF), and matrix metalloproteinase (MMP) may also expedite the islet vascularization process. [69, 70] Basterrechea *et al.* applied a plasma-based scaffold containing fibroblasts to augment subcutaneous IT function in mice. [71]

1.3.5 Brief Overview of Oxidative Stress

Oxidative stress is associated with release of free radicals especially reactive oxygen species (ROS). [72] Islets are especially vulnerable to pro-inflammatory cytokines (e.g. TNFα), free radicals (i.e. H_2O_2 or peroxynitrite) and superoxide dismutases (SODs). [73] Treatment of islet preparations with potent antioxidants may mitigate oxidative stress. Supplementation with glutathione (GSH) was able to decrease apoptosis and reduce intracellular ROS during islet isolation. [74] This supplementation may have the converse detrimental effect of disrupting VEGF synthesis and thereby impede neovascularization. [75] An antioxidant metalloporphyrin analog BMX-010 improved islet function and survival and was non-toxic to islets. [72] A pilot study using BMX-010 is currently underway at the University of Alberta to evaluate the impact of this agent in improving single donor islet engraftment. [76] Controlling oxidative stress could provide promise for improved islet survival.

1.3.6 Mesenchymal Stem Cells to Improve Islet Engraftment

Mesenchymal stem cells (MSCs) were first identified by Friedenstein *et al.* in rat bone marrow in 1966. [77] MSCs are non-hematopoietic precursor cells that can differentiate into mesoderm lineages: osteocytes, chondrocytes, myocytes, and adipocytes. [78] MSCs may be

isolated from amniotic fluid, [79] skeletal muscle, [80] adipose tissue, [81] umbilical cord, [82] and human umbilical cord perivascular cells. [83] MSCs may be transdifferentiated into insulin-producing cells, but have yet to be rendered as fully functional β-like cells. [84] MSCs also secrete trophic factors that may stimulate and support tissue regeneration, [85] and also hold immune regulatory properties that could also suppress allograft rejection. [86] (**Fig. 1.2**)

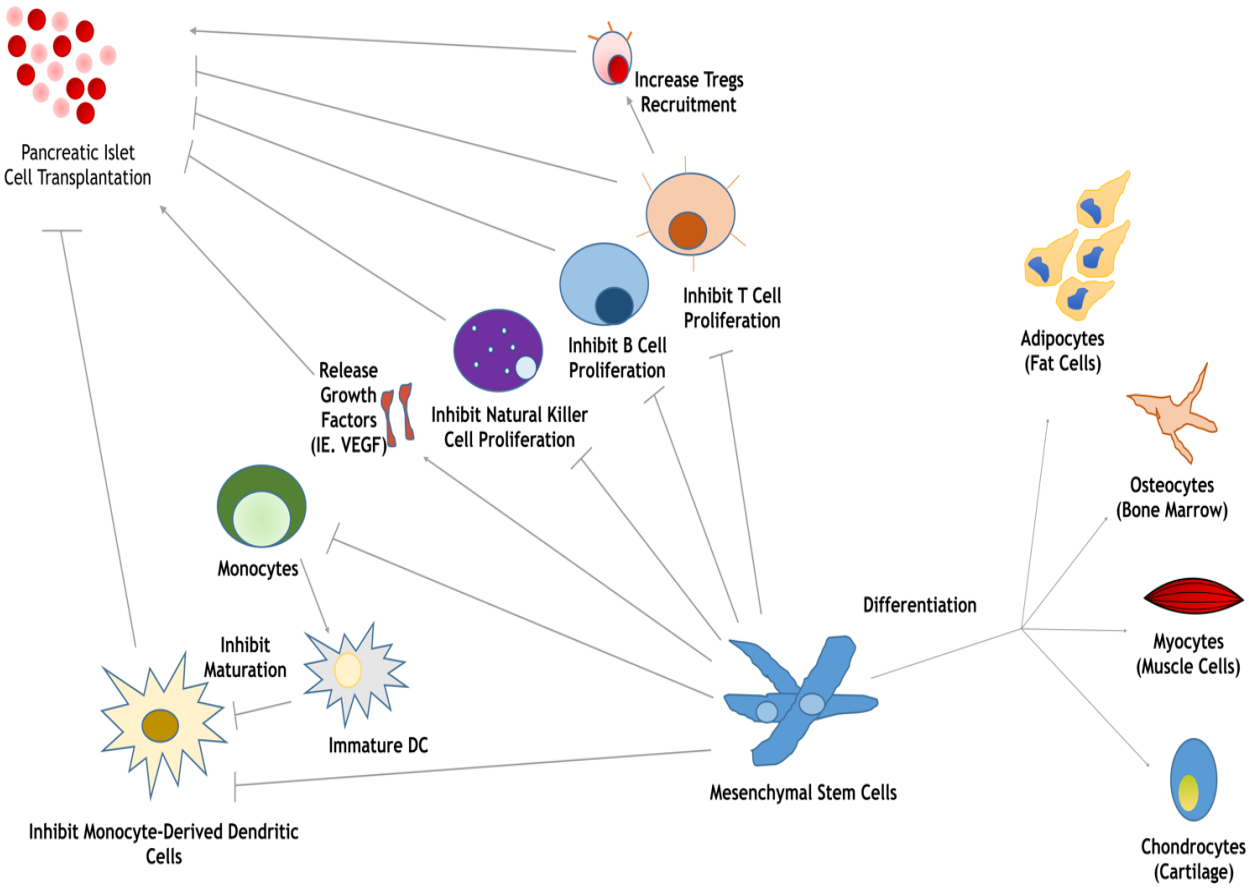

Figure 1.1: Mesenchymal stem cell differentiation and co-transplantation

The benefits of MSCs effectiveness to ameliorate islet cell transplantation and MSCs capability to differentiate is displayed. The multipotent capability of MSC can differentiate into mesodermal lineages such as adipocytes, osteocytes, myocytes, and chondrocytes. MSCs co-transplanted with pancreatic islets can decrease the proliferation of natural killer cells, dendritic cells, monocytes, B cells and T cells. The inhibition of T cells promotes regulatory T cells (Tregs). Alongside, MSCs release trophic factors that can improve islet engraftment.

1.3.6.1 Trophic Factors

MSCs may promote angiogenesis through gene expression of cytokines, including VEGF, fibroblast growth factor (FGF), Angiopoietin-1 (Ang-1), matrix metalloproteinase (MMPs) and transforming growth factor-β (TGF-β). [87, 88] Such growth factors may facilitate islet survival. MSCs can migrate to sites of injury and release paracrine factors that regulate local inflammation, and may promote revascularization and repair at the transplant site. [89, 90,91] Ongoing studies will help define the potential role of MSC co-transplantation aimed at improving islet survival. [92]

1.3.6.2 Immunomodulatory Effects of MSCs

MSCs may immunomodulate both innate and adaptive immune responses in experimental islet transplantation, both through direct and indirect antigen presentation. [88, 93, 94] MSCs suppress T lymphocyte proliferation and have low human leukocyte antigen (HLA) Class I expression. Low but inducible Class II MHC expression by MSCs could further modulate allogeneic rejection. [95] MSCs may decrease B cell proliferation, [96] natural killer cells, [97] and monocyte-derived dendritic cells. [93] MSCs suppress T cell reactivity and proliferation, and increase recruitment of T-regulatory cells (Tregs). [98] MSC secretion of matrix metalloproteinases 2 and 9 may block T cell expansion and activation. [99] Tregs are master regulators of immune reactivity and involution, and could potentially facilitate immune tolerance induction or minimize the need for chronic long term immunosuppression in transplant recipients. [100] Berman *et al*. noted Tregs recruitment occurred with co-transplantation of allogeneic islets and third-party MSCs, and led to prolonged islet survival. [101] MSCs decrease T cells through reduced differentiation, maturation, and dendritic cell (DC) function. [102] CD11c (DCs phenotype derived from monocytes) and CD83 (mature DCs phenotype) can be down-regulated in mice using co-transplantation of pancreatic islets with MSCs. [103] Co-transplantation of bone marrow MSCs co-cultured with different agonist antibodies including anti-CD40, or anti-IL-4 markedly inhibited B cell and immunoglobulin production.

[96] MSCs alter natural killer (NK) cell function by suppressing proliferation and cytotoxicity. Spaggiari *et al.* found that MSCs inhibited NK cell function. [97] The role of MSCs in cross-regulation of cytokine production and immune cell function merits ongoing intense research. [104]

1. 4 Reversing Autoimmunity in Type 1 Diabetes

All future strategies that aim to reverse diabetes with cellular replacement of insulin secreting cells will require some adjunctive strategy to prevent recurrent autoimmune destruction of the newly transplanted cells. TrialNet is a group of international scientists that have focused efforts in reversing autoimmunity in T1DM. Over 500 strategies have proven effective in NOD autoimmune mouse models, but very few have translated to clinical benefit. This suggests that the mouse model is an inadequate representation of the human disease, and that strategies that interrupt autoimmunity in mice are inadequate when applied to the far more complex human immune system. Haller *et al*. gave newly diagnosed T1DM subjects autologous umbilical cord blood and reported lower HbA_{1c} and reduced insulin requirement. [105] This study is small and underpowered, and lacked appropriate control groups to demonstrate clear efficacy in the intervention arm. Voltorelli *et al.*, and more recently Couri *et al.* infused with hematopoietic stem cells after myeloablative conditioning in children with new onset T1DM, and reported remarkably high rates of insulin independence with restoration of endogenous C-peptide production. [7] This approach was associated with infectious and other complications related to the conditioning regimen, including sterility. Bluestone *et al.* infused autologous polyclonal reactive *ex vivo* expanded Tregs into patients with new onset T1DM and markedly prolonged the honeymoon period with that approach. [106,107]

1.5 Alternative Islet Cell Sources:

The available organ donor supply will never be sufficient to match the potential demand if cellular replacement therapies are to play a greater role in the treatment of all patients with T1DM and T2DM. Thus, alternative strategies including gene therapy, xenotransplantation and stem

cell transplantation are being explored to bridge this gap. Transfecting non-islet cells to contain and express glucose-regulated insulin is an attractive approach that has been tested in keratinocytes, [108] adipose-derived stem cells, [109] and hepatocytes. [110] Hepatocytes share a common endodermal origin with pancreatic islets, and hepatocyte adenoviral transfection with genes encoding for human proinsulin have demonstrated ability to secrete human insulin and C-peptide and maintained normoglycemia in small and large animal models. [111] If a glucose-sensitive promoter could be included in these constructs, and if the vectors were less antigenic, their potential could be substantial in the future management of all forms of clinical diabetes.

1.5.1 Xenotransplantation

Xenotransplant sources of islet replacement have been considered for many years. Neonatal or adult pig islets provide an attractive source. [112] First-in-human trials by Carl Groth and colleagues in 1994 involved transplantation of fetal pig islets placed beneath the kidney capsule of human kidneys in patients with diabetes and renal failure. [113] These studies were remarkable as porcine C-peptide was detectable for >300 days in many subjects, and no serious side effects were observed. However, no reduction in insulin requirement and no insulin independence was ever observed. The opportunity to genetically manipulate the pig genome initially with knock-out constructions for decay accelerating factor, and Gal epitopes, and more recently the potential to humanize the pig genome using CRISP-Cas9 technologies, offers great potential. [114, 115,116,117] These technologies have been used recently to eliminate porcine endogenous retroviruses from the pig genome. CRISPR-Cas9 was used to inactivate 62 copies of the PERV pol gene in a porcine cell line and resulted in >1000-fold reduction in PERV transmission to human cells. [118] Nui *et al.* formulated PERV-inactivated pigs with this approach. In New Zealand, encapsulated neonatal porcine islets have been transplanted into non-immunosuppressed T1DM patients but with minimal if any detectable function to date. [119] Detectable porcine C-peptide has been strikingly absent in these subjects, and

insulin requirement reduction has been modest, suggesting that these cells are non-functional to date. A similar study in Argentina with encapsulated porcine islets showed similar findings, but claimed modest reduction in HbA_{1c} and some correction of hypoglycemic unawareness, but these studies lack sufficiently rigorous controls to validate that the function is all derived from the transplanted xenogenic cells. [120] Ongoing studies are required to validate such approaches, and the further application of CRISP-Cas9 to humanize the porcine genome will generate more promise, but additional ethical challenges too.

1.5.2 Pluripotent Stem Cell Transplantation

1.5.2.1 Human Embryonic Stem Cells (hESCs)

Human embryonic stem cell (hESC) and induced pluripotent stem cells (iPSC) are being intensively investigated for their ability to differentiate into insulin producing cells. Essential expression of a series of transcription factors including pancreatic homeodomain transcription (PDX1), the homeobox transcription factor NKX61, and MafA have been used to generate pancreatic progenitor cells. [121, 122, 123] In 2004, Kubo *et al.* [124] successfully differentiated hESCs into pancreatic endoderm cells (PEC). In 2006, a California-based company called ViaCyte Inc. generated PEC-01 cells that experimentally displayed positive C-peptide, proinsulin, and key transcription factors that led to regulated insulin production after transplantation and *in vivo* differentiation. [125] ViaCyte Inc. utilizes a single pluripotent embryonic stem cell line, termed CyT49, that differentiates into PEC-01 cells. The PEC-01 cell population is intended to mature into glucose-responsive and insulin-producing cells and continues to differential *in vivo* after implantation. Two clinical trials of Viacyte's PEC-Encap™ (VC-01™) and PEC-Direct™ (VC-02™) utilize these hESC-derived pancreatic endoderm cells contained in a macroencapsulation device. In trial VC-01, the device has an intact membrane that prevents direct immune cell-to-cell contact, whereas in VC-02 the device has laser microperforations designed to improve

neovascularization and stem cell survival, but recipient subjects in this second trial require full dose immunosuppression. Ongoing data is eagerly awaited to validate the safety and preliminary efficacy of these promising approaches.

ViaCyte cells are considered 'Stage 4' and are immature at the time of transplantation. The advantage of this approach is that the metabolic demands of cells at this stage may be less than their fully mature metabolically active counterparts. Furthermore, expression of Class II HLA antigens may be less, and so the cells may be less immunogenic. This remains to be proven; however, such cells take 2-3 months to fully mature in mice, and are not expected to work instantly when transplanted into patients. Those with longstanding T1DM may be more than happy to wait the 2-3-month maturation period which may be inconsequential. However, other groups including Rezania *et al.* have further differentiated these types of cells to a more mature 'Stage 7' phenotype, which are more mature and engraft faster after implantation in mice. [121] Paglucia *et al.* used similar 'Stage 7' cells to avert diabetes onset in mice, and demonstrated more robust function *in vitro*. [126] Russ *et al.* confirmed earlier diabetes reversal with similar cellls. [127] Doug Melton and colleagues within the Harvard Stem Cell Institute and Semma Therapeutics have used a 6-step protocol to create more mature human β cells from hESC-derived cells. [126, 128] The potential risk of teratogenicity warrants caution in all approaches that use hESC-derived product. Whether a benign teratoma would have serious consequences in patients, or whether unregulated growth would pose high risk of hypoglycemia remains to be tested, but these remain of concern in first-in-human trials. There are potential ethical and religious considerations when hESCs are used, as the starting cell population is derived from discarded human embryos taken at the blastocyst stage. ViaCyte's Cyt49 and subsequent PEC-01 derivation was obtained from just one human discarded embryo. Many, but not all might consider that a small ethical price to pay for the potential to provide a limitless cell source for future diabetes treatments.

1.5.2.2 Induced pluripotent stem cells (iPSCs)

In 2006, Shinya Yamanaka's group from Kyoto Japan developed a protocol for dedifferentiating and transdifferentiating adult human pluripotential stem cells. [129] The Yamanaka genetic factors Oct3/4, Sox2, Klf4 and c-Myc allowed human skin fibroblasts to dedifferentiate and to mature into human cardiomyocytes. Ongoing intense research will determine the utility of generating patients' own β cells with the iPS approach. Controlling recurrence of islet autoimmunity will be key to the success of this approach. Patients will not require immunosuppression, but the costs associated with good manufacturing practice (GMP) manufacture of individualized stem cells could be astronomical, and there are still many hurdles to cross.

1.6 Post-Transplant Limitations

1.6.1 Protecting Against Immunosuppressant-Related Toxicity

Islet and future stem cell therapies will not be considered truly 'curative' until such treatments can be delivered and maintained without need for chronic immunosuppression. Antirejection drugs paralyze immune responses to alloantigens effectively, but also increase the risk of life-threatening infection or malignancy. Furthermore, the most potent antirejection drugs (tacrolimus and cyclosporine) have direct toxicity to β cells. [130,131, 132] Approaches that limit need for immunosuppression will lower the barrier for future patients with diabetes being considered for cellular therapy. Gala-Lopez *et al.* found that an anti-aging glycopeptide (AAGP) protected human islets from tacrolimus toxicity and promoted graft survival and function in animal models. [133] Ongoing work in the area of tolerance induction using myeloablative chemotherapy or non-ablative cellular therapeutics including facilitating cells or Tregs could transform future opportunities across all aread of transplantation.

1.7 Conclusion

T1DM remains a chronic autoimmune disease resulting from permanent destruction of β-cells. Improvements in new insulin formulations, continuous insulin and now coupled glucagon infusion pumps, and continuous glucose monitoring systems represent advances in care, but are still cumbersome, imprecise and costly. Cellular replacement with IT has also advanced considerably, and this therapy is of proven benefit in protecting against hypoglycemia, correcting HbA1C, and in many cases providing sustained periods of insulin independence. The need for lifelong immunosuppressive therapy and attendant risks of infection, cancer and nephrotoxicity pose their own unique additional challenges, making this treatment unattractive for all but those with severe risk of brittle hypoglycemia. IT success is also hampered by limited islet survival after implantation, resulting from a combination of innate immune attack through IBMIR, recurrent autoimmune islet destruction or alloimmune rejection. Optimizing neovascularization with better control of angiogenesis, suppressing inflammation and reducing oxidative stress all offer to further improve outcomes with IT. Access to human islets from available scarce organ donors makes cellular replacement therapy impractical if indications are to be broadened to include patients with both T1DM and T2DM. Alternative cell sources are therefore required. Intense efforts to improve islets derived from xenogenic sources are underway, and in parallel remarkable progress has occurred in the science and application of pluripotential stem cells, which are now entering early pilot clinical trials. The possibility that cellular transplantation could be accomplished with less need for immunosuppressant's remains a tangible possibility, and advances in immune regulation control with Treg infusions, MSC co-transplantation and other innovative approaches are underway. Further, advances in vascularized macroencapsulated oxygenated devices offer potential to shield transplanted cells from immune attach. It remains to be seen whether any of these approaches will prove to be as promising in patients as they have offered to date in mice.

1.7 References

1. Guariguata L, Whiting DR, Hambleton I, Beagley J, Linnenkamp U, Shaw JE. Global estimates of diabetes prevalence for 2013 and projections for 2035. Diabetes Res Clin Pract 2014; 103:137-49.

2. Vanikar AV, Trivedi HL, Thakkar UG. Stem cell therapy emerging as the key player in treating type 1 diabetes mellitus. Cytotherapy 2016; 18:1077-86.

3. Harjutsalo V, Sjoberg L, Tuomilehto J. Time trends in the incidence of type 1 diabetes in Finnish children: a cohort study. Lancet 2008; 371:1777-82.

4. Miller RG, Secrest AM, Sharma RK, Songer TJ, Orchard TJ. Improvements in the life expectancy of type 1 diabetes: the Pittsburgh Epidemiology of Diabetes Complications study cohort. Diabetes 2012; 61:2987-92.

5. Skyler JS, Greenbaum CJ, Lachin JM, Leschek E, Rafkin-Mervis L, Savage P, et al. Type 1 Diabetes TrialNet--an international collaborative clinical trials network. Ann N Y Acad Sci 2008; 1150:14-24.

6. Malmegrim KC, de Azevedo JT, Arruda LC, Abreu JR, Couri CE, de Oliveira GL, et al. Immunological Balance Is Associated with Clinical Outcome after Autologous Hematopoietic Stem Cell Transplantation in Type 1 Diabetes. Front Immunol 2017; 8:167.

7. Couri CE, Oliveira MC, Stracieri AB, Moraes DA, Pieroni F, Barros GM, et al. C-peptide levels and insulin independence following autologous nonmyeloablative hematopoietic stem cell transplantation in newly diagnosed type 1 diabetes mellitus. JAMA 2009; 301:1573-9.

8. Voltarelli JC, Couri CE, Stracieri AB, Oliveira MC, Moraes DA, Pieroni F, et al. Autologous nonmyeloablative hematopoietic stem cell transplantation in newly diagnosed type 1 diabetes mellitus. JAMA 2007; 297:1568-76.

9. Banting FG, Best CH, Collip JB, Campbell WR, Fletcher AA. Pancreatic Extracts in the Treatment of Diabetes Mellitus. Can Med Assoc J 1922; 12:141-6.

10. Williams P.
Notes on diabetes treated with extract and by grafts of sheep's pancreas. Br Med J 1894; 2:1303-4.
11. Ballinger WF, Lacy PE. Transplantation of intact pancreatic islets in rats. Surgery 1972; 72:175-86.
12. Najarian JS, Sutherland DE, Baumgartner D, Burke B, Rynasiewicz JJ, Matas AJ, et al. Total or near total pancreatectomy and islet autotransplantation for treatment of chronic pancreatitis. Ann Surg 1980; 192:526-42.
13. Ricordi C, Lacy PE, Scharp DW. Automated islet isolation from human pancreas. Diabetes 1989; 38 Suppl 1:140-2.
14. Scharp DW, Lacy PE, Santiago JV, McCullough CS, Weide LG, Falqui L, et al. Insulin independence after islet transplantation into type I diabetic patient. Diabetes 1990; 39:515-8.
15. Brendel M HB, Shulz A, Bretzel R. . International Islet Transplant Registry Report. University of Giessen 1999:1–20.
16. Shapiro AM, Lakey JR, Ryan EA, Korbutt GS, Toth E, Warnock GL, et al. Islet transplantation in seven patients with type 1 diabetes mellitus using a glucocorticoid-free immunosuppressive regimen. The New England journal of medicine 2000; 343:230-8.
17. Ryan EA, Paty BW, Senior PA, Bigam D, Alfadhli E, Kneteman NM, et al. Five-year follow-up after clinical islet transplantation. Diabetes 2005; 54:2060-9.
18. Collaborative Islet Transplant Registry. Rockville, MD, 2016.
19. Shapiro AM, Pokrywczynska M, Ricordi C. Clinical pancreatic islet transplantation. Nat Rev Endocrinol 2017; 13:268-77.
20. Kawahara T, Kin T, Kashkoush S, Gala-Lopez B, Bigam DL, Kneteman NM, et al. Portal vein thrombosis is a potentially preventable complication in clinical islet transplantation. Am J Transplant 2011; 11:2700-7.

21. Yamamoto T, Horiguchi A, Ito M, Nagata H, Ichii H, Ricordi C, et al. Quality control for clinical islet transplantation: organ procurement and preservation, the islet processing facility, isolation, and potency tests. J Hepatobiliary Pancreat Surg 2009; 16:131-6.

22. Berney T. Islet culture and counter-culture. Commentary on: Effect of short-term culture on functional and stress-related parameters in isolated human islets by Ihm et al. Transpl Int 2009; 22:531-3.

23. Shapiro AM, Ryan EA, Lakey JR. Diabetes. Islet cell transplantation. Lancet 2001; 358 Suppl:S21.

24. Kanak MA, Takita M, Kunnathodi F, Lawrence MC, Levy MF, Naziruddin B. Inflammatory response in islet transplantation. Int J Endocrinol 2014; 2014:451035.

25. Biarnes M, Montolio M, Nacher V, Raurell M, Soler J, Montanya E. Beta-cell death and mass in syngeneically transplanted islets exposed to short- and long-term hyperglycemia. Diabetes 2002; 51:66-72.

26. Nilsson B, Ekdahl KN, Korsgren O. Control of instant blood-mediated inflammatory reaction to improve islets of Langerhans engraftment. Curr Opin Organ Transplant 2011; 16:620-6.

27. Koh A, Senior P, Salam A, Kin T, Imes S, Dinyari P, et al. Insulin-heparin infusions peritransplant substantially improve single-donor clinical islet transplant success. Transplantation 2010; 89:465-71.

28. Johansson H, Goto M, Dufrane D, Siegbahn A, Elgue G, Gianello P, et al. Low molecular weight dextran sulfate: a strong candidate drug to block IBMIR in clinical islet transplantation. Am J Transplant 2006; 6:305-12.

29. McCall M, Shapiro AM. Update on islet transplantation. Cold Spring Harb Perspect Med 2012; 2:a007823.

30. Al-Jazaeri A, Xu BY, Yang H, Macneil D, Leventhal JR, Wright JR, Jr. Effect of glucose toxicity on intraportal tilapia islet xenotransplantation in nude mice. Xenotransplantation 2005; 12:189-96.

31. Szot GL, Koudria P, Bluestone JA. Transplantation of pancreatic islets into the kidney capsule of diabetic mice. J Vis Exp 2007:404.

32. Weber CJ, Hardy MA, Pi-Sunyer F, Zimmerman E, Reemtsma K. Tissue culture preservation and intramuscular transplantation of pancreatic islets. Surgery 1978; 84:166-74.

33. Stagner JI, Rilo HL, White KK. The pancreas as an islet transplantation site. Confirmation in a syngeneic rodent and canine autotransplant model. JOP 2007; 8:628-36.

34. al-Abdullah IH, Anil Kumar MS, Kelly-Sullivan D, Abouna GM. Site for unpurified islet transplantation is an important parameter for determination of the outcome of graft survival and function. Cell Transplant 1995; 4:297-305.

35. Adeghate E, Donath T. Morphological findings in long-term pancreatic tissue transplants in the anterior eye chamber of rats. Pancreas 1990; 5:298-305.

36. Ferguson J, Scothorne RJ. Extended survival of pancreatic islet allografts in the testis of guinea-pigs. J Anat 1977; 124:1-8.

37. Sakata N, Tan A, Chan N, Obenaus A, Mace J, Peverini R, et al. Efficacy comparison between intraportal and subcapsular islet transplants in a murine diabetic model. Transplant Proc 2009; 41:346-9.

38. Pepper AR, Pawlick R, Bruni A, Gala-Lopez B, Wink J, Rafiei Y, et al. Harnessing the Foreign Body Reaction in Marginal Mass Device-less Subcutaneous Islet Transplantation in Mice. Transplantation 2016; 100:1474-9.

39. Pepper AR, Gala-Lopez B, Pawlick R, Merani S, Kin T, Shapiro AM. A prevascularized subcutaneous device-less site for islet and cellular transplantation. Nat Biotechnol 2015; 33:518-23.

40. Pepper AR, Pawlick R, Bruni A, Wink J, Rafiei Y, O'Gorman D, et al. Transplantation of Human Pancreatic Endoderm Cells Reverses Diabetes Post Transplantation in a Prevascularized Subcutaneous Site. Stem Cell Reports 2017; 8:1689-700.

41. Gala-Lopez B. L. PAR, Dinyari P., Malcolm A. J., Kin T., Pawlick L. R., Senior P. A., and Shapiro A.M. J. Subcutaneous clinical islet transplantation in a prevascularized subcutaneous pouch – preliminary experience. CellR4 2016; 4:e2132.

42. Pepper AR, Pawlick R, Gala-Lopez B, MacGillivary A, Mazzuca DM, White DJ, et al. Diabetes Is Reversed in a Murine Model by Marginal Mass Syngeneic Islet Transplantation Using a Subcutaneous Cell Pouch Device. Transplantation 2015; 99:2294-300.

43. Kin T, Korbutt GS, Rajotte RV. Survival and metabolic function of syngeneic rat islet grafts transplanted in the omental pouch. Am J Transplant 2003; 3:281-5.

44. Baidal DA, Ricordi C, Berman DM, Alvarez A, Padilla N, Ciancio G, et al. Bioengineering of an Intraabdominal Endocrine Pancreas. The New England journal of medicine 2017; 376:1887-9.

45. Christoffersson G, Henriksnas J, Johansson L, Rolny C, Ahlstrom H, Caballero-Corbalan J, et al. Clinical and experimental pancreatic islet transplantation to striated muscle: establishment of a vascular system similar to that in native islets. Diabetes 2010; 59:2569-78.

46. Rafael E, Tibell A, Ryden M, Lundgren T, Savendahl L, Borgstrom B, et al. Intramuscular autotransplantation of pancreatic islets in a 7-year-old child: a 2-year follow-up. Am J Transplant 2008; 8:458-62.

47. Desai T, Shea LD. Advances in islet encapsulation technologies. Nat Rev Drug Discov 2016.

48. Vaithilingam V, Bal S, Tuch BE. Encapsulated Islet Transplantation: Where Do We Stand? Rev Diabet Stud 2017; 14:51-78.

49. Algire GH, Weaver JM, Prehn RT. Growth of cells in vivo in diffusion chambers. I. Survival of homografts in immunized mice. J Natl Cancer Inst 1954; 15:493-507.

50. Desai T, Shea LD. Advances in islet encapsulation technologies. Nat Rev Drug Discov 2017; 16:367.

51. Scharp DW, Marchetti P. Encapsulated islets for diabetes therapy: history, current progress, and critical issues requiring solution. Adv Drug Deliv Rev 2014; 67-68:35-73.

52. Brauker J, Martinson LA, Young SK, Johnson RC. Local inflammatory response around diffusion chambers containing xenografts. Nonspecific destruction of tissues and decreased local vascularization. Transplantation 1996; 61:1671-7.

53. Kumagai-Braesch M, Jacobson S, Mori H, Jia X, Takahashi T, Wernerson A, et al. The TheraCyte device protects against islet allograft rejection in immunized hosts. Cell Transplant 2013; 22:1137-46.

54. Ludwig B, Reichel A, Steffen A, Zimerman B, Schally AV, Block NL, et al. Transplantation of human islets without immunosuppression. Proc Natl Acad Sci U S A 2013; 110:19054-8.

55. Barkai U, Weir GC, Colton CK, Ludwig B, Bornstein SR, Brendel MD, et al. Enhanced oxygen supply improves islet viability in a new bioartificial pancreas. Cell Transplant 2013; 22:1463-76.

56. Chang TM. Semipermeable Microcapsules. Science 1964; 146:524-5.

57. Lim FS, A. M. . Microencapsulated islets as bioartificial endocrine pancreas. Science 1980; 210:908–10.

58. Wang T, Lacik I, Brissova M, Anilkumar AV, Prokop A, Hunkeler D, et al. An encapsulation system for the immunoisolation of pancreatic islets. Nat Biotechnol 1997; 15:358-62.

59. Vegas AJ, Veiseh O, Gurtler M, Millman JR, Pagliuca FW, Bader AR, et al. Long-term glycemic control using polymer-encapsulated human stem cell-derived beta cells in immune-competent mice. Nat Med 2016; 22:306-11.

60. Calafiore R, Basta G, Luca G, Boselli C, Bufalari A, Bufalari A, et al. Transplantation of pancreatic islets contained in minimal volume microcapsules in diabetic high mammalians. Ann N Y Acad Sci 1999; 875:219-32.

61. Fung J, Wong T, Chok K, Chan A, Cheung TT, Dai J, et al. Long Term Outcomes of Entecavir Monotherapy for Chronic Hepatitis B after Liver Transplantation: Results up to 8 years. Hepatology 2017.

62. Fu L, Wei N, Wang JS, Wu L, Wang YN, Huang DY, et al. [The clinical characteristics of adult hemophagocytic lymphohistiocytosis treated with haploidentical donor hematopoietic stem cell transplantation]. Zhonghua Nei Ke Za Zhi 2017; 56:273-8.

63. Stendahl JC, Kaufman DB, Stupp SI. Extracellular matrix in pancreatic islets: relevance to scaffold design and transplantation. Cell Transplant 2009; 18:1-12.

64. Figliuzzi M, Bonandrini B, Silvani S, Remuzzi A. Mesenchymal stem cells help pancreatic islet transplantation to control type 1 diabetes. World J Stem Cells 2014; 6:163-72.

65. Emamaullee JA, Shapiro AM. Factors influencing the loss of beta-cell mass in islet transplantation. Cell Transplant 2007; 16:1-8.

66. Yancopoulos GD, Davis S, Gale NW, Rudge JS, Wiegand SJ, Holash J. Vascular-specific growth factors and blood vessel formation. Nature 2000; 407:242-8.

67. Cheng K, Fraga D, Zhang C, Kotb M, Gaber AO, Guntaka RV, et al. Adenovirus-based vascular endothelial growth factor gene delivery to human pancreatic islets. Gene Ther 2004; 11:1105-16.

68. Haynesworth SE, Baber MA, Caplan AI. Cytokine expression by human marrow-derived mesenchymal progenitor cells in vitro: effects of dexamethasone and IL-1 alpha. J Cell Physiol 1996; 166:585-92.

69. Golocheikine A, Tiriveedhi V, Angaswamy N, Benshoff N, Sabarinathan R, Mohanakumar T. Cooperative signaling for angiogenesis and neovascularization by VEGF and HGF following islet transplantation. Transplantation 2010; 90:725-31.

70. Dubois S, Madec AM, Mesnier A, Armanet M, Chikh K, Berney T, et al. Glucose inhibits angiogenesis of isolated human pancreatic islets. J Mol Endocrinol 2010; 45:99-105.

71. Perez-Basterrechea M, Esteban MM, Alvarez-Viejo M, Fontanil T, Cal S, Sanchez Pitiot M, et al. Fibroblasts accelerate islet revascularization and improve long-term graft survival in a mouse model of subcutaneous islet transplantation. PLoS One 2017; 12:e0180695.

72. Bruni A, Pepper AR, Gala-Lopez B, Pawlick R, Abualhassan N, Crapo JD, et al. A novel redox-active metalloporphyrin reduces reactive oxygen species and inflammatory markers but does not improve marginal mass engraftment in a murine donation after circulatory death islet transplantation model. Islets 2016; 8:e1190058.

73. Ramkumar KM, Sekar TV, Bhakkiyalakshmi E, Foygel K, Rajaguru P, Berger F, et al. The impact of oxidative stress on islet transplantation and monitoring the graft survival by non-invasive imaging. Curr Med Chem 2013; 20:1127-46.

74. do Amaral AS, Pawlick RL, Rodrigues E, Costal F, Pepper A, Galvao FH, et al. Glutathione ethyl ester supplementation during pancreatic islet isolation improves viability and transplant outcomes in a murine marginal islet mass model. PLoS One 2013; 8:e55288.

75. Grzenkowicz-Wydra J, Cisowski J, Nakonieczna J, Zarebski A, Udilova N, Nohl H, et al. Gene transfer of CuZn superoxide dismutase enhances the synthesis of vascular endothelial growth factor. Mol Cell Biochem 2004; 264:169-81.

76. L. G-LB. The metalloporphyrin BMX-010 in human islet isolation and clinical transplantation. CellR4 2016; 4:2066.

77. Friedenstein AJ, Piatetzky S, II, Petrakova KV. Osteogenesis in transplants of bone marrow cells. J Embryol Exp Morphol 1966; 16:381-90.

78. Brusko TM. Mesenchymal stem cells: a potential border patrol for transplanted islets? Diabetes 2009; 58:1728-9.

79. Loukogeorgakis SP, De Coppi P. Stem cells from amniotic fluid--Potential for regenerative medicine. Best Pract Res Clin Obstet Gynaecol 2016; 31:45-57.

80. Williams JT, Southerland SS, Souza J, Calcutt AF, Cartledge RG. Cells isolated from adult human skeletal muscle capable of differentiating into multiple mesodermal phenotypes. Am Surg 1999; 65:22-6.

81. Mahmoudifar N, Doran PM. Mesenchymal Stem Cells Derived from Human Adipose Tissue. Methods Mol Biol 2015; 1340:53-64.

82. Lazarus HM, Koc ON, Devine SM, Curtin P, Maziarz RT, Holland HK, et al. Cotransplantation of HLA-identical sibling culture-expanded mesenchymal stem cells and hematopoietic stem cells in hematologic malignancy patients. Biol Blood Marrow Transplant 2005; 11:389-98.

83. Sarugaser R, Lickorish D, Baksh D, Hosseini MM, Davies JE. Human umbilical cord perivascular (HUCPV) cells: a source of mesenchymal progenitors. Stem Cells 2005; 23:220-9.

84. Xin Y, Jiang X, Wang Y, Su X, Sun M, Zhang L, et al. Insulin-Producing Cells Differentiated from Human Bone Marrow Mesenchymal Stem Cells In Vitro Ameliorate Streptozotocin-Induced Diabetic Hyperglycemia. PLoS One 2016; 11:e0145838.

85. Zhang Y, Li C, Jiang X, Zhang S, Wu Y, Liu B, et al. Human placenta-derived mesenchymal progenitor cells support culture expansion of long-term culture-initiating cells from cord blood CD34+ cells. Exp Hematol 2004; 32:657-64.

86. Fiorina P, Shapiro AM, Ricordi C, Secchi A. The clinical impact of islet transplantation. Am J Transplant 2008; 8:1990-7.

87. Gruber R, Kandler B, Holzmann P, Vogele-Kadletz M, Losert U, Fischer MB, et al. Bone marrow stromal cells can provide a local environment that favors migration and formation of tubular structures of endothelial cells. Tissue Eng 2005; 11:896-903.

88. Liu M, Han ZC. Mesenchymal stem cells: biology and clinical potential in type 1 diabetes therapy. J Cell Mol Med 2008; 12:1155-68.

89. Sordi V, Malosio ML, Marchesi F, Mercalli A, Melzi R, Giordano T, et al. Bone marrow mesenchymal stem cells express a restricted set of functionally active chemokine receptors capable of promoting migration to pancreatic islets. Blood 2005; 106:419-27.

90. Caplan AI, Dennis JE. Mesenchymal stem cells as trophic mediators. J Cell Biochem 2006; 98:1076-84.

91. Chen L, Tredget EE, Wu PY, Wu Y. Paracrine factors of mesenchymal stem cells recruit macrophages and endothelial lineage cells and enhance wound healing. PLoS One 2008; 3:e1886.

92. Yeung TY, Seeberger KL, Kin T, Adesida A, Jomha N, Shapiro AM, et al. Human mesenchymal stem cells protect human islets from pro-inflammatory cytokines. PLoS One 2012; 7:e38189.

93. Le Blanc K, Tammik L, Sundberg B, Haynesworth SE, Ringden O. Mesenchymal stem cells inhibit and stimulate mixed lymphocyte cultures and mitogenic responses independently of the major histocompatibility complex. Scand J Immunol 2003; 57:11-20.

94. Vija L, Farge D, Gautier JF, Vexiau P, Dumitrache C, Bourgarit A, et al. Mesenchymal stem cells: Stem cell therapy perspectives for type 1 diabetes. Diabetes Metab 2009; 35:85-93.

95. Ryan JM, Barry FP, Murphy JM, Mahon BP. Mesenchymal stem cells avoid allogeneic rejection. J Inflamm (Lond) 2005; 2:8.

96. Corcione A, Benvenuto F, Ferretti E, Giunti D, Cappiello V, Cazzanti F, et al. Human mesenchymal stem cells modulate B-cell functions. Blood 2006; 107:367-72.

97. Spaggiari GM, Capobianco A, Becchetti S, Mingari MC, Moretta L. Mesenchymal stem cell-natural killer cell interactions: evidence that activated NK cells are capable of killing MSCs, whereas MSCs can inhibit IL-2-induced NK-cell proliferation. Blood 2006; 107:1484-90.

98. De Miguel MP, Fuentes-Julian S, Blazquez-Martinez A, Pascual CY, Aller MA, Arias J, et al. Immunosuppressive properties of mesenchymal stem cells: advances and applications. Curr Mol Med 2012; 12:574-91.

99. Ding Y, Xu D, Feng G, Bushell A, Muschel RJ, Wood KJ. Mesenchymal stem cells prevent the rejection of fully allogenic islet grafts by the immunosuppressive activity of matrix metalloproteinase-2 and -9. Diabetes 2009; 58:1797-806.

100. Cabrera SM, Rigby MR, Mirmira RG. Targeting regulatory T cells in the treatment of type 1 diabetes mellitus. Curr Mol Med 2012; 12:1261-72.

101. Berman DM, Willman MA, Han D, Kleiner G, Kenyon NM, Cabrera O, et al. Mesenchymal stem cells enhance allogeneic islet engraftment in nonhuman primates. Diabetes 2010; 59:2558-68.

102. Parekkadan B, Tilles AW, Yarmush ML. Bone marrow-derived mesenchymal stem cells ameliorate autoimmune enteropathy independently of regulatory T cells. Stem Cells 2008; 26:1913-9.

103. Li FR, Wang XG, Deng CY, Qi H, Ren LL, Zhou HX. Immune modulation of co-transplantation mesenchymal stem cells with islet on T and dendritic cells. Clin Exp Immunol 2010; 161:357-63.

104. Spaggiari GM, Capobianco A, Abdelrazik H, Becchetti F, Mingari MC, Moretta L. Mesenchymal stem cells inhibit natural killer-cell proliferation, cytotoxicity, and cytokine

production: role of indoleamine 2,3-dioxygenase and prostaglandin E2. Blood 2008; 111:1327-33.

105. Haller MJ, Viener HL, Wasserfall C, Brusko T, Atkinson MA, Schatz DA. Autologous umbilical cord blood infusion for type 1 diabetes. Exp Hematol 2008; 36:710-5.

106. Bluestone JA, Buckner JH, Fitch M, Gitelman SE, Gupta S, Hellerstein MK, et al. Type 1 diabetes immunotherapy using polyclonal regulatory T cells. Sci Transl Med 2015; 7:315ra189.

107. Tang Q, Bluestone JA. Regulatory T-cell therapy in transplantation: moving to the clinic. Cold Spring Harb Perspect Med 2013; 3.

108. Mauda-Havakuk M, Litichever N, Chernichovski E, Nakar O, Winkler E, Mazkereth R, et al. Ectopic PDX-1 expression directly reprograms human keratinocytes along pancreatic insulin-producing cells fate. PLoS One 2011; 6:e26298.

109. Lin G, Wang G, Liu G, Yang LJ, Chang LJ, Lue TF, et al. Treatment of type 1 diabetes with adipose tissue-derived stem cells expressing pancreatic duodenal homeobox 1. Stem Cells Dev 2009; 18:1399-406.

110. Bouwens L, Houbracken I, Mfopou JK. The use of stem cells for pancreatic regeneration in diabetes mellitus. Nat Rev Endocrinol 2013; 9:598-606.

111. Gan SU, Notaridou M, Fu ZY, Lee KO, Sia KC, Nathwani AC, et al. Correction of Murine Diabetic Hyperglycaemia With A Single Systemic Administration of An AAV2/8 Vector Containing A Novel Codon Optimized Human Insulin Gene. Curr Gene Ther 2016; 16:65-72.

112. Zhu HT, Wang WL, Yu L, Wang B. Pig-islet xenotransplantation: recent progress and current perspectives. Front Surg 2014; 1:7.

113. Groth CG, Korsgren O, Tibell A, Tollemar J, Moller E, Bolinder J, et al. Transplantation of porcine fetal pancreas to diabetic patients. Lancet 1994; 344:1402-4.

114. Hering BJ, Walawalkar N. Pig-to-nonhuman primate islet xenotransplantation. Transpl Immunol 2009; 21:81-6.

115. van der Windt DJ, Bottino R, Kumar G, Wijkstrom M, Hara H, Ezzelarab M, et al. Clinical islet xenotransplantation: how close are we? Diabetes 2012; 61:3046-55.

116. Samy KP, Martin BM, Turgeon NA, Kirk AD. Islet cell xenotransplantation: a serious look toward the clinic. Xenotransplantation 2014; 21:221-9.

117. Phelps CJ, Koike C, Vaught TD, Boone J, Wells KD, Chen SH, et al. Production of alpha 1,3-galactosyltransferase-deficient pigs. Science 2003; 299:411-4.

118. Yang L, Guell M, Niu D, George H, Lesha E, Grishin D, et al. Genome-wide inactivation of porcine endogenous retroviruses (PERVs). Science 2015; 350:1101-4.

119. Matsumoto S, Tan P, Baker J, Durbin K, Tomiya M, Azuma K, et al. Clinical porcine islet xenotransplantation under comprehensive regulation. Transplant Proc 2014; 46:1992-5.

120. Matsumoto S, Abalovich A, Wechsler C, Wynyard S, Elliott RB. Clinical Benefit of Islet Xenotransplantation for the Treatment of Type 1 Diabetes. EBioMedicine 2016; 12:255-62.

121. Rezania A, Bruin JE, Arora P, Rubin A, Batushansky I, Asadi A, et al. Reversal of diabetes with insulin-producing cells derived in vitro from human pluripotent stem cells. Nat Biotechnol 2014; 32:1121-33.

122. Hori Y, Rulifson IC, Tsai BC, Heit JJ, Cahoy JD, Kim SK. Growth inhibitors promote differentiation of insulin-producing tissue from embryonic stem cells. Proc Natl Acad Sci U S A 2002; 99:16105-10.

123. Lumelsky N, Blondel O, Laeng P, Velasco I, Ravin R, McKay R. Differentiation of embryonic stem cells to insulin-secreting structures similar to pancreatic islets. Science 2001; 292:1389-94.

124. Kubo A, Shinozaki K, Shannon JM, Kouskoff V, Kennedy M, Woo S, et al. Development of definitive endoderm from embryonic stem cells in culture. Development 2004; 131:1651-62.

125. D'Amour KA, Bang AG, Eliazer S, Kelly OG, Agulnick AD, Smart NG, et al. Production of pancreatic hormone-expressing endocrine cells from human embryonic stem cells. Nat Biotechnol 2006; 24:1392-401.

126. Pagliuca FW, Millman JR, Gurtler M, Segel M, Van Dervort A, Ryu JH, et al. Generation of functional human pancreatic beta cells in vitro. Cell 2014; 159:428-39.

127. Russ HA, Parent AV, Ringler JJ, Hennings TG, Nair GG, Shveygert M, et al. Controlled induction of human pancreatic progenitors produces functional beta-like cells in vitro. EMBO J 2015; 34:1759-72.

128. Zhou Q, Brown J, Kanarek A, Rajagopal J, Melton DA. In vivo reprogramming of adult pancreatic exocrine cells to beta-cells. Nature 2008; 455:627-32.

129. Takahashi K, Yamanaka S. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell 2006; 126:663-76.

130. Nir T, Melton DA, Dor Y. Recovery from diabetes in mice by beta cell regeneration. J Clin Invest 2007; 117:2553-61.

131. Oetjen E, Baun D, Beimesche S, Krause D, Cierny I, Blume R, et al. Inhibition of human insulin gene transcription by the immunosuppressive drugs cyclosporin A and tacrolimus in primary, mature islets of transgenic mice. Mol Pharmacol 2003; 63:1289-95.

132. Rostambeigi N, Lanza IR, Dzeja PP, Deeds MC, Irving BA, Reddi HV, et al. Unique cellular and mitochondrial defects mediate FK506-induced islet beta-cell dysfunction. Transplantation 2011; 91:615-23.

133. Gala-Lopez BL, Pepper AR, Pawlick RL, O'Gorman D, Kin T, Bruni A, et al. Antiaging Glycopeptide Protects Human Islets Against Tacrolimus-Related Injury and Facilitates Engraftment in Mice. Diabetes 2016; 65:451-62.

Chapter 2

Improved Islet Recovery and Efficacy Through Co-Culture and Co-Transplantation of Islets with Human Adipose-Derived Mesenchymal Stem Cells

Note: This manuscript is near completion and intended for submission to the journal **PLOS ONE** with Gamble A. as first author.

Chapter 2: Improved Islet Recovery and Efficacy Through Co-Culture and Co-Transplantation of Islets with Human Adipose-Derived Mesenchymal Stem Cells

Anissa Gamble[1,3,4], Rena Pawlick[1,2], Andrew R. Pepper[1,2,3,4], Antonio Bruni [1,2,3,4], Adetola Adesida[3], Peter A. Senior[1,2,4,5], Gregory S. Korbutt[1,3,6], A.M. James Shapiro[1,2,3,4,5,6*]

[1]Alberta Diabetes Institute, University of Alberta, Edmonton, AB, Canada

[2]Clinical Islet Transplant Program, University of Alberta, Edmonton, AB, Canada

[3]Department of Surgery, University of Alberta, Edmonton, Alberta, Canada

[4]Members of the Canadian National Transplant Research Project (CNTRP)

[5]Department of Medicine, University of Alberta, Edmonton, Alberta, Canada

[6]Denotes co-senior authorship

*Corresponding Author:

A.M. James Shapiro, MD PhD FRCS(Eng) FRCSC MSM FRSC FCAHS

Canada Research Chair in Transplant Surgery and Regenerative Medicine

Professor of Surgery, Medicine and Surgical Oncology

University of Alberta, 2000 College Plaza, 8215-112th St, Edmonton AB T6G 2C8

Phone: 780-407-7330 Fax: 780-407-6933 Email: amjs@islet.ca

2.1 Chapter Overview

Islet transplantation is an established clinical procedure for select patients with type 1 diabetes and severe hypoglycemia to stabilize glycemic control. Post-transplant, substantial beta cell mass is lost, necessitating multiple donors to maintain euglycemia. A potential strategy to augment islet engraftment is the co-transplantation of islets with multipotent mesenchymal stem cells to capitalize upon their pro-angiogenic and anti-inflammatory properties. Herein, we examine the *in vitro* and *in vivo* effect of co-culturing murine islets with human adipose-derived mesenchymal stem cells (Ad-MSCs). Islets co-cultured with Ad-MSCs for 48 hours had decreased cell death, superior viability as measured by membrane integrity, improved glucose stimulated insulin secretion and reduced apoptosis compared to control islets. These observations were recapitulated with human islets, albeit tested in a limited capacity. Recipients of marginal mouse islet mass grafts, co-transplanted with Ad-MSCs without a co-culture period, did not reverse to normoglycemia as efficiently as islets alone. However, utilizing a 48-hour co-culture period, marginal mouse islets grafts with Ad-MSCs achieved a superior percent euglycemia rate when compared to islets cultured and transplanted alone. A co-culture period of human islets with human Ad-MSCs may have a clinical benefit improving engraftment outcomes.

2.2 Introduction

Islet transplantation is a therapeutic procedure that can restore endogenous insulin production and maintain euglycemia for a sustained period in patients with difficult to control type 1 diabetes mellitus (T1DM). The recent Clinical Islet Transplant Consortium's, National Institute of Health (NIH) sponsored phase 3 trial demonstrated islet transplantation's ability to stabilize glycemic control in select patients with T1DM presenting hypoglycemia unawareness where the primary end-point revealed 88% and 71% of recipients maintained euglycemia for 1-year and 2-years post-islet transplant respectively. [1] This Federal Drug Administration (FDA)-Biologics License Application enabling study may allow product licensure for islet transplant, facilitating reimbursement through insurance in the USA. Despite its apparent success, this procedure is not without limitations. A major challenge is overcoming suboptimal acute engraftment, where up to 60% of the initial transplanted islet mass is potentially lost due to innate instant blood-mediated inflammatory reaction (IBMIR), delayed re-vascularization, or hypoxic stress. [2-5] Multiple islet infusions are therefore often required to maintain periods of insulin independence. Co-transplantation of islets with multipotent stem cells (MSCs) is a potential strategy to mitigate early islet cell loss in culture and after transplantation. [6, 7] MSCs are ubiquitous throughout cell types, and their capacity for self-renewal and differentiation into cells of mesoderm lineage includes adipocytes, chondrocytes, osteoblast and myocytes. [8] Previous studies have demonstrated the ability of MSCs to augment islet function, in part due to MSC's immunomodulatory and trophic properties, and their ability to secrete several paracrine factors. [9-11] Notably, MSCs modulate angiogenesis through gene expression of cytokines, including vascular endothelial growth factor (VEGF), fibroblast growth factors (FGFs), transforming growth factor-βs (TGF-βs) and Annexin-1 (ANXA1). [12-14] MSCs can modulate the secretion of cytokines and promote the concentration of growth factors at the islet engraftment site and may aid neovascularization. [15, 16]

In the clinical islet allograft setting, islets undergo an obligate culture period of up to 72 hours before transplantation. The culture period facilitates recipient conditioning, and may allow for transplantation of a more immunologically quiescent graft. [17, 18] Conversely, culture may be detrimental to islet survival due to limited nutrients, and exposure to oxidative, hypoxic, and inflammatory stressors. [19] These stressors lead to impaired islet viability and decreased cell mass. In addition, islet endothelial cells are compromised during the islet isolation and culture process that diminishes islet recovery and function prior to transplantation. [20, 21] During isolation, islets are stripped of their native vascularization and rely on diffusion of nutrients and oxygen to survive. Moreover, the intra-islet endothelial cells rapidly decline to 5% by 4 days post culture. [20] The disrupted vascular supply hinders the post-transplant revascularization process. Over this period, insufficient vascularization causes increased cell death and graft failure due to inadequate nutrient delivery and prolonged ischemia. [21] In consequence, multiple donors and infusions are often required to maintain recipient insulin independence. [19, 22, 23] To decrease donor demand, it has been shown co-culturing islets with human bone marrow-derived MSCs or mouse adipose-derived MSCs (Ad-MSCs), for an extended period, can augment islet function and improve islet engraftment post-transplant. [24-26]

In the present study, we explored the use of human Ad-MSCs co-cultured with murine and human islets for 48 hours at islet to Ad-MSC ratios of 1:300 [24] and 1:2000. [25] We hypothesize co-culturing islets with human Ad-MSCs will improve islet recovery, islet function and augment engraftment outcomes.

2.3 Methods

2.3.1 Murine Pancreatectomy and Islet Isolation

Pancreatic islets were isolated from 8- to 12-week-old BALB/c mice (Jackson Laboratories, CA) and housed under conventional conditions in accordance with the Canadian Council on Animal Care. All experimental procedures were approved by the University of Alberta Research Ethics and Animal Use Committee (Study ID: AUP00000331). Prior to pancreatectomy, the common bile duct was cannulated and the pancreas was distended with 0.125 mg/mL cold Liberase TL Research Grade enzyme (Roche Diagnostics, Laval, QC, CA) in Hanks balanced salt solution (Sigma, St. Louis, MO, USA). Pancreas digestion was continued in a 37°C water bath for 14 minutes with light agitation. After the pancreatic digestion phase, islets were purified using histopaque-density gradient centrifugation (1.108, 1.083 and 1.069 g/mL, Sigma, St. Louis, MO, USA). Upon purification, islets were placed in Connaught Medical Research Laboratories (CMRL-1066) (Corning-cellgro, Manassas, VA, USA) supplemented with 10% fetal bovine serum, 1% L-glutamine (200 mmol/L, Sigma, St. Louis, MO, USA), 1% sodium pyruvate (100 mmol/L, Sigma, St. Louis, MO, USA), 1% non-essential amino acid 100x (Sigma, St. Louis, MO, USA) and 100 U/mL penicillin-G and100 μg/mL streptomycin (Sigma Aldrich Canada Co., Oakville, ON, CA) at 37°C/5%CO_2 at pH 7.4.

2.3.2 Human islet isolation, purification and culture

Human islet preparations were isolated from deceased consenting multi-organ deceased donors, as described, [19] with intent for clinical transplantation. Islets were only used for research when yields failed to meet that required for a listed recipient and with research consent from donor families. Permission for these studies was granted by the University of Alberta Health Research Ethics Board (REB), Edmonton, Alberta, Canada. The REB ensures that individual research

projects involving human participants, identifiable data and/or human biological material meet the requirements of the current *Tri-Council Policy Statement: Ethical Conduct of Research Involving Humans* and University policy as well as provincial, federal and other legislation and regulations, as applicable.

2.3.3 Adipose Derived Mesenchymal Stem Cell Expansion and Culture Conditions

To prepare the human adipose-derived MSCs, infrapatellar fat pad was removed from 2 donors (18 and 25 year old males) undergoing orthopedic knee surgery at the University Hospital of Alberta, Edmonton, Canada, and processed as described before [27]. Ethics committee waived the need for written informed consent of patients, as specimens used in this study were intended for discard in the normal course of the surgical procedure. Extensive precautions were taken to preserve the privacy of the participants donating specimens. All research involving human participants were reviewed and approved by the University of Alberta Health REB (Study ID: PRO 00001416 and PRO 000018778).

Cryopreserved Ad-MSCs containing $2x10^6$ cells per vial were thawed upon second passage and population doubling of 4. For expansion, cells were plated in flasks containing Eagle's minimum essential medium (MEM) (Sigma Aldrich Canada Co., Oakville, ON, CA) supplemented with 2.5 ng/mL basic fibroblast growth factor (Millipore, Etobicoke, ON, Canada), 10% fetal bovine serum, L-glutamine (2 mmol/L) (Sigma, St. Louis, MO, USA), penicillin (50 000 units) and streptomycin (50 mg) (Sigma Aldrich Canada Co., Oakville, ON, CA), HEPES (5 mmol/L) and sodium pyruvate (5 mmol/L) (Sigma, St. Louis, MO, USA) at 37°C/5%CO_2 and a pH 7.4. The medium was changed within the first 24-hours, and thereafter in 48-hours intervals. Once confluent, the cell monolayer was washed with Versene (1% EDTA in PBS; Life technologies Inc. Burlington, ON, Canada) and enzymatically detached with 0.5% volume for volume trypsin-EDTA. Cells were counted by a hemocytometer and aliquoted ($4 x10^5$ cells) onto 60 mm ×

15 mm ultra-low adherence culture dishes (Corning, Corning, NY, USA). Experimental groups contained 200 BALB/c islets, and all groups (islets alone, islet: Ad-MSCs 1:300 and 1:2000) contained 5 mL of CMRL culture media for 48 hours. The MSCs isolated abided by the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy guidelines.

2.3.4 Percent Islet Recovery

Post isolation and subsequently post 48-hour culture, islets were harvested and counted to determine islet yield. Aliquots from respective samples were stained with dithizone (Sigma Aldrich Canada Co., Oakville, ON, CA) and counted in triplet. The percentage of islet recovery was determined by ratio of total islets harvested 48 hours' post-culture relative to the number of islets harvested immediately post-isolation.

2.3.5 Insulin Secretion Assessment

Following isolation and post 48-hour culture, triplicates of 50 mouse and human islet equivalents were collected for respective groups and a static glucose-stimulated insulin secretion (s-GSIS) assay and dynamic insulin perfusion was performed. s-GSIS was performed by washing islets of residual glucose in glucose free medium, followed by incubation in RPMI-1640 (Sigma Aldrich Canada Co., Oakville, ON, CA) containing low (2.8 mmol/L) glucose, followed by high (16.7 mmol/L) glucose, each, for one hour at 37ºC. The supernatant was harvested and stored at 20ºC. Insulin levels were measured by an enzyme-linked immunosorbent assay (ELISA) (Mercodia, Uppsala, Sweden).

Dynamic insulin perifusion [28] was assessed by an automated perifusion system (Bio rep® Perifusion). Islets were exposed to Krebs solution containing low glucose (2.8 mmol/L), high glucose (28 mmol/L) and KCL solution (20 mmol/L KCl in 2.8 mmol/L glucose) for respective

time periods. The perfusate was collected in an automated multiwell plate format and stored at -20^{o}C until analyzed for insulin quantification by an ELISA (Alpco, Salem, NH, USA).

2.3.6 Apoptosis TUNEL Staining

Apoptosis of islets was assessed quantitatively using Tdt-mediated dUTP nick-end labeling (TUNEL) staining. Prior and post-cultured islets were fixed in 4% paraformaldehyde, embedded in agar, processed and embedded in paraffin. Tissue sections were co-stained with anti-insulin antibody at 1:200 concentration (Agilent Technologies, Mississauga, ON, CA) and labeled with Rhodamine (TRITC) conjugated anti-guinea pig IgG (1:200, Jackson ImmunoResearch, West Grove, PA, USA). To identify the apoptosis, fluorescein isothiocyanate-dUTP with TdT enzyme (Promega, Madison, WI, USA) was added and counterstained with the nuclear stain, DAPI (ProLong Gold DAPI, Invitrogen, Calrsbadm CA, USA). Apoptosis was determined by analyzing the number of positive TUNEL-stained cells as a percentage of both insulin and nuclei positive cells utilizing FIJI ImageJ Software (National Institute of Health, USA).

2.3.7 Mesenchymal Stem/Stromal Cell Tracking

Following isolation, MSCs were stained with a vybrant^{TM} DiO cell labeling dye (ThermoFisher Scientific, Waltham, MA) dosed at 5 µl per 10^{6} cells. Stained islets were distributed amongst respective groups of MSCs alone, 1:300 and 1:2000 islet to MSC ratios. Islets cultured alone did not contain MSCs. Microscopic evaluation was performed prior to culture (0 hours) and post culture (48 hours). MSCs microscopic evaluation and quantification was performed by FIJI ImageJ Software (National Institute of Health, USA).

2.3.8 Pro-inflammatory Cytokine Assessment

Pro-inflammatory cytokines were analyzed from culture media post 48-hour culture. Media was assessed for mouse tumor necrosis factor (TNF)-α, KC-GRO, interferon (IFN)-γ,

interleukin (IL)-1β, IL-10, IL-6 and IL-12p70 using a Mouse ProInflammatory 7-Plex Tissue Culture Kit (Meso Scale Diagnostics, Rockville, Maryland, USA). Human IL-1β, IL-12p70, IL-6, IL-8, IL-10, IL-13, TNF-α and IFN-γ cytokine analysis was demonstrated using a Human ProInflammatory Panel kit (Meso Scale Diagnostics, Rockville, Maryland, USA). The plates were loaded into an MSD-SECTOR® instrument for electrochemiluminescence analysis.

2.3.9 Islet Transplantation

To induce diabetes, female and male adult (8-10 weeks, 20-30 gm) immunodeficient mice (B6.129S7-Rag1$^{tm1Mom/J}$; Jackson Laboratories, CA) were administered an intraperitoneal injection of streptozotocin (175mg/kg i.p) (Sigma, St. Louis, MO, USA) in acetate phosphate buffer (Sigma Aldrich Canada Co., Oakville, ON, CA) at pH 4.5. Mice with blood glucose levels ≥18 mmol/L for two daily consecutive non-fasting blood glucose readings, were considered diabetic.

The mice were transplanted with post-isolation or post-culture mouse islets with or without the presence of Ad-MSCs (islet to Ad-MSC ratio: 1:2000) or islets alone. Islets were aspirated into polyethylene (PE-50) tubing and centrifuged. A left lateral paralumbar subcostal incision was made and islets were delivered under the left kidney capsule. Islet engraftment was assessed through non-fasting blood glucose measurements, three times per week for 60 days. Blood glucose monitoring was conducted using a portable glucometer (OneTouch Ultra 2, LifeScan, CA, USA). Two consecutive readings maintained at <11.1 mmol/L confirmed graft function and reversal of diabetes. At day 60, a recovery nephrectomy of the grafted kidney confirmed graft dependent efficacy, when animals returned to hyperglycemia (≥18 mmol/L).

2.3.10 Intraperitoneal Glucose Tolerance Test (IPGTT)

In vivo glucose tolerance and islet function was assessed by an intraperitoneal glucose tolerance test (IPGTT) in euglycemic mice 6 weeks' post-transplant. The mice were fasted overnight and administered 25% dextrose intraperitoneally at a dose of 3 g/kg. Naïve

normoglycemic mice served as controls. Blood glucose was monitored at baseline (t=0), 15, 30, 60, 90 and 120 minutes' post injection. Significance was measured as area under the curve amongst groups.

2.3.11 Insulin Graft Assessment

Sixty days' post islet transplant, kidney bearing grafts were removed and placed in -80°C. Kidneys were homogenized and sonicated with 2mM of acetic acid in 0.5% of bovine serum albumin (Thermo Fisher Scientific, Waltham, MA, USA). Supernatant was collected and insulin levels were measured by an enzyme-linked immunosorbent assay (ELISA) (Mercodia, Uppsala, Sweden).

Immunohistochemistry was performed on a subset of kidneys bearing the islet grafts post 60 days' post islet transplant and which were fixed in 10% formalin. The tissue was sectioned and upon heat retrieval, were blocked with 20% goat serum (Sigma-Aldrich, St. Louis, MI, USA), and subsequently incubated overnight at 4^{o}C primary antibodies guinea pig anti-insulin (1:200, Dako, Mississauga, ON, Canada) and rabbit anti-glucagon (1:100, Abcam, Cambridge, MA, USA). The following day sections were washed and incubated with secondary antibodies for 1 hour at room temperature utilizing goat anti-guinea pig Rhodamine and goat anti-rat fluorescein (1:200 Jackson ImmunoResearch, West Grove, PA, USA) counterstained with DAPI (ProLong Gold DAPI, Invitrogen, Calrsbadm CA, USA).

2.3.12 Statistical Analysis

Data were analyzed using GraphPad Prism (GraphPad Software, La Jolla, CA, USA). Student *t* test or analysis of variance (ANOVA) was used to compare islet yield, membrane integrity, TUNEL, sGSIS and AUC for IPGTT's and represented as scatter plots. Kaplan-Meier graft survival function curves were compared using the log-rank (Mantel-Cox) statistical method. Statistical significance was considered when p-values < 0.05. Graphical representation of

data is as mean ± standard error of the mean (s.e.m) for *in vivo* analysis where *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

2.4 Results

2.4.1 Mouse Islets Co-Cultured with Human Adipocyte derived Mesenchymal Stem Cells Improve *In Vitro* Function

Murine islets co-cultured with human Ad-MSCs improved islet recovery, viability, survival and function. After 48-hour of co-culture, islets were quantified to determine islet yield. Ad-MSCs improved islet yield (**Fig. 2.1a**) and demonstrated ability to adhere to the islet surface (**Fig. 2.1b**). Control islets cultured alone exhibited significant islet loss (22.1 ± 10.5% islet loss) compared to both co-cultured Ad-MSC groups: 1:300 (2.7 ± 1.9%) and 1:2000 (1.1 ± 0.81%) ($p<0.0001$ respectively). Dual-fluorescence staining assessing islet viability revealed islets co-cultured with Ad-MSCs, 1:300 (5.9 ± 1.3%) and 1:2000 (7.1 ± 0.09%), maintained greater viability, less percentage of cell death, relative to islets cultured alone (11.7 ± 0.9%) ($p<0.05$, $p<0.01$, respectively, **Fig. 2.2a**). Insulin secretory function was assessed by a static glucose stimulated insulin secretion (sGSIS) assay and revealed islets co-cultured with a 1:2000 islet to Ad-MSC ratio had significantly higher stimulation index relative to islets cultured alone (islets: 0.95 ± 0.15 vs. 1:2000: 3.29 ± 0.76, $p<0.05$) (**Table 2.1**). A dynamic islet perifusion assay, completed after 48-hour culture (**Fig. 2.2b**), illustrated no statistical difference for area under the curve amongst groups (**Fig. 2.2c**). Islets cultured alone had significantly increased apoptosis compared to both Ad-MSC groups. The percentage apoptosis of islets cultured alone was 34.9 ± 4.6% compared to islets cultured with MSCs at 1:300, at 19.9 ± 3.7% vs. islets cultured with MSCs at 1:2000, at 17.0 ± 3.6% ($p<0.05$ and $p<0.001$ respectively, **Fig. 2.2 d,e**).

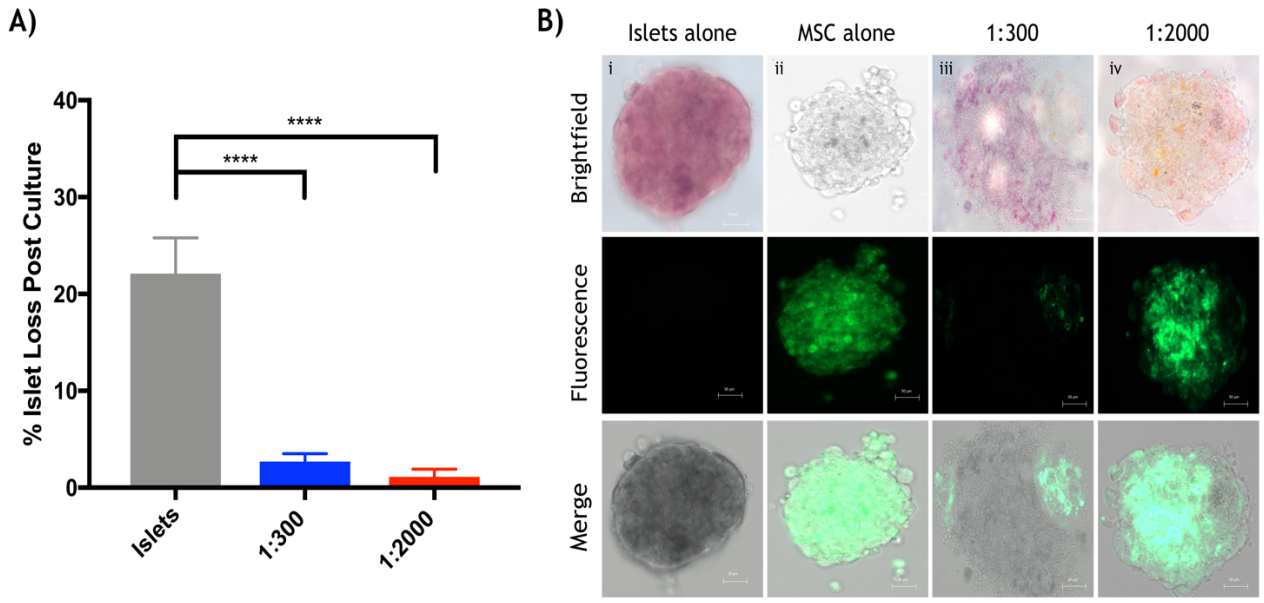

Figure 2. 1 Quantitative and qualitative analysis of human adipose derived mesenchymal stem cells co-cultured with murine islets.

In vitro assessment of experimental groups post 48-hour co-culture period. (**a**) The percentage of islet cell loss post 48-hour co-culture for islets cultured alone (grey) was significantly less than both the islet:Ad-MSCs at 1:300 (blue) (****$p<0.0001$, ANOVA) and 1:2000 (red) (****$p<0.0001$, ANOVA). (**b**) Microscopic imaging of labelled human adipose derived mesenchymal stem cells accumulation around mouse islet cells after culture. Column (i) Islets cultured alone, (ii) Ad-MSCs cultured alone, (iii) 1:300 islet to Ad-MSC ratio and (iv) 1:2000 islet to Ad-MSC ratio. Rows (from top to bottom) include bright field microscopic imaging with islets stained with dithizone, fluorescence imaging displaying cell labelled mesenchymal stem cells (DiO staining) displayed in green and combined brightfield and fluorescence microscopic images. The scale bar represents 50µm and data is represented as (mean ± s.e.m).

Cellular Insulin

Group	Condition and Culture Period	2.8 mM Glucose (ng/mL)	16.7 mM Glucose (ng/mL)	Stimulation Index
Mouse Islets Alone	0-hour culture	11.63 ± 1.15 †	19.01 ± 1.78* ‖	1.79 ± 0.23
	48-hour culture	2.62 ± 0.57 †	2.65 ± 0.43 *§	1.17 ± 0.33 §
Mouse Islets + Ad-MSCs	1:300 48-hour culture	5.07 ± 1.32	9.89 ± 2.03 ‖	1.17 ± 0.33
	1:2000 48-hour culture	6.91 ± 1.81	13.20 ± 2.49 §	2.93 ± 0.85 §
Human Islets Alone	0-hour culture	11.64 ± 3.90	39.30 ± 7.44 º ∫	5.13 ± 1.92
	48-hour culture	3.75 ± 1.49	8.29 ± 2.03 º	1.48 ± 0.29
Human Islets + Ad-MSCs	1:300 48-hour culture	10.49 ± 3.96	26.53 ± 6.36	4.36 ± 1.47
	1:2000 48-hour culture	6.67 ± 2.52	17.28 ± 3.29 ∫	4.54 ± 1.15

Table 2. 1- Mouse and Human Static Glucose Stimulated Insulin Secretion Assay

Data are mean ± s.e.m. of three independent experiments. In each experiment, islets were collected post isolation (0-hour culture) and post 48-hour co-culture, where groups consist of islets alone, islets with 1:300 and 1:2000 islet to human adipose-derived mesenchymal stem cell ratios. Static glucose insulin secretion assay were performed in triplets of 50 islets per group. Stimulation indices were calculated by dividing the amount of insulin released at high glucose (16.7 mM) by that release at low glucose (2.8 mM). Insulin secreted is measured in ng/mL.

* $p < 0.0001$ mouse islets (0 hrs culture) vs. islets (48 hrs culture)

† $p < 0.05$ mouse islets (0 hrs culture) vs. islets (48 hrs culture)

‖ $p < 0.01$ mouse islets (0 hrs culture) vs. islets + 1:300 Ad-MSCs

§ $p < 0.05$ mouse islets (48 hrs culture) vs. islets + 1:2000 Ad-MSCs

º $p < 0{,}001$ human islets (0 hrs culture) vs. islets (48 hrs culture)

∫ $p < 0{,}05$ human islets (0 hrs culture) vs. islets + 1:2000 Ad-MSCs

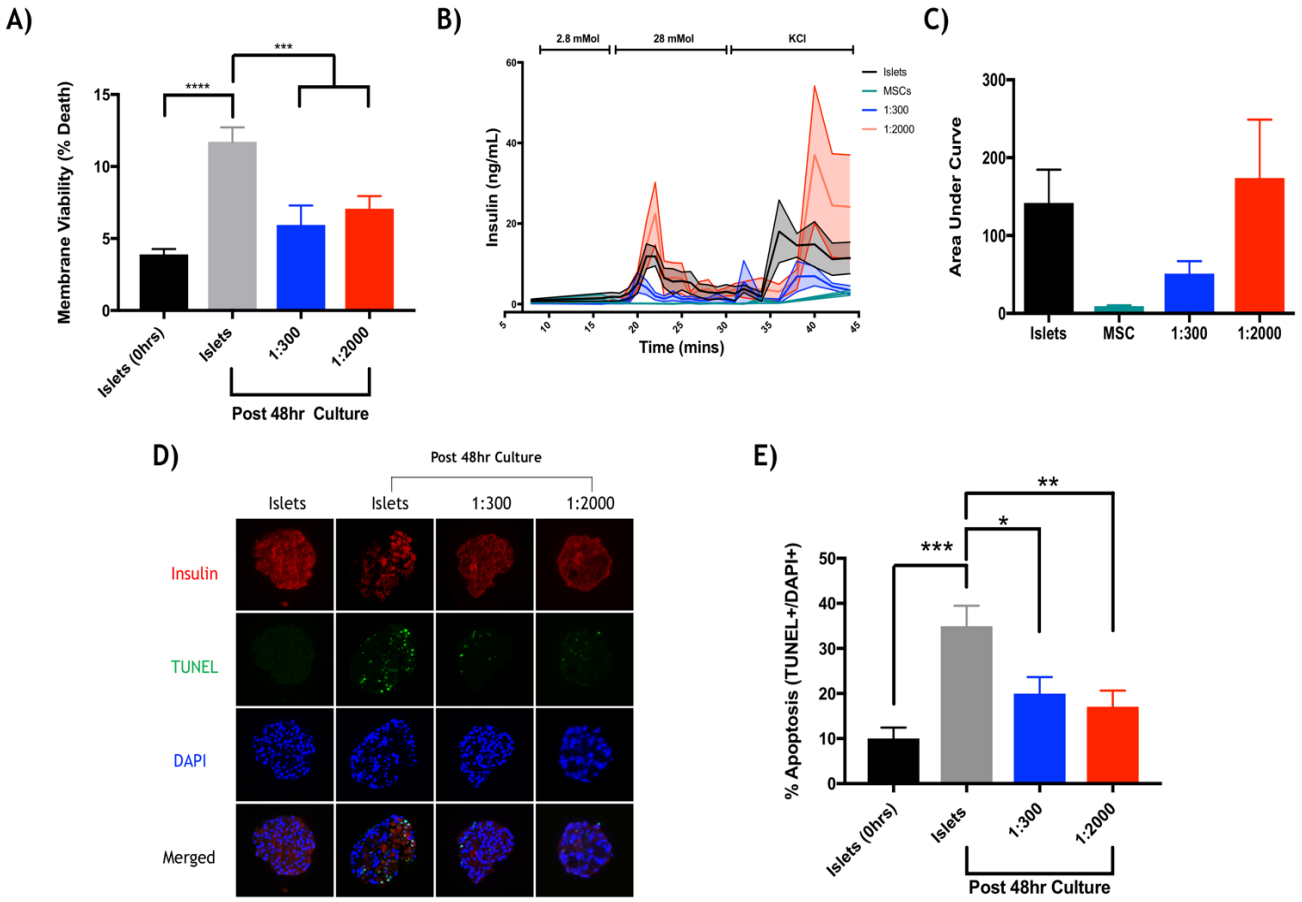

Figure 2. 2 In vitro assessment of mouse islet viability and function after co-culture with adipose mesenchymal cells.

(**a**) Islet and Ad-MSC Live/dead staining by dual-fluorescence revealed mouse islets cultured alone (grey) exhibited significantly reduced islet viability compared to when cultured with human Ad-MSCs after 48 hours in culture, islets to Ad-MSCs at 1:300 (blue) and 1:2000 (red) (***$p<0.001$, ANOVA). There was no significant difference when Ad-MSCs with islets were compared to islets alone immediately after isolation (black). (**b**) A dynamic perifusion assay was also completed after the 48-hour culture period for islets alone (black), mesenchymal stem cells alone (teal), and the combination at 1:300 (blue) and 1:2000 (red). (**c**) Area under the curve for the perifusion assay displayed no significant difference amongst all groups (*$p>0.05$, ANOVA). (**d**) Cell apoptosis was assessed by TUNEL staining of insulin (red), apoptosis (green) and nucleus/ DAPI (blue). (**e**) Upon analysis, there was significant cell death for islets and Ad-MSCs cultured alone (grey)

compared to those cultured with Ad-MSCs at 1:300 (*$p<0.05$, ANOVA) and 1:2000 (**$p<0.01$, ANOVA). Apoptosis percentage was analyzed by FIJI software by the surface area of TUNEL positive over DAPI positive. Data represented as (mean ± s.e.m).

2.4.2 Pro Inflammatory Cytokine Expression after Islet Transplant

Post 48-hour culture media was collected and analyzed for murine and human pro-inflammatory cytokines (**Fig. 2.3**). Murine interleukin (IL)-2p70 expression was reduced by the presence of Ad-MSCs during culture, whereas islets cultured alone revealed increased expression ($p<0.05$, **Fig. 2.3b**). Human and mouse IL-1β, IL-6, IL-10, and tumor necrosis factor (TNF)-α were detected but their expression amongst respective groups did not provide statistical significance (islets vs. islets +Ad-MSCs) (**Fig. 2.3 a,c-e**).

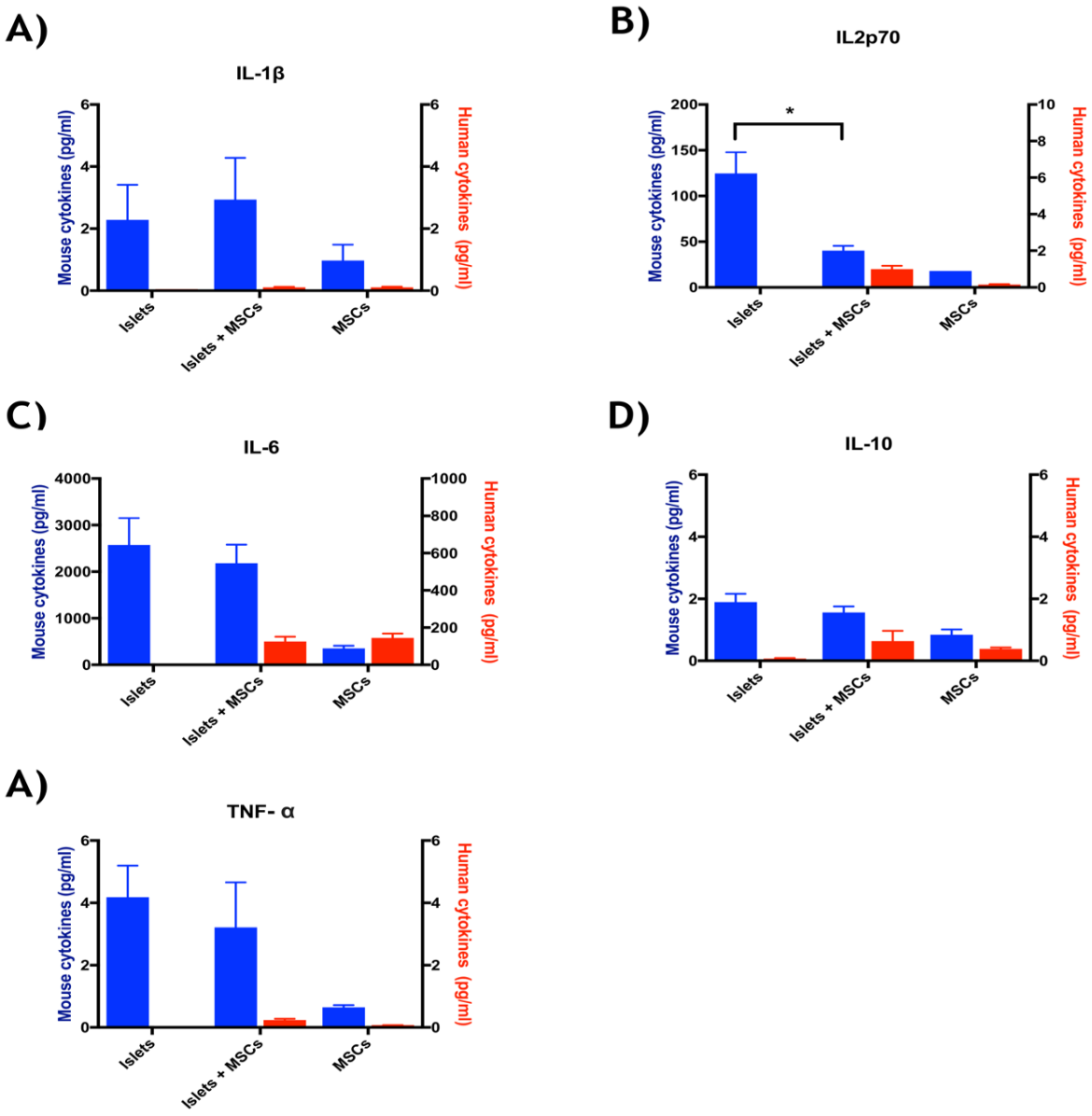

Figure 2. 3 Murine and human proinflammatory cytokine analysis.

Murine and human proinflammatory profiles from the medium after 48-hour culture (n=3). Mouse cytokines are displayed on the left y-axis in blue and human cytokines on the right y-axis in red. Upon analysis, (**b**) murine IL2p70 expression was significantly downregulated for islets co-cultured with Ad-MSCs (1:2000) compared to control islets (*$p<0.05$, ANOVA) (mean ± s.e.m). IL-1β, IL-6, IL-10, and tumor necrosis factor (TNF)-α expression was detected, but no significance difference was demonstrated amongst groups ($p>0.05$, ANOVA).

2.4.3 Co-Transplantation of Islets and Ad-MSCs without Prior Co-Culture is Detrimental to Engraftment

To determine if a period of islet-MSC co-culture was needed, we co-transplanted islets + Ad-MSCs without prior culture. One hundred and fifty mouse islets (marginal islet mass) were isolated and co-transplanted under diabetic murine kidney capsule with or without 1:2000 Ad-MSCs combined at the time of transplant. Ten of 13 (76.9%) control islet recipients without MSCs became euglycemic at 21.0 ± 6.4 days after transplant, whereas 7 of 13 (53.8%, $p<0.001$) islets + Ad-MSCs became euglycemic by 38.6 ± 6.1 days (**Fig. 2.4a, b**). All euglycemic animals became hyperglycemic after graft recovery nephrectomy 60 days after transplant, confirming graft dependent euglycemia. Seven weeks' post-transplant, an intraperitoneal glucose tolerance test (IPGTT) was performed on all euglycemic mice (**Fig. 2.4c**) and demonstrated no significance amongst the mean area under the curve (**Fig. 2.4d**).

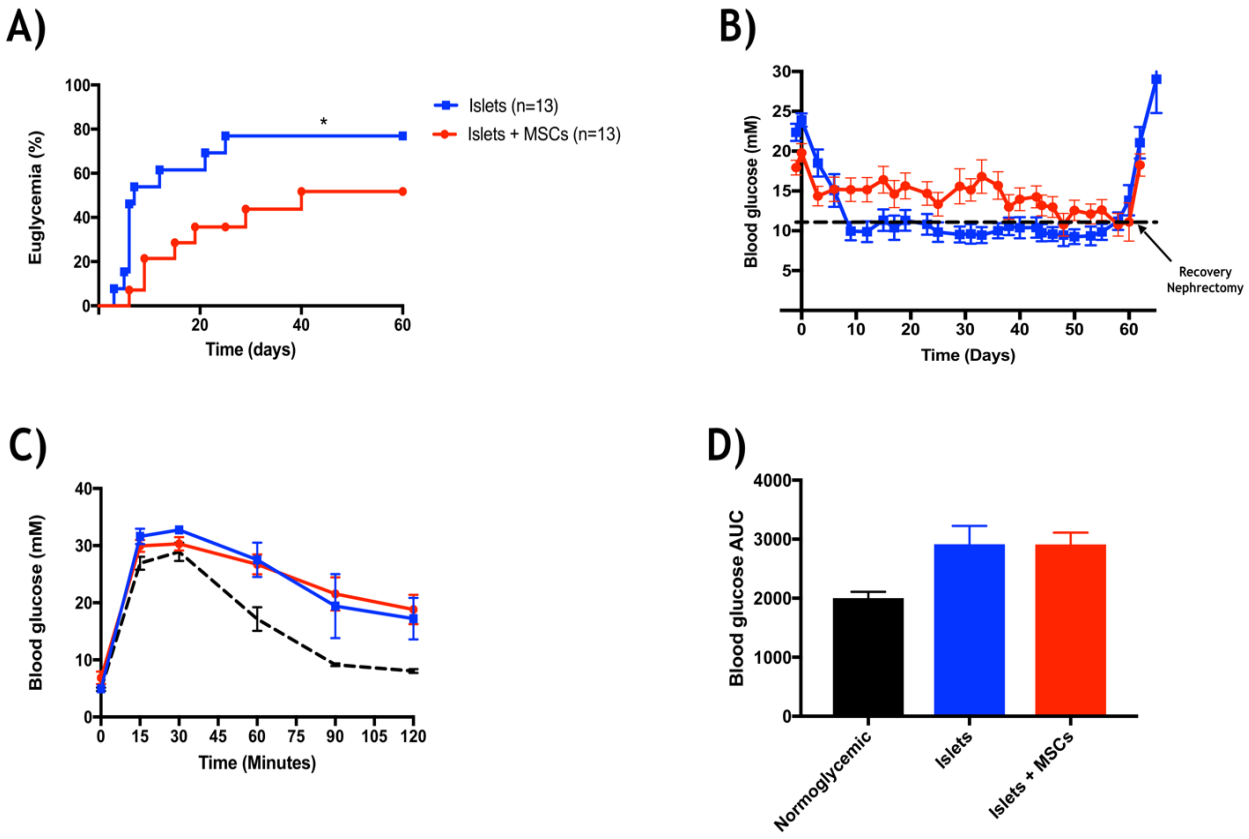

Figure 2. 4 Marginal mass islet co-transplantation and glucose tolerance.

Efficacy of a marginal mass (150 BALB/c mouse islets per recipient) under the kidney capsule of diabetic Rag$^{-/-}$ mice with or without the presence of human Ad-MSCs (1:2000). (**a**) Percent euglycemia exhibited improved diabetes reversal of diabetes for the control group (blue, n= 10 of 13) relative to co-transplant groups (red, n= 7 of 13) (*$p<0.05$, log-rank). (**b**) Weekly non-fasting blood glucose measurements, regardless of euglycemia, illustrated improved graft function for islets transplanted alone (blue) relative to islets co-transplanted with Ad-MSCs (red). (**c**) Intraperitoneal glucose tolerance test (**d**) and blood glucose area under the curve (AUC) demonstrated a similar trend for respective groups (*$p>0.05$, ANOVA). Naïve normoglycemic mice served as controls (n=6) and data represented as (mean ± s.e.m).

2.4.4 Co-Culture of Mouse Islets and Ad-MSCs for 48 hours followed by Co-Transplantation Improves Engraftment

Post-isolation, islet aliquots were placed into non-adherent petri dishes and co-cultured with or without the presence of Ad-MSCs (1:2000 islet to Ad-MSC ratio, 48hrs co-culture period) and subsequently transplanted into diabetic mice under the kidney capsule (200 islets per recipient). Mice co-transplanted with Ad-MSCs (1:2000 islet to Ad-MSC ratio) reversed to euglycemia at a faster rate (22.3 ± 4.7 days) than the islets alone group (38.5 ± 7.6 days) (**Fig. 2.5a,b**). As well, percent engraftment was improved in those mice transplanted with islets co-cultured with 1:2000 Ad-MSCs (islets: 9 out of 19 (47%) vs. 1:2000: 18 out of 21 (86%)) ($p<0.05$) (**Fig. 2.5.a,b**). Sixty days post-transplant, all euglycemic mice returned to hyperglycemia post recovery nephrectomy. IPGTTs were performed on all euglycemic recipients 7 weeks post-transplant (**Fig. 2.5c**) and there was no significant difference for area under the curve amongst respective groups (**Fig. 2.5d**).

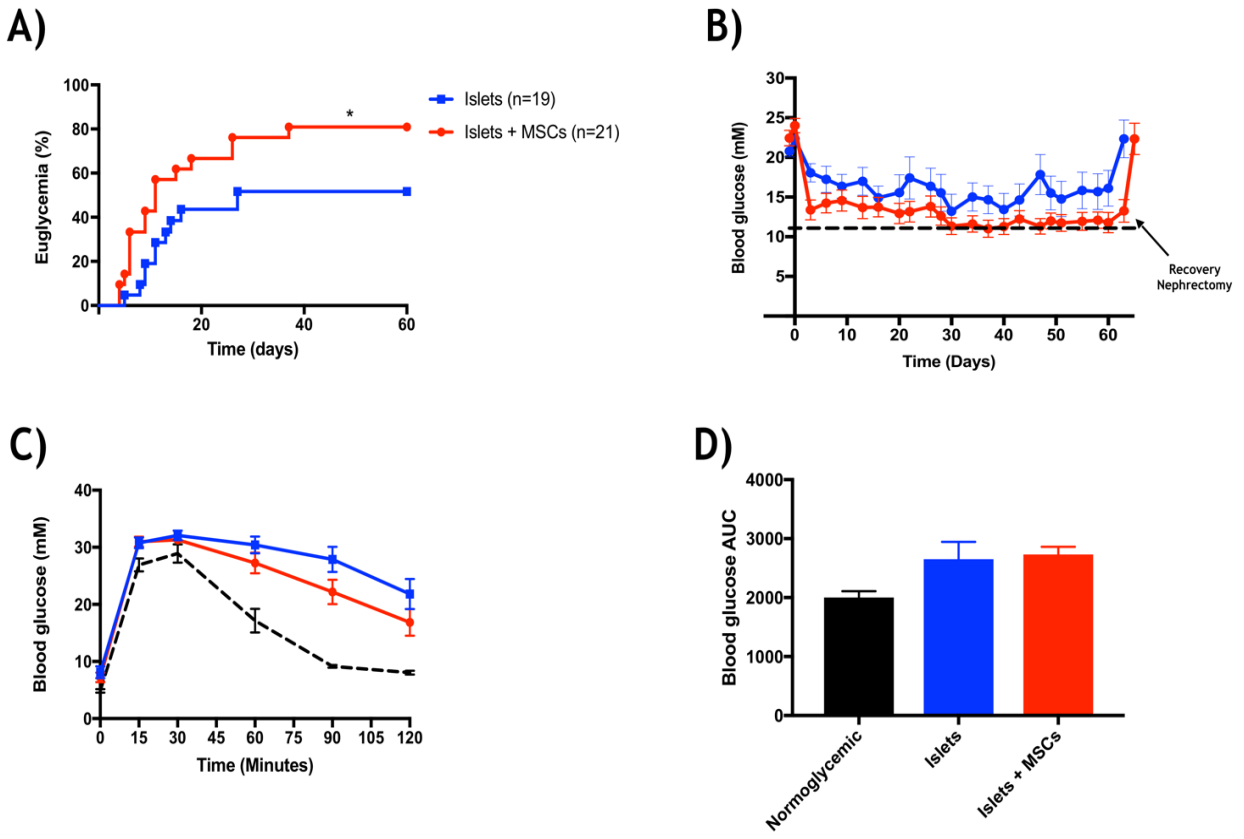

Figure 2. 5 Efficacy of 48-hour co-culture period.

Subsequent to 48-hour co-culture period, 200 BALB/c islets were counted for respective groups and co-transplanted with human Ad-MSCs under the kidney capsule of diabetic $Rag^{-/-}$ mice. (**a**) Percent euglycemia displayed significant diabetes reversal for the islet-Ad-MSC group, 1:2000 (red, n=18 of 21) versus the control group (blue, n= 9 of 19) (*$p<0.05$, log-rank). (**b**) Weekly non-fasting blood glucose measurements, regardless of euglycemia, demonstrated similar glucose profile amongst the control (blue) and 1:2000 (red) groups. (**c**) 7 weeks' post-transplant an intraperitoneal glucose tolerance test was similar between groups and blood glucose area under the curve (AUC) (**d**) supported no significance amongst respective groups (*$p>0.05$, ANOVA). Naïve normoglycemic mice served as controls (n=6) and data represented as (mean ± s.e.m).

2.4.5 Corrected Group Glucose Tolerance to Assess Transplant Outcomes

Intraperitoneal glucose tolerance tests (IPGTT) were performed only on euglycemic mice from all *in vivo* groups (**Fig. 2.4.c,d and Fig. 2.5.d**). Taking into consideration that a minimal islet mass was transplanted, a corrected calculation was used to evaluate the glucose tolerance as a function of the proportion of animals that became normoglycemic after transplant. This was to correct for selection bias since non-euglycemic mice did not undergo IPGTT. The corrected group glucose tolerance (CGGT) was calculated by comparing the area under the curve for glucose of each experimental group divided by the corresponding proportion of normoglycemic mice in the respective groups **(Fig. 2.6)** with lower scores indicating superior outcome. Corrected group glucose tolerance for islets transplanted alone was superior compared to islets co-transplanted with Ad-MSCs without prior culture (corrected glucose tolerance islets: 3786 ± 403.8 mmol/L/120min vs. 1:2000: 5415 ± 369.7 mmol/L/120min, $p<0.05$, ANOVA, **Fig. 2.6a**). In contrast, with islets co-transplanted with Ad-MSCs after 48-hour culture displayed improved glucose tolerance compared to islets cultured alone transplanted alone respectively (corrected glucose tolerance islets: 6632 ± 396.2 mmol/L/120min vs. 1:2000: 3441 ± 187.7 mmol/L/120min, $p>0.05$, ANOVA, **Fig. 2.6b**).

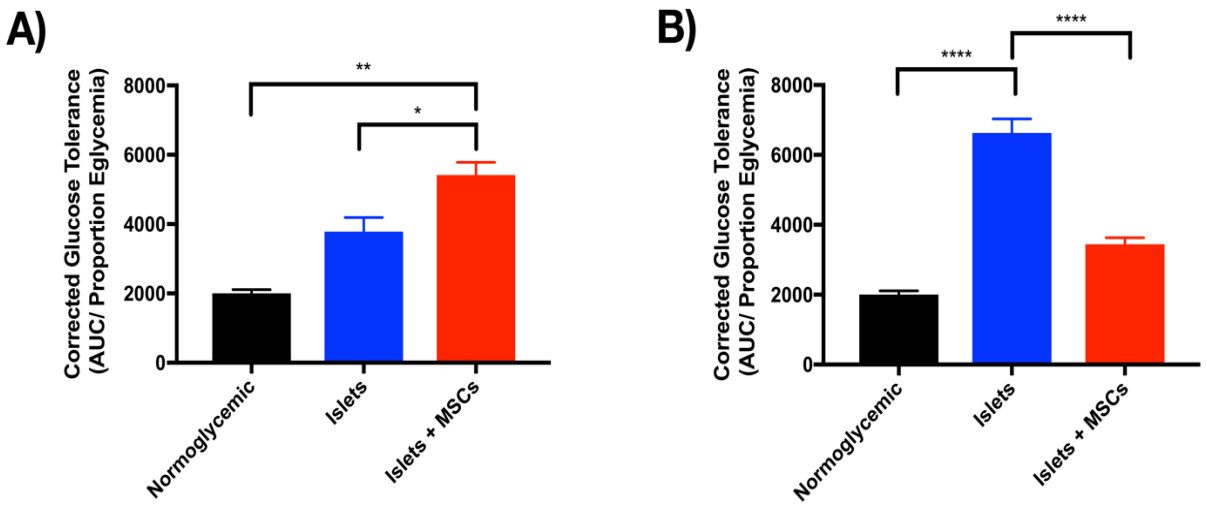

Figure 2. 6 Corrected Group Glucose Tolerance

Intraperitoneal glucose tolerance tests were performed only on euglycemic mice 7 week's post-transplant. The corrected glucose tolerance was calculated by comparing the area under the curve for the glycemic tolerance over the proportion of euglycemic mice. (**a**) Marginal mass islet co-transplant recipients (150 islets, no culture period) with Ad-MSCs (red), with no culture period, had hindered glucose tolerance compared to islets alone (blue) recipients (*$p<0.05$, ANOVA). (**b**) Islets co-cultured for 48 hours (200 islets) with Ad-MSCs followed by co-transplant (blue) had improved glucose tolerance compared to islets transplanted alone (red) (***$p<0.0001$, ANOVA).

Insulin Content of Kidney Islet Engraftment

At 60 days' post islet transplant, kidney bearing grafts were removed from both normoglycemic and hyperglycemic mice and measured for insulin content. The insulin content in the marginal mass of 150 islets transplanted without a culture period did not differ amongst groups (islets: 0.9 ± 0.04ng/g of kidney tissue vs. 1:2000: 1.3 ± 0.14ng/g, **Fig. 2.7a**). Similarly, islets cultured for 48-hours and co-transplanted did not differ amongst respective groups (islets: 2.1 ± 0.8ng/g vs. 1:2000: 3.1 ± 0.12ng/g, **Fig. 2.7b**). Immunohistochemistry analyzing insulin (islets: 1.8 ± 0.3% vs. 1:2000: 1.38 ± 0.21%) and glucagon (islets: 1.4 ± 0.2% vs. 1:2000: 1.4 ± 0.3%) content did not display significance amongst respective groups (**Fig. 2.8**).

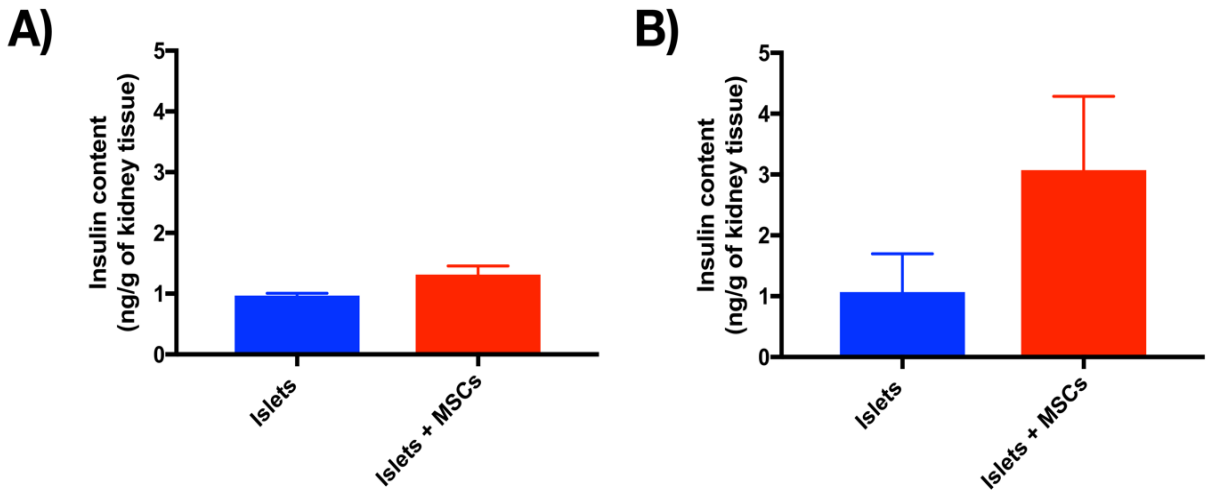

Figure 2. 7 Kidney Islet Engraftment Insulin Content

Kidney bearing grafts were removed from mice ≥ 60 days post islet transplant. (**a**) Insulin content in islet grafts were similar between those animals that had marginal mass (150 islets) islet co-transplants with Ad-MSCs, with no culture period (n=2, red), compared to islets transplanted alone (n=2, blue) ($p>0.05$, t-test). (**b**) Graft insulin content for islets co-cultured for 48 hours (200 islets) followed by co-transplant demonstrated similar insulin content for islets alone (n=3, blue) and islets with Ad-MSCs (n=3, blue) ($p>0.05$, t-test). Data represented (mean ± s.e.m).

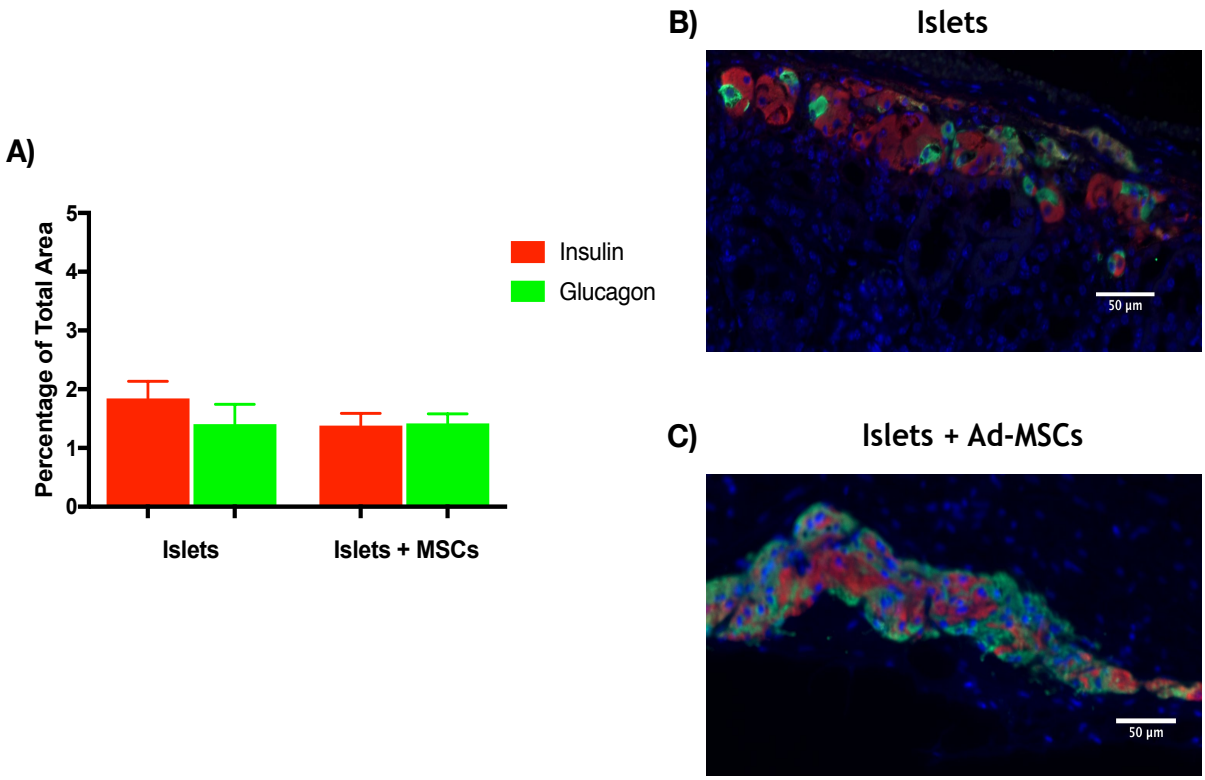

Figure 2. 8 Immunohistochemistry of Islet Graft Transplanted under the Kidney Capsule of an Immunodeficient Mouse

Kidney bearing grafts were removed from euglycemic mice ≥ 60 days' post islet transplant. (**a**) Insulin and glucagon content in islet grafts were similar amongst islets transplanted with or without Ad-MSCs (200 islets, 1:2000 Ad-MSCs: islets alone n=4 and Ad-MSCs n= 5) ($p>0.05$, t-test). Fluorescent staining of islet grafts positive for insulin (red), glucagon (green), and nuclei (blue) for islets transplanted alone (**b**) and islets co-transplanted with Ad-MSCs (**c**). Data represented (mean ± s.e.m).

2.4.6 Human Islets Co-Cultured with Mesenchymal Cells Maintain *In Vitro* Function

Human islets were examined under the same *in vitro* conditions as mouse islets. Although not significant, human islets cultured alone exhibited increased islet loss (12.2 ± 12.8% islet loss) compared to both Ad-MSC groups: 1:300 (2.3 ± 2.9%) and 1:2000 (0.3 ± 0.2 %) (**Fig. 2.9a**). Comparable to mouse islets, Ad-MSCs adhered to human islets (**Fig. 2.9b**). Membrane viability was significantly higher in islets co-cultured with 1:2000 Ad-MSCs (12.1 ± 1.6%) relative to both the 1:300 and islets cultured alone groups (23.5 ± 4.7% and 25.5 ± 2.5%, respectively) ($p<0.01$, **Fig. 2.9c**). Insulin stimulation index from sGSIS revealed no significant difference amongst groups (islets: 1.3 ± 0.3 vs. 1:300: 3.4 ± 0.9 and 1:2000: 4.5 ± 1.2) (**Table 2.1).**

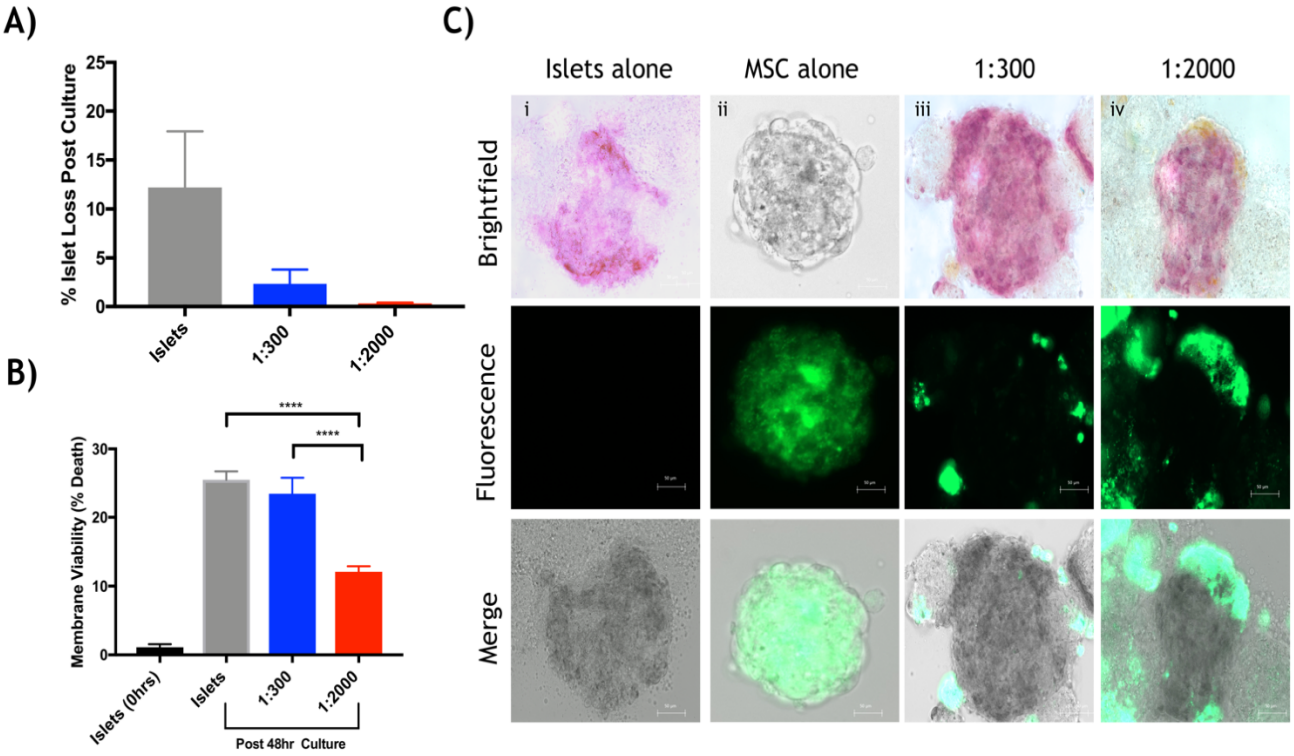

Figure 2. 9 In *Vitro* Assessment of Human Islet Recovery, Viability and Function after Co-Culture with Adipose Mesenchymal Cells.

In vitro assessment of human islets co-cultured with human adipose derived mesenchymal stem cells (n=3, isolations). (**a**) The percentage islet loss post 48-hour co-culture was not statistically different amongst groups ($p>0.05$, ANOVA) (**b**) Live/dead staining by dual-fluorescence revealed human islets cultured with 1:2000 Ad-MSCs (red) had significantly improved islet viability compared to islets cultured alone (grey) and cultured with 1:300 Ad-MSCs (blue) respectively (**$p<0.01$, ANOVA). (**c**) Microscopic imaging of labelled human adipose derived mesenchymal stem cells adhered to human islet cells after culture. Column (i) Islets cultured alone, (ii) Ad-MSCs cultured alone, (iii) 1:300 islet to Ad-MSC ratio and (iv) 1:2000 islet to Ad-MSC ratio. Rows (from top to bottom) include bright field microscopic imaging with islets stained with dithizone, fluorescence imaging displaying cell labelled mesenchymal stem cells (DiO staining) displayed in green and combined brightfield and fluorescence microscopic images. White bar represents 50µm.

2.5 Discussion

In this study, we show that co-culturing mouse islets for 48 hours with human Ad-MSCs improves islet function and efficacy relative to islets cultured alone. Furthermore, we demonstrated at a limited capacity human islets co-cultured with Ad-MSCs may improve *in vitro* islet recovery and function. Collectively, these findings provide a fundamental basis for applications within a clinical setting. Clinical islet transplantation is restricted currently to patients with brittle T1DM that cannot be stabilized by alternative measures. Multiple intraportal islet infusions are often required to sustain euglycemia. Human islet loss during culture and intraportal transplantation is substantial, related in part to IBMIR, hypoxia, apoptosis, and other inflammatory or immune activating events. [29-32] Supplementation of islets with additives during culture may support islet function, morphology, and vitality. [33-35] It has been shown previously that human and mouse islet function can be maintained for up to 30 days after co-culture with MSCs. [36, 37] During the culture period, high doses of mouse derived MSCs and islets adhere to the edges of islets and can penetrate the islet core to form MSC-islet composites. [26] Consequentially, most studies evaluate the utilization of bone marrow derived MSCs and limited research evaluates the use of Ad-MSCs. [38, 39] Herein, this study examined the supplementation of mouse or human islets with human-derived Ad-MSCs during a 48-hour co-culture period to evaluate their potential clinical relevance and therapeutic benefits for human islet survival and engraftment.

Our *in vitro* observations demonstrated the ability to increase mouse islet yield, maintain vitality, cell survival and insulin secretion with the supplementation of Ad-MSCs during co-culture relative to islets cultured alone. Our observations are potentially applicable for future translation to the clinic as we demonstrated the capacity of human Ad-MSCs to also improve human islet *in vitro* potency when co-cultured. Our findings support previous literature where human islets with human-derived Ad-MSCs improved islet function *in vitro* after 72-hour culture on an engineered cell sheet [40] and improved islet efficacy in mice by pre-48-hour-hypoxic cultured human-derived Ad-

MSCs intraperitoneally injected into mice. [41] Likewise, rat islets following a 72-hour post-culture with human-derived Ad-MSCs had improved islet engraftment upon intraportal co-transplantation in rats. [42] Direct static co-culture with MSCs has the potential to release local cytokines, including VEGF and ANAX1, to promote cell survival. [13, 14, 43] We did not measure such trophic factors but in an effort to comprehend and analyze the activity of Ad-MSCs we collected media after 48h co-culture to quantify proinflammatory expression. [44-46] Mouse and human cytokine profiles was neither elevated nor down regulated, except for murine IL2p70. IL2p70 is a lymphocyte membrane glycoprotein (p70, IL-2RP) which aids IL-2 recruitment of lymphocytes and natural killer activity to the islet engraftment. [47, 48] The down regulation of IL2p70 may be potentially advantageous in dampening early immune responses. From these data, we interpret that MSCs are not harmful for *in vitro* islet survival and function, and the ability to downregulate pro-inflammatory cytokines could be dependent on *in vivo* function. [3, 49]

Based on our *in vitro* observations, the 1:2000 islet to Ad-MSC ratio was used for *in vivo* transplantations due to their superior *in vitro* islet function. Although islet mass transplanted was different, we have shown improved graft efficacy when islets were co-cultured with Ad-MSCs relative to islets co-transplanted with Ad-MSCs immediately after isolation without prior co-culture. We speculate improved graft function was due to the trophic effect of Ad-MSCs during a prolonged direct cell contact of islets and Ad-MSC. In addition, Ad-MSCs are single cells when transplanted without a culture period and they could migrate away from the transplant site, whereas, during a co-culture, Ad-MSCs adhere to islets and clump together and are likely to remain at the engraftment site. [50] Extended culture times can reduce exocrine tissue and endotoxin carry-over [49] which may account for our observed marked improvement in islet potency after co-culture. We and others have demonstrated that MSCs can decrease islet loss, maintain islet morphology and function during co-culture with islets compared to culture of islets alone. [24] In this study, as a result of the islet loss in the control group compared to the islet and Ad-MSC group, the number of donor pancreata in

the control group had to be increased to ensure that all groups were transplanted with a similar islet mass after 48-hour culture.

These findings are potentially readily translatable to the clinical setting, where human islets routinely undergo up to 72 hours of culture. Addition of human Ad-MSCs manufactured under good manufacturing conditions (GMP) during this period could be highly protective, and may decrease the need for multiple donors and risk of Human Leukocyte Antigen sensitization. Autologous MSCs offer potential promise in mitigating allo-rejection, perhaps allowing transplants to proceed with less immunosuppression and therefore less potential risk. To further elucidate the cytoprotective capacity of human Ad-MSCs our future research efforts will further explore the impact that co-culture on human islet function and engraftment. Herein, we highlight a potential translatable strategy to protect islets in culture and early after transplantation that can increase the frequency of donor engraftment rates with the supplementation of Ad-MSC during culture.

2.7 References

1. Hering BJ, Clarke WR, Bridges ND, Eggerman TL, Alejandro R, Bellin MD, et al. Phase 3 Trial of Transplantation of Human Islets in Type 1 Diabetes Complicated by Severe Hypoglycemia. Diabetes Care 2016; 39:1230-40.

2. Emamaullee JA, Shapiro AM. Factors influencing the loss of beta-cell mass in islet transplantation. Cell transplantation 2007; 16:1-8.

3. Kanak MA, Takita M, Kunnathodi F, Lawrence MC, Levy MF, Naziruddin B. Inflammatory response in islet transplantation. Int J Endocrinol 2014; 2014:451035.

4. Biarnes M, Montolio M, Nacher V, Raurell M, Soler J, Montanya E. Beta-cell death and mass in syngeneically transplanted islets exposed to short- and long-term hyperglycemia. Diabetes 2002; 51:66-72.

5. Eich T, Eriksson O, Lundgren T, Nordic Network for Clinical Islet T. Visualization of early engraftment in clinical islet transplantation by positron-emission tomography. N Engl J Med 2007; 356:2754-5.

6. Hematti P, Kim J, Stein AP, Kaufman D. Potential role of mesenchymal stromal cells in pancreatic islet transplantation. Transplant Rev (Orlando) 2013; 27:21-9.

7. Park KS, Shim EY, Choi BK, Moon C, Kim SH, Kim YS, et al. Cartilage oligomeric matrix protein-angiopoientin-1 enhances angiogenesis of isolated islet and maintains normoglycemia following transplantation. Transplant Proc 2010; 42:2653-7.

8. Phinney DG, Prockop DJ. Concise review: mesenchymal stem/multipotent stromal cells: the state of transdifferentiation and modes of tissue repair--current views. Stem Cells 2007; 25:2896-902.

9. Yeung TY, Seeberger KL, Kin T, Adesida A, Jomha N, Shapiro AM, et al. Human mesenchymal stem cells protect human islets from pro-inflammatory cytokines. PLoS One 2012; 7:e38189.

10. Ding Y, Xu D, Feng G, Bushell A, Muschel RJ, Wood KJ. Mesenchymal stem cells prevent the rejection of fully allogenic islet grafts by the immunosuppressive activity of matrix metalloproteinase-2 and -9. Diabetes 2009; 58:1797-806.

11. Ito T, Itakura S, Todorov I, Rawson J, Asari S, Shintaku J, et al. Mesenchymal stem cell and islet co-transplantation promotes graft revascularization and function. Transplantation 2010; 89:1438-45.

12. Rackham CL, Vargas AE, Hawkes RG, Amisten S, Persaud SJ, Austin AL, et al. Annexin A1 Is a Key Modulator of Mesenchymal Stromal Cell-Mediated Improvements in Islet Function. Diabetes 2016; 65:129-39.

13. Gruber R, Kandler B, Holzmann P, Vogele-Kadletz M, Losert U, Fischer MB, et al. Bone marrow stromal cells can provide a local environment that favors migration and formation of tubular structures of endothelial cells. Tissue Eng 2005; 11:896-903.

14. Liu M, Han ZC. Mesenchymal stem cells: biology and clinical potential in type 1 diabetes therapy. J Cell Mol Med 2008; 12:1155-68.

15. Caplan AI, Dennis JE. Mesenchymal stem cells as trophic mediators. J Cell Biochem 2006; 98:1076-84.

16. Chen L, Tredget EE, Wu PY, Wu Y. Paracrine factors of mesenchymal stem cells recruit macrophages and endothelial lineage cells and enhance wound healing. PLoS One 2008; 3:e1886.

17. Yamamoto T, Horiguchi A, Ito M, Nagata H, Ichii H, Ricordi C, et al. Quality control for clinical islet transplantation: organ procurement and preservation, the islet processing facility, isolation, and potency tests. J Hepatobiliary Pancreat Surg 2009; 16:131-6.

18. Yamamoto T, Ricordi C, Messinger S, Sakuma Y, Miki A, Rodriguez R, et al. Deterioration and variability of highly purified collagenase blends used in clinical islet isolation. Transplantation 2007; 84:997-1002.

19. Kin T, Senior P, O'Gorman D, Richer B, Salam A, Shapiro AM. Risk factors for islet loss during culture prior to transplantation. Transpl Int 2008; 21:1029-35.

20. Nyqvist D, Kohler M, Wahlstedt H, Berggren PO. Donor islet endothelial cells participate in formation of functional vessels within pancreatic islet grafts. Diabetes 2005; 54:2287-93.

21. Korsgren O, Lundgren T, Felldin M, Foss A, Isaksson B, Permert J, et al. Optimising islet engraftment is critical for successful clinical islet transplantation. Diabetologia 2008; 51:227-32.

22. Shapiro AM, Pokrywczynska M, Ricordi C. Clinical pancreatic islet transplantation. Nat Rev Endocrinol 2017; 13:268-77.

23. Fraker C, Montelongo J, Szust J, Khan A, Ricordi C. The use of multiparametric monitoring during islet cell isolation and culture: a potential tool for in-process corrections of critical physiological factors. Cell transplantation 2004; 13:497-502.

24. Hayward JA, Ellis CE, Seeberger K, Lee T, Salama B, Mulet-Sierra A, et al. Cotransplantation of Mesenchymal Stem Cells With Neonatal Porcine Islets Improve Graft Function in Diabetic Mice. Diabetes 2017; 66:1312-21.

25. Rackham CL, Dhadda PK, Le Lay AM, King AJ, Jones PM. Preculturing Islets With Adipose-Derived Mesenchymal Stromal Cells Is an Effective Strategy for Improving Transplantation Efficiency at the Clinically Preferred Intraportal Site. Cell Med 2014; 7:37-47.

26. Rackham CL, Dhadda PK, Simpson SJS, Godazgar M, King AJF, Jones PM. Composite Mesenchymal Stromal Cell Islets: Implications for Transplantation via the Clinically Preferred Intraportal Route. Transplant Direct 2018; 4:e354.

27. Cabrera O, Jacques-Silva MC, Berman DM, Fachado A, Echeverri F, Poo R, et al. Automated, high-throughput assays for evaluation of human pancreatic islet function. Cell transplantation 2008; 16:1039-48.

28. Merani S, Toso C, Emamaullee J, Shapiro AM. Optimal implantation site for pancreatic islet transplantation. Br J Surg 2008; 95:1449-61.

29. Linn T, Schmitz J, Hauck-Schmalenberger I, Lai Y, Bretzel RG, Brandhorst H, et al. Ischaemia is linked to inflammation and induction of angiogenesis in pancreatic islets. Clin Exp Immunol 2006; 144:179-87.

30. Brissova M, Powers AC. Revascularization of transplanted islets: can it be improved? Diabetes 2008; 57:2269-71.

31. Zhang N, Richter A, Suriawinata J, Harbaran S, Altomonte J, Cong L, et al. Elevated vascular endothelial growth factor production in islets improves islet graft vascularization. Diabetes 2004; 53:963-70.

32. Gala-Lopez BL, Pepper AR, Pawlick RL, O'Gorman D, Kin T, Bruni A, et al. Antiaging Glycopeptide Protects Human Islets Against Tacrolimus-Related Injury and Facilitates Engraftment in Mice. Diabetes 2016; 65:451-62.

33. Nilsson B, Ekdahl KN, Korsgren O. Control of instant blood-mediated inflammatory reaction to improve islets of Langerhans engraftment. Curr Opin Organ Transplant 2011; 16:620-6.

34. Berney T. Islet culture and counter-culture. Commentary on: Effect of short-term culture on functional and stress-related parameters in isolated human islets by Ihm et al. Transpl Int 2009; 22:531-3.

35. J C. Mesenchymal Stem Cells Promote Islet Survival In Vitro and Function In Vivo. CellR4 2013; 1:382.

36. Park KS, Kim YS, Kim JH, Choi B, Kim SH, Tan AH, et al. Trophic molecules derived from human mesenchymal stem cells enhance survival, function, and angiogenesis of isolated islets after transplantation. Transplantation 2010; 89:509-17.

37. He Y, Zhang D, Zeng Y, Ma J, Wang J, Guo H, et al. Bone Marrow-Derived Mesenchymal Stem Cells Protect Islet Grafts Against Endoplasmic Reticulum Stress-Induced Apoptosis During the Early Stage After Transplantation. Stem Cells 2018.

38. Jung EJ, Kim SC, Wee YM, Kim YH, Choi MY, Jeong SH, et al. Bone marrow-derived mesenchymal stromal cells support rat pancreatic islet survival and insulin secretory function in vitro. Cytotherapy 2011; 13:19-29.

39. Imamura H, Adachi T, Kin T, Ono S, Sakai Y, Adachi T, et al. An engineered cell sheet composed of human islets and human fibroblast, bone marrow-derived mesenchymal stem cells, or adipose-derived mesenchymal stem cells: An in vitro comparison study. Islets 2018:1-11.

40. Schive SW, Mirlashari MR, Hasvold G, Wang M, Josefsen D, Gullestad HP, et al. Human Adipose-Derived Mesenchymal Stem Cells Respond to Short-Term Hypoxia by Secreting Factors Beneficial for Human Islets In Vitro and Potentiate Antidiabetic Effect In Vivo. Cell Med 2017; 9:103-16.

41. Cavallari G, Olivi E, Bianchi F, Neri F, Foroni L, Valente S, et al. Mesenchymal stem cells and islet cotransplantation in diabetic rats: improved islet graft revascularization and function by human adipose tissue-derived stem cells preconditioned with natural molecules. Cell transplantation 2012; 21:2771-81.

42. Johansson U, Rasmusson I, Niclou SP, Forslund N, Gustavsson L, Nilsson B, et al. Formation of composite endothelial cell-mesenchymal stem cell islets: a novel approach to promote islet revascularization. Diabetes 2008; 57:2393-401.

43. Rackham CL, Dhadda PK, Chagastelles PC, Simpson SJ, Dattani AA, Bowe JE, et al. Pre-culturing islets with mesenchymal stromal cells using a direct contact configuration is beneficial for transplantation outcome in diabetic mice. Cytotherapy 2013; 15:449-59.

44. Karaoz E, Genc ZS, Demircan PC, Aksoy A, Duruksu G. Protection of rat pancreatic islet function and viability by coculture with rat bone marrow-derived mesenchymal stem cells. Cell Death Dis 2010; 1:e36.

45. de Souza BM, Boucas AP, Oliveira FD, Reis KP, Ziegelmann P, Bauer AC, et al. Effect of co-culture of mesenchymal stem/stromal cells with pancreatic islets on viability and function outcomes: a systematic review and meta-analysis. Islets 2017; 9:30-42.

46. Sharon M, Klausner RD, Cullen BR, Chizzonite R, Leonard WJ. Novel interleukin-2 receptor subunit detected by cross-linking under high-affinity conditions. Science 1986; 234:859-63.

47. Siegel JP, Sharon M, Smith PL, Leonard WJ. The IL-2 receptor beta chain (p70): role in mediating signals for LAK, NK, and proliferative activities. Science 1987; 238:75-8.

48. Sandler S, Bendtzen K, Eizirik DL, Welsh M. Interleukin-6 affects insulin secretion and glucose metabolism of rat pancreatic islets in vitro. Endocrinology 1990; 126:1288-94.

Chapter 3:

Bioengineered Human Pseudoislets Form Efficiently From Donated Tissue, Outperform Native Islets In Vitro, and Restore Normoglycemia In Mice.

Chapter 3: Bioengineered human pseudoislets form efficiently from donated tissue, outperform native islets in vitro, and restore normoglycemia in mice.

Diabetologia
https://doi.org/10.1007/s00125-018-4672-5

ARTICLE

Bioengineered human pseudoislets form efficiently from donated tissue, compare favourably with native islets in vitro and restore normoglycaemia in mice

Yang Yu[1,2] · Anissa Gamble[2] · Rena Pawlick[2,3] · Andrew R. Pepper[2,3] · Bassem Salama[2,3] · Derek Toms[2,4] · Golsa Razian[2,4] · Cara Ellis[2] · Antonio Bruni[2,3] · Boris Gala-Lopez[2,3] · Jia (Lulu) Lu[2,4] · Heather Vovko[2,4] · Cecilia Chiu[2,4] · Shaaban Abdo[2,4] · Tatsuya Kin[5] · Greg Korbutt[2] · A. M. James Shapiro[2,3,5] · Mark Ungrin[1,2,4,6]

Received: 15 February 2018 / Accepted: 23 May 2018

Abstract

Aims/hypothesis Islet transplantation is a treatment option that can help individuals with type 1 diabetes become insulin independent, but inefficient oxygen and nutrient delivery can hamper islet survival and engraftment due to the size of the islets and loss of the native microvasculature. We hypothesised that size-controlled pseudoislets engineered via centrifugal-forced-aggregation (CFA-PI) in a platform we previously developed would compare favourably with native islets, even after taking into account cell loss during the process.

Methods Human islets were dissociated and reaggregated into uniform, size-controlled CFA-PI in our microwell system. Their performance was assessed in vitro and in vivo over a range of sizes, and compared with that of unmodified native islets, as well as islet cell clusters formed by a conventional spontaneous aggregation approach (in which dissociated islet cells are cultured on ultra-low-attachment plates). In vitro studies included assays for membrane integrity, apoptosis, glucose-stimulated insulin secretion assay and total DNA content. In vivo efficacy was determined by transplantation under the kidney capsule of streptozotocin-treated *Rag1*$^{-/-}$ mice, with non-fasting blood glucose monitoring three times per week and IPGTT at day 60 for glucose response. A recovery nephrectomy, removing the graft, was conducted to confirm efficacy after completing the IPGTT. Architecture and composition were analysed by histological assessment via insulin, glucagon, pancreatic polypeptide, somatostatin, CD31 and von Willebrand factor staining.

Results CFA-PI exhibit markedly increased uniformity over native islets, as well as substantially improved glucose-stimulated insulin secretion (8.8-fold to 11.1-fold, even after taking cell loss into account) and hypoxia tolerance. In vivo, CFA-PI function similarly to (and potentially better than) native islets in reversing hyperglycaemia (55.6% for CFA-PI vs 20.0% for native islets at 500 islet equivalents [IEQ], and 77.8% for CFA-PI vs 55.6% for native islets at 1000 IEQ), and significantly better than spontaneously aggregated control cells (55.6% for CFA-PI vs 0% for spontaneous aggregation at 500 IEQ, and 77.8% CFA-PI vs 33.4% for spontaneous aggregation at 1000 IEQ; $p < 0.05$). Glucose clearance in the CFA-PI groups was improved over that

Greg Korbutt, A.M. James Shapiro and Mark Ungrin are joint senior authors.

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s00125-018-4672-5) contains peer-reviewed but unedited supplementary material, which is available to authorised users.

✉ Mark Ungrin
mark.ungrin@ucalgary.ca

[1] Biomedical Engineering Graduate Program, University of Calgary, Calgary, AB, Canada

[2] Alberta Diabetes Institute, University of Alberta, Edmonton AB, Canada

[3] Canadian National Transplant Research Program, Edmonton

[4] Department of Comparative Biology and Experimental Medicine, Faculty of Veterinary Medicine, University of Calgary, Heritage Medical Research Building Room 412, 3330 Hospital Drive NW, Calgary, AB T2N 4N1, Canada

[5] Clinical Islet Transplant Program, University of Alberta, Edmonton, AB, Canada

[6] Alberta Children's Hospital Research Institute, University of Calgary, Calgary, AB, Canada

2-5

A version of this manuscript is published in *Diabetologia 2018* Feb; e1428511.

ORIGINAL ARTICLE

Bioengineered human pseudoislets form efficiently from donated tissue, outperform native islets in vitro, and restore normoglycemia in mice.

Yang Yu[1,2], Anissa Gamble[2], Rena Pawlick[2,3], Andrew Pepper[2,3], Bassem Salama[2,3], Derek Toms[2,4], Golsa Razian[2,4], Cara Ellis[2], Antonio Bruni[2,3], Boris Gala-Lopez B[2,3], Lulu Lu[2,4], Heather Vovko[2,4], Cecilia Chiu[2,4], Shaaban Abdo[2,4], Tatsuya Kin[5], Greg Korbutt[2,6], A.M. James Shapiro[2,3,5,6], Mark Ungrin[1,2,4,6,*]

[1]Biomedical Engineering Graduate Program, University of Calgary, Calgary, AB, Canada

[2]Alberta Diabetes Institute, University of Alberta, Edmonton, AB, Canada

[3]Canadian National Transplant Research Program, Edmonton, AB, Canada

[4]Department of Comparative Biology and Experimental Medicine, Faculty of Veterinary Medicine, University of Calgary, Calgary, AB, Canada

[5]Clinical Islet Transplant Program, University of Alberta, Edmonton, AB, Canada

[6]Denotes joint senior authorship

*Corresponding Author:

Mark Ungrin

HMRB 412, 3330 Hospital Drive NW

University of Calgary, Calgary, Alberta

Canada, T2N 4N1

Tel: 403.210.6203

Email: mark.ungrin@ucalgary.ca

Note: Chapter 3 was conducted in collaboration with Dr. Mark Ungrin and colleagues from the University of Calgary. Yang Yu is the first author of the project, and the article constitutes part of his doctor of philosophy degree. Yang Yu contributed to experimental design, *in vitro*

analysis, data formulation, figure creation and writing of the manuscript. Combined efforts of our entire team, where Rena Pawlick and I had leading roles in the design and execution of the experiments, data analysis, generation of figures, and monitoring. As the second author of the project and publication, this paper represents a substantial component of my MSc work.

3.1 Chapter Overview

3.1.1 Aims/hypothesis

Islet transplantation is a treatment option that can help patients with type 1 diabetes become insulin independent, but inefficient oxygen and nutrient delivery can hamper islet survival and engraftment due to the size of the islets and loss of the native microvasculature. We hypothesized that size-controlled pseudoislets engineered via centrifugal-forced-aggregation (CFA-PI) in a platform we previously developed would outperform native islets, even after taking into account cell loss during the process.

3.1.2 Methods

Human islets were dissociated and re-aggregated into uniform, size-controlled CFA-PI in our microwell system. Their performance was assessed *in vitro* and *in vivo* over a range of sizes, and compared to that of unmodified native islets, as well as islet cell clusters formed by a conventional spontaneous-aggregation approach (where dissociated islet cells are cultured on ultra-low-attachment plates). *In vitro* studies included assays for membrane integrity, apoptosis, glucose-stimulated insulin secretion assay and total DNA content. *In vivo* efficacy was determined by transplantation under the kidney capsule of streptozotocin-treated $Rag^{-/-}$ mice, with weekly non-fasting blood glucose monitoring and intraperitoneal glucose tolerance test for glucose response. A recovery nephrectomy at day 60, removing the graft, was conducted to confirm efficacy. Architecture and composition were analyzed by histological assessment via insulin, glucagon, pancreatic polypeptide, somatostatin, CD31 and von Willebrand factor staining.

3.1.3 Results

CFA-PI exhibit markedly increased uniformity over native islets, as well as substantially improved glucosestimulated insulin secretion (nine to eleven fold, even after taking cell loss into

account) and hypoxia tolerance. In vivo, CFA-PI function similarly to (and potentially better than) native islets in reverting hyperglycemia (55.6% CFA-PI at 500 IEQ versus 20.0% NI, and 77.8% CFA-PI versus 55.6% NI at 1000 IEQ), and significantly better than spontaneous-aggregation (SA) controls (55.6% CFA-PI versus 0% SA at 500 IEQ, and 77.8% CFA-PI versus 33.4% SA at 1000 IEQ, $p<0.05$). Glucose clearance in the CFA-PI groups was improved over the native islet groups (CFA-PI 18.1 mmol/l versus NI 29.7 mmol/l at 60 minutes, $p<0.05$) to the point where they were comparable to the non-transplanted naïve normoglycemic control mice at low islet equivalence, 500 IEQ (17.2 mmol/l at 60 minutes).

3.1.4 Conclusion

The ability to efficiently re-format dissociated islet cells into engineered pseudoislets with improved properties has high potential for both research and therapeutic applications.

3.2 Introduction

Type 1 diabetes is a major health problem affecting tens of millions of patients, and its prevalence has been increasing globally. [1,2] Conventional insulin-replacement therapies are only palliative, and fail to correct excursions in glycemic control, with consequent secondary diabetic complications of blindness, kidney failure, limb amputation, heart attack, stroke and up to a 15-year reduction in life expectancy. [3,4]

Islet transplantation represents a promising and effective treatment approach in selected patients with risk of hypoglycemia, [5-7] but this treatment currently fails to provide a cure. Substantial islet loss in the immediate post-transplant results from ischemia and an instant blood mediated inflammatory response (IBMIR) coupled with poor vascularization after implantation. [8] The islet microvasculature is disconnected during isolation, and degenerates during culture, and the post-transplant process of recruiting host endothelial cells is slow, even more so in the human system than the mouse. [10] Prior to revascularization, delivery of oxygen and nutrients occurs only via diffusion, which is insufficient to support cells in the core of the islet. [9,10] Even after a prolonged period, the native vasculature is not fully restored, [11-13] which is likely to contribute to long-term islet loss due to chronic stress, [14] and reduced oxygen levels negatively affect insulin secretion capacity. [15]

A widely-researched approach for the induction of immune tolerance in islet transplantation is to conceal the transplanted material from the host immune system using encapsulation, [16] and strategies targeting improved islet survival and engraftment must be compatible with this concept. This approach provides an additional diffusive barrier to the flow of nutrients and oxygen, reducing delivery to the islet and consequently the slope of the concentration gradient driving delivery to cells in the interior. [10] Further, in fulfilling the objective of blocking access by cells of the host immune system to the transplanted tissue, the capsule also prevents access by host endothelium, permanently forestalling reconstitution of the microvasculature and full

integration with the host blood system. In this case, engrafted islets will be completely dependent on diffusive delivery of oxygen and nutrients for the duration of the graft. Quantitative modeling of oxygen delivery shows significant benefits to smaller islets (whether encapsulated or not), [10,17] and consistent with this concept, smaller human islets are reported to perform better than larger ones both in a clinical setting [18] and in culture. [19] There is thus potential for engineering size-controlled islet cell clusters ("pseudoislets") to overcome some of these challenges.

Many attempts have been made to dissociate native islets and re-aggregate them. [20-26] The most common method has employed spontaneous aggregation, where the dissociated islet cell suspension is cultured in ultra-low binding plates. [26-28] This method results in pseudoislets that are heterogeneous in size, and yields are low. Alternatively, a hanging-drop approach has been used to form pseudoislets from rat [26] and human islet sources. [30] While this approach yields pseudoislets of uniform size, the method is labor-intensive and difficult to scale. More recently, there have been several reports of the formation of pseudoislets utilizing a microwell technique. [31-33] However, these approaches have relied on a variety of customized devices, which restricts widespread reproduction of the work and may impose limits on scalability. Moreover, the clinical relevance and quantitative *in vivo* and *in vitro* performance have not yet been fully investigated.

We have previously established a scalable microwell platform for the generation of large numbers of uniform cellular aggregates, [34] now widely available under the AggreWell name. In the present study, we apply a scalable centrifugal-forced-aggregation approach to generate large numbers of uniform, size-controlled pseudoislets, and characterize them *in vitro* and *in vivo*. We find that pseudoislet performance is significantly better than both spontaneous aggregates and native islets.

3.2 Methods

3.2.1 Human islet isolation

Human islet preparations were provided by the Alberta Diabetes Institute IsletCore and the Clinical Islet Transplant Program at the University of Alberta in Edmonton with the assistance of the Human Organ Procurement and Exchange (HOPE) program, Trillium Gift of Life Network (TGLN) and Canadian Organ Procurement Organizations (OPO), as well as the NIDDK-funded Integrated Islet Distribution Program (IIDP) at City of Hope, NIH Grant #2UC4DK098085-02, United States. While we saw no evidence of source-specific or purity-specific effects on our findings, *in vivo* experiments were carried out entirely with islets sourced from the ADI IsletCore to maximize consistency and minimize transport effects. *In vitro* experiments were performed in both Calgary and Edmonton under approvals for the use of human tissue from the respective Health Research Ethics Boards.

3.2.2 Human islet dissociation and re-aggregation

Islets were suspended in TrypLE Select (Thermo Fisher Scientific, Waltham, MA, USA) and incubated at 37°C in a shaking water bath for 8-10 minutes, followed by trituration to break up remaining clumps. After centrifugation (200g, 2 minutes) the cells were re-suspended in culture medium pre-warmed in a 37ºC water bath. Culture media consisted of CMRL-1066 (Corning, Manassas, VA, USA) supplemented with 1.2 g nicotinamide, 10 ml ITS, 2 mmol/l $ZnSO_4$, 50 ml sodium pyruvate, 10 ml 100X Glutamax (Gibco, California, USA), 10 ml penicillin/streptomycin, 25 ml HEPES and 10% FBS (Sigma-Aldrich, Oakville, ON, Canada).

10% fetal bovine serum, L-glutamine (2 mmol/L) (Sigma, St. Louis, MO, USA), penicillin (50 000 units) and streptomycin (50 mg) (Sigma Aldrich Canada Co., Oakville, ON, CA), HEPES (5 mmol/L)

CFA-PI were formed in 24-well or 6-well AggreWell 400 plates (STEMCELL Technologies, Vancouver, BC, Canada) as P1000, P750 and P500 (where P1000 represents CFA-PI formed from 1,000 cells apiece, P750 from 750 cells, etc.), with centrifugation (200g, 5 minutes) and cultured at density equivalent to 750, 563, 375 IEQ/ml respectively, with one IEQ defined as 1,600 cells. [32] Native islet controls were cultured in 24-well or 6-well ultra-low attachment plates (Corning, Manassas, VA, USA), as were dissociated single cells for spontaneous-aggregation controls, both plated at 500 IEQ/ml and cultured at 37ºC and 5% CO_2 for 3-5 days prior to transplant and further assay. Medium volumes used were 1ml/well for 24-well plates and 2.5ml/well for 6-well plates.

3.2.3 Static glucose-stimulated insulin secretion (s-GSIS)

An s-GSIS assay was performed for pre-cultured native islets and dissociated single cells (**SM Fig. 3.7.3.1**), and post 3-5 days of culture for all groups at 200 IEQ/group. The islets were washed of residual glucose three times in glucose free medium, incubated in RPMI-1640 containing low (2.8 mmol/l) glucose for one hour, followed by high (16.7 mmol/l) glucose for an additional hour at 37ºC and 5% CO2. The supernatant was harvested post glucose incubation and insulin levels measured by ELISA (Cat#10-1113-01, Mercodia, Uppsala, Sweden).

3.2.4 Hypoxic culture and viability analysis

CFA-PI and islets were cultured as described above at 37°C, 5% CO_2 for 7 days in both ambient and hypoxic (5% O_2 incubator) conditions. Islet and CFA-PI viability was then assessed using the inclusion and exclusion dyes fluorescein diacetate (FDA) and propidium iodide (PI) [33]. Islets were aliquoted into a 10 x 35 mm culture dish containing 460 µl Dulbecco's phosphate

buffered saline (DPBS). 10 µl of PI (750 µmol/l in DPBS) + 10 µl of FDA (24 µmol/l in acetone). Imaging by Zeiss AxioObserver Z1 inverted microscope.

3.2.5 Apoptosis TUNEL staining

Apoptosis of islets and pseudoislets was assessed by TUNEL assay (Promega, Madison, WI, USA). Tissue sections were co-stained with anti-insulin antibody at 1:200 concentration (Dako, Mississauga, ON, Canada) and DAPI to identify nuclei. Apoptosis was determined by analyzing the number of positive TUNEL-stained cells as a percentage nuclei within the insulin-positive. Sections analyzed were distributed blinded.

3.2.5 Real-time qPCR

RNA was isolated using a Total RNA Purification Kit (Norgen Biotek, Thorold, Canada), quantified on a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific), and reverse transcribed using the iScript Reverse Transcription Supermix (BioRad, Hercules, USA). Quantitative PCR (qPCR) was carried out (see Table 3.7.2.1 for primer sequences) on an Applied Biosystems Cycler (Thermo Fisher Scientific), and analyzed with the ΔΔCT method [36] with normalization against two stable internal reference genes (*POLR2A* and *EIF2B1)*. [37]

3.2.6 Islet transplantation

Female and male adult (8-10 weeks, 20-30 gm) immunodeficient mice (B6.129S7-Rag1$^{tm1Mom/J}$) were obtained from Jackson Laboratories, Canada. The care of the animals was in accordance with the guidelines approved by the Canadian Council on Animal Care. All in vivo experiments were carried out at the Alberta Diabetes Institute under approvals from the Research Ethics Board of the University of Alberta. Animals were housed in cages with no more than 5 animals per cage in a temperature-controlled environment, on a light/dark cycle with access to food and water *ad libitum*. Animals were randomized to groups and within cages. Islets from each

isolation were distributed between groups. Native islets, CFA-PI (P500, P750 and P1000), and spontaneous aggregates were transplanted under the capsule of the left kidney of STZ-induced diabetic mice. The islet graft-function was assessed through non-fasting blood glucose measurements, three times per week for 60 days, at which point the graft was retrieved from normoglycemic mice (<11.1 mmol/l) via a recovery nephrectomy of the left kidney and reversion to hyperglycemia confirmed (≥18 mmol/l).

3.2.7 Intraperitoneal glucose tolerance test (IPGTT)

In vivo glucose tolerance and islet function in mice regardless of euglycemia was assessed by IPGTT 60 days post-transplant. The mice were fasted overnight, and 25% dextrose was administered intraperitoneally at 3 g/kg body weight (Hospira, Lake Forest, IL, USA). Animal group identifications were blinded and blood glucose measurements were monitored at baseline (t=0), 15, 30, 60, 90 and 120 minutes.

3.2.8 Immunohistochemistry

Islet and CFA-PI transplant grafts were removed from mice. Immediately after explantation, the kidney bearing the islet graft was fixed in 10% formalin. The tissue was dissected, embedded in paraffin, and sectioned. Following de-paraffinization and antigen heat retrieval, sections were blocked with 20% goat serum in DPBS for 1.5 hours at room temperature. For vessel staining an additional enzymatic antigen retrieval step was performed prior to blocking (Proteinase K at 20 μg/ml for 20 minutes at 37°C). Sections were incubated with primary antibodies overnight at 4°C: guinea pig anti-insulin diluted 1:200 (Dako), rabbit anti-glucagon diluted 1:100 (Abcam, Cambridge, MA, USA), rabbit anti-human pancreatic polypeptide diluted 1:100 (Abcam), rat anti-human somatostatin diluted 1:100 (Abcam), or rabbit anti-CD31 and anti-von Willebrand factor (vWF) diluted 1:50 (Abcam). The following day sections were washed with Tween buffer followed by incubation with secondary antibodies 1:200 for 1 hour at room

temperature utilizing goat anti-guinea pig Rhodamine, goat anti-rat fluoroscein (Jackson ImmunoResearch, West Grove, PA, USA), or goat anti-rabbit Alexa Fluro 568 (Abcam). Samples were counterstained with DAPI (Invitrogen).

Imaging was carried out on a Zeiss COLIBRI inverted fluorescence microscope unless otherwise specified and analysis was via ImageJ software (http://rsb.info.nih.gov/ij). Spatial statistical analysis of vascular element distribution was performed using the "Spatial statistics 2D/3D" plugin, implementing the previously published F-function, [37,38] with independent evaluation points set to 10,000, hardcore distance set to 0, pattern samples set to 10.

3. 3 Results

3.3.1 CFA-PI form effectively and show substantially increased geometric consistency when compared to native islets

CFA-PI form and coalesce into spherical structures (**Fig. 3.1a-c**), as expected from well-understood free-energy-minimization models. [40,41] Varying the number of input cells provided precise control over CFA-PI size, and dramatically enhanced symmetry and size consistency as compared to native islets (**Fig. 3.1d,e** – note the P# terminology, where P1000 represents CFA-PI formed from 1,000 cells apiece, P750 from 750 cells, etc.). Comparison of diameter in all three axes via micromanipulation of individual CFA-PI confirmed this symmetry extends to all three dimensions (**Fig. 3.1f**). In the process we were also able to confirm the accessibility of the individual cells prior to CFA-PI assembly for genetic modification (**Fig. 3.7.3.2**) which may prove useful in future applications.

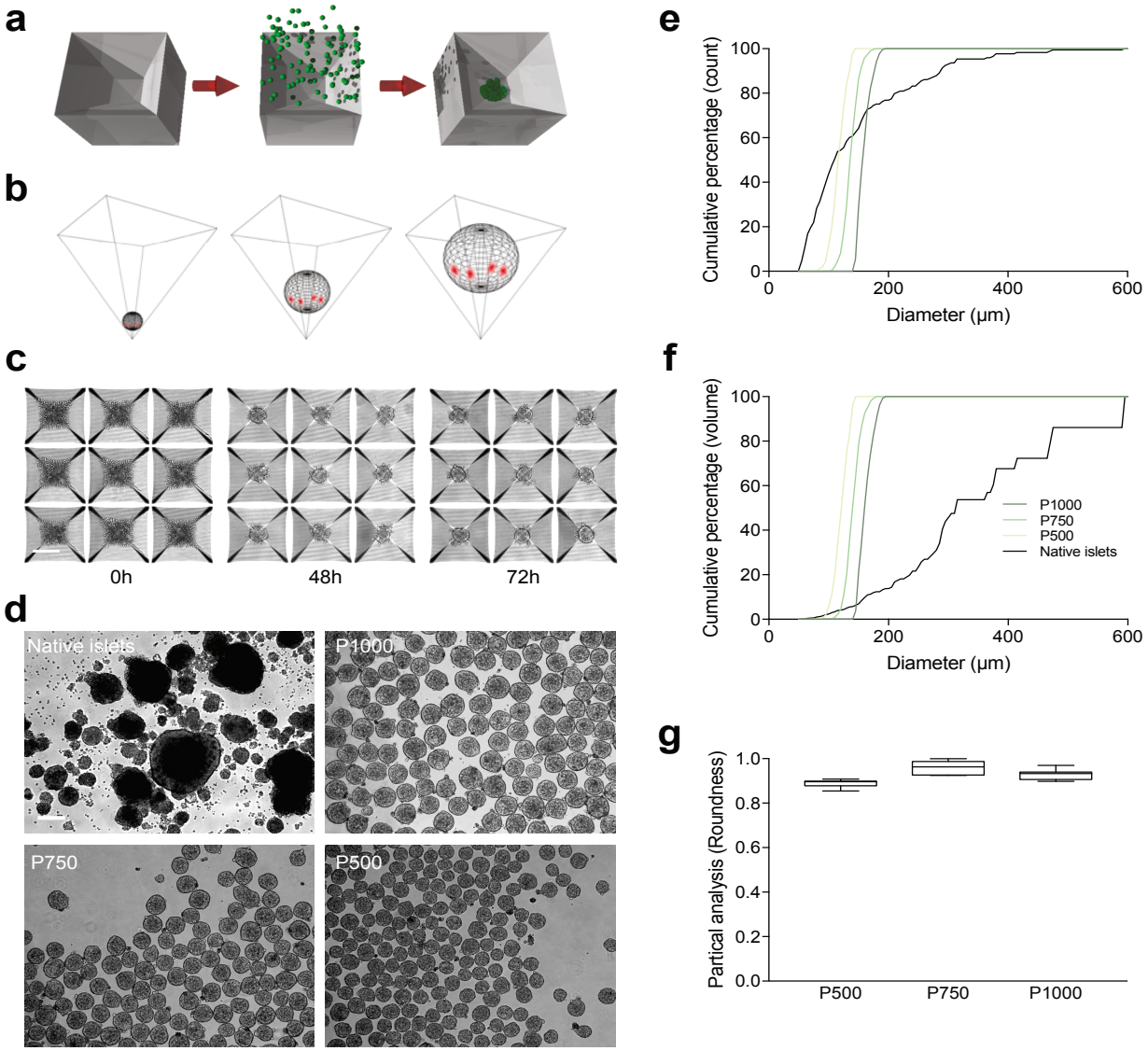

Figure 3. 1: *In Vitro* Pseudoislet Formation

CFA-PIs are formed via centrifugation of a suspension of single cells into square-pyramidal microwells (**a**). Geometric relationship between the aggregate, the microwell, and the underlying medium space (red highlights indicate contact points) is independent of aggregate size (**b**). CFA-PI form over 48 to 72 hours (**c**) in 400-micron microwells (scale bar represents 200 microns), and exhibit controlled sizes (P1000, P750, P500 represent CFA-PI formed from clusters of 1000, 750 or 500 cells respectively) and enhanced consistency over native islets (**d**). Quantifying these images, the cumulative size distribution of CFA-PIs (dark green, P1000; light green, P750;

blue, P500) emphasizes the dramatic improvement in consistency when compared to native islets (black) (**e**). The mass distribution vs size (cumulative proportion of total volume contained in structures of a given size or smaller) (dark green, P1000; light green, P750; blue, P500; black, native islets). (**f**). Sphericity of CFA-PI was confirmed by quantifying roundness (defined as $4 \times [\text{Area}]/(\pi \times [\text{Major axis}]^2)$ of randomly selected CFA-PI imaged before and after 90 degrees rotation (n=10) using a micromanipulator (roundness = 1.0 indicates perfectly round aggregates) (**g**).

3.3.2 CFA-PI outperform native islets in vitro

The selection of a suitable readout is vital for bioprocess assessment. In the case of islet transplantation for the treatment of insulin-dependent diabetes, the primary constraint is a shortage of transplantable pancreatic islet material, while the desired product is the capacity to secrete insulin upon exposure to glucose. Accordingly, we developed a quantitative parameter, termed the "Efficacy Ratio" (**ER**), based on cell numbers and s-GSIS results, which represents the amount of glucose-regulated insulin secretion obtained from a known quantity of starting material:

$$Efficacy\ Ratio(ER) = \left(\frac{cells\ per\ pseudoislet}{input\ cells\ per\ pseudoislet}\right) \times \left(\frac{insulin\ production}{number\ of\ cells\ assayed}\right)$$

This quantity represents the amount of insulin-generating capacity that results per primary pancreatic islet cell utilized in the process.

When comparing the ER of CFA-PI to native islets, we observed a trend of increase in CFA-PI groups where the improvements of P500 and P750 are statistically significant, exhibiting 8.8 and 11.1-fold increase over native islets (**Fig. 3.2a**). Comparing the Stimulation Index (SI) of CFA-PI to native islets and spontaneous aggregates, we found a significant increase in the P1000, P750 and P500 when compared to the native islets and the spontaneous aggregates (**Fig. 3.2b**). CFA-PI retained their SI advantage over native islets for at least 15 days under standard culture conditions (**Fig. 3.7.3.3)**. Additional data on insulin release in response to glucose stimulation are presented in **Fig. 3.7.3. 4-7** and **Tables 3.7.2. 2-7**.

The effects of hypoxic culture on CFA-PI and native islets were assessed after a 7-day culture period. Viability estimation using exclusion and inclusion fluorescent dyes (FDA/PI) revealed substantially higher cell death in native islets even under ambient atmospheric conditions, in sharp

contrast to CFA-PI, and this effect is further exacerbated when cultured in a 5% O_2 atmosphere (**Fig. 3.2d**).

There was no significant difference in the percentage of apoptotic cells (TUNEL assay) between CFA-PI and native islets cultured under standard conditions, however spontaneous aggregates did show a statistically significant increase in apoptosis compared to CFA-PI P1000 group (**Fig. 3.2c,e**). Immunolocalization of insulin and glucagon positive cells within native islets was heterogeneous, whereas interestingly CFA-PI exhibited a peripheral localization of insulin-positive cells, around a core composed primarily of glucagon-positive cells (**Fig. 3.2f**).

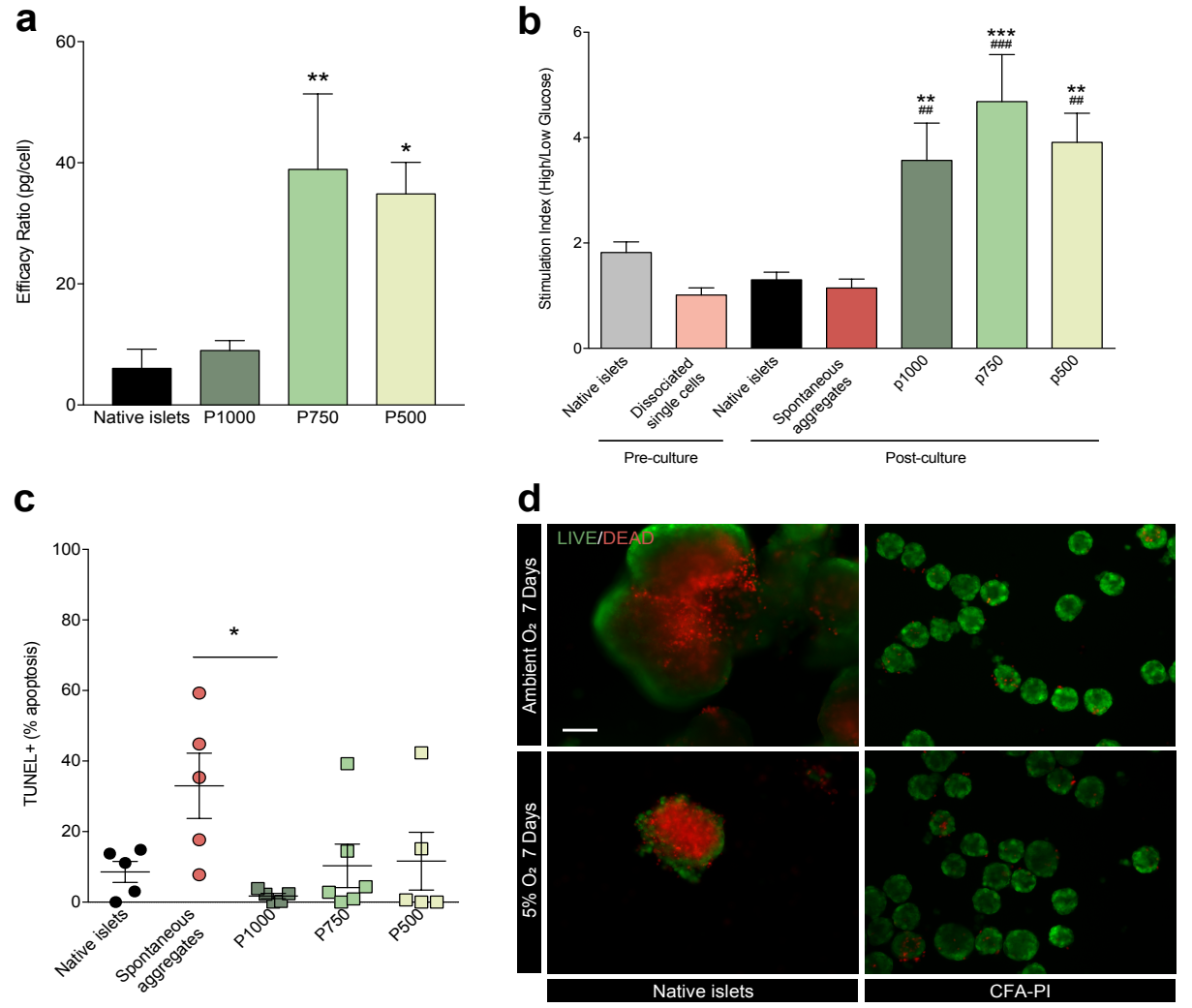
a
Efficacy Ratio (pg/cell)
60
40
20
0
**
*
Native islets
P1000
P750
P500
b
Stimulation Index (High/Low Glucose)
6
4
2
0
**
##
***
###
**
##
Native islets
Dissociated single cells
Native islets
Spontaneous aggregates
p1000
p750
p500
Pre-culture
Post-culture
c
TUNEL+ (% apoptosis)
100
80
60
40
20
0
*
Native islets
Spontaneous aggregates
P1000
P750
P500
d
LIVE/DEAD
Ambient O2 7 Days
5% O2 7 Days
Native islets
CFA-PI
e
Insulin TUNEL DAPI
Native islets
P1000
P750
P500
Spontaneous aggregates
f
Insulin Glucagon DAPI
Native islets
P1000
P750
P500

Figure 3. 2: Qualitative and Quantitative Assessment of Pseudoislets

Overall CFA-PI showed enhanced glucose stimulated insulin secretion per input cell, and the Efficacy Ratio of P750 and P500 was significantly improved compared to native islets 105.70 ± 39.24 fg/cell and 84.19 ± 19.79 fg/cell versus 9.53 ± 3.87 fg/cell, *$p<0.05$ for P500, **$p<0.01$ for P750, n = 15 per group, one-way ANOVA with Dunnett's correction for multiple comparisons) (**a**). Stimulation index (insulin secretion under high glucose divided by secretion under low glucose) was assessed (**b**) for islets upon receipt (grey), and immediately following dissociation to single cells (pink); and after 3-5 days of culture as islets (black), spontaneous aggregates (red) and CFA-PI (green). CFA-PI showed significant improvements over both native islets (***$p<0.001$ for P750, **$p<0.01$ for P1000 and P500) and spontaneous aggregates (†††$p<0.001$ for P750, ††$p<0.01$ for P1000 and P500, n≥12 per group, Kruskal-Wallis test with Dunn's correction for multiple comparisons). TUNEL staining after 3-5 days in culture showed significantly increased apoptosis in the spontaneous aggregates compared to the P1000 CFA-PI (32.98% ± 9.24% versus 1.75% ± 0.73%, *$p<0.05$, n=5, Kruskal-Wallis test), other comparisons were not statistically significant (**c**). Representative images show differences in cell death in native islets and CFA-PI cultured for seven days under ambient and hypoxic (5% O_2) atmospheric conditions and stained for live (green) and dead (red) cells (**d**). Immunostaining of cultured native islets, CFA-PI (P1000, P750 and P500) and spontaneous aggregates prior to transplant for insulin (red), TUNEL+ (green / arrows) and nuclei (DAPI, grey) shows the increased apoptosis in spontaneous aggregates (**e**). Immunolocalization of insulin (red) and glucagon (green) shows potential structural differences in the location α- and β-cells between native islets (intermingled) and CFA-PI (β-cells at periphery) (**f**).

3.3.3 CFA-PI show similar gene expression profiles to native islets

To investigate whether the dissociation and re-aggregation process during CFA-PI formation alters gene expression levels in comparison to intact, native islet cells, we conducted real-time PCR comparing P750 CFA-PI 48-hours post-formation to native islet controls. Genes from four categories were assessed: cell communication, secretory function, oxidative stress and apoptosis (**Table 3.7.2.1**). CFA-PI and native islet controls from eight clinical human donor islet isolations received from three isolation centers were tested. A reduction in NOS2 gene expression in CFA-PI was the only statistically significant difference observed (**Fig. 3.3**).

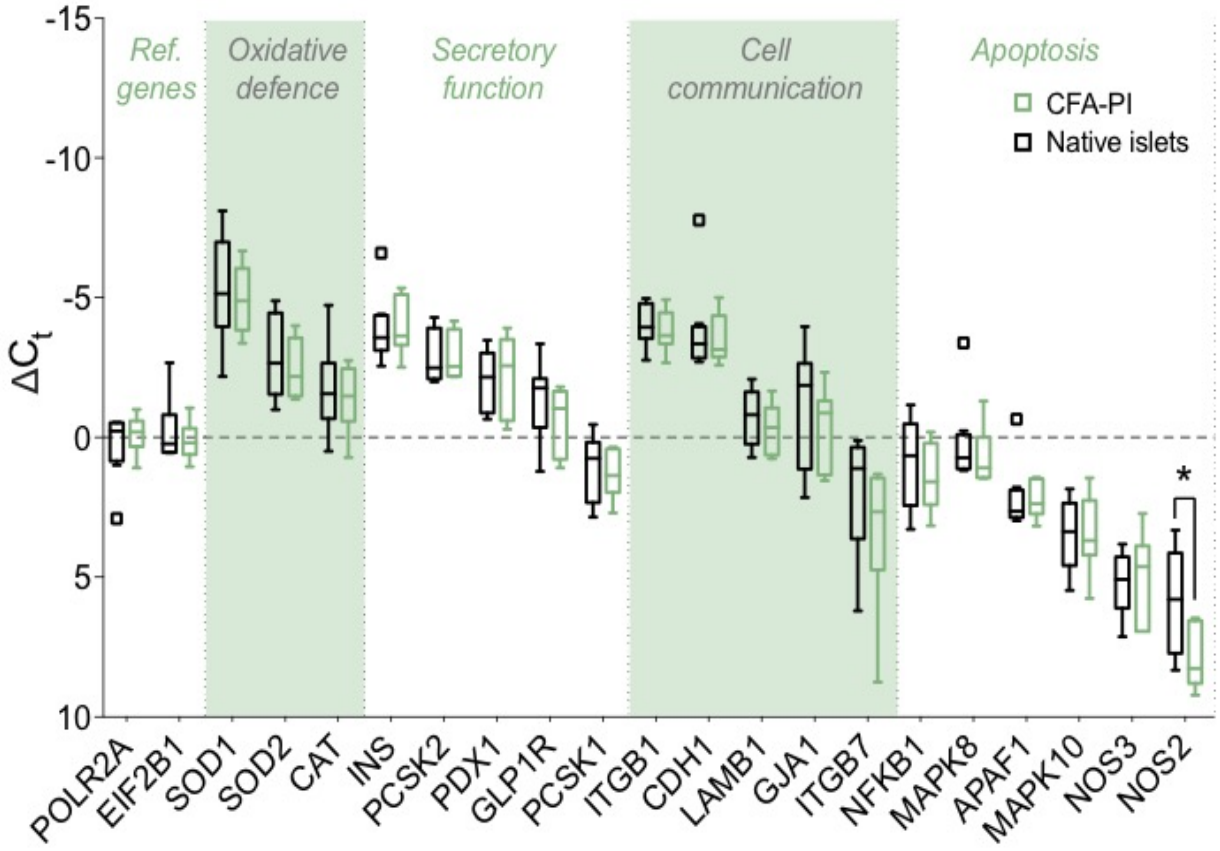

Figure 3. 3: Real-time RT-PCR

Real-time quantitative RT-PCR was performed to identify differences between the gene expression profiles of native islets (n=8) and P750 CFA-PI (n=7) 48 hours post-aggregation. Results were normalized to reference genes *POLR2A* and *EIF2B1* and are presented as delta Ct values to allow comparison between native islets (black) and CFA-PI (green) on a gene-by-gene basis. Data compared by Mann-Whitney test between groups within each gene.

3.3.4 CFA-PI are highly functional in vivo

Islet material from six human donors was employed in a marginal-mass transplant model. Samples of native islets, spontaneous aggregates, P1000, P750 and P500 CFA-PI were aliquoted into two doses, 500 IEQ and 1000 IEQ for transplant under the kidney capsule (**Table 3.7.2.8**). For all tissue transplanted DNA content was measured to determine recovery of native islets post-culture and CFA-PI formation (76.3% ± 16.6% versus 74.1% ± 15.9%, respectively, mean ± SD) and to confirm equivalent mass was transplanted (**Table 3.7.2.9**). While the native islet and CFA-PI groups were not significantly different, as expected the spontaneous aggregate group received significantly less material (~0.43x) due to the low efficiency of spontaneous aggregation. Cell loss during CFA-PI formation was primarily during dissociation (recovery 78.3% ± 11.7%).

At 500 IEQ marginal-mass transplant dose, significantly more mice reversed diabetes (defined as maintaining a blood glucose reading <11.1 mmol/l) in both the P1000 and P750 CFA-PI groups when compared to spontaneous aggregates, with median reversal time at day 7 (**Fig. 3.4a,b**). They also appeared to show improved reversal rates over native islets, however this effect did not reach statistical significance (55.6% versus 20%, *p=0.09*). There was no significant difference between the P500 group compared to native islets and spontaneous aggregates (**Fig. 3.4c**). At an increased, marginal dose, 1000 IEQ, the results followed a similar pattern with significant improvement in diabetes reversal in the P1000 group compared to spontaneous aggregates; and a potential improvement when compared to native islets transplanted alone (**Fig. 3.4d**) that failed to reach statistical significance (77.8% versus 55.6%, *p=0.13*). The proportion of mice that reversed diabetes at 1000 IEQ was similar between the P1000 and P750 CFA-PI and native islets, with P500 CFA-PI having a potentially somewhat lower reversal rate (**Fig. 3.4d-f**).

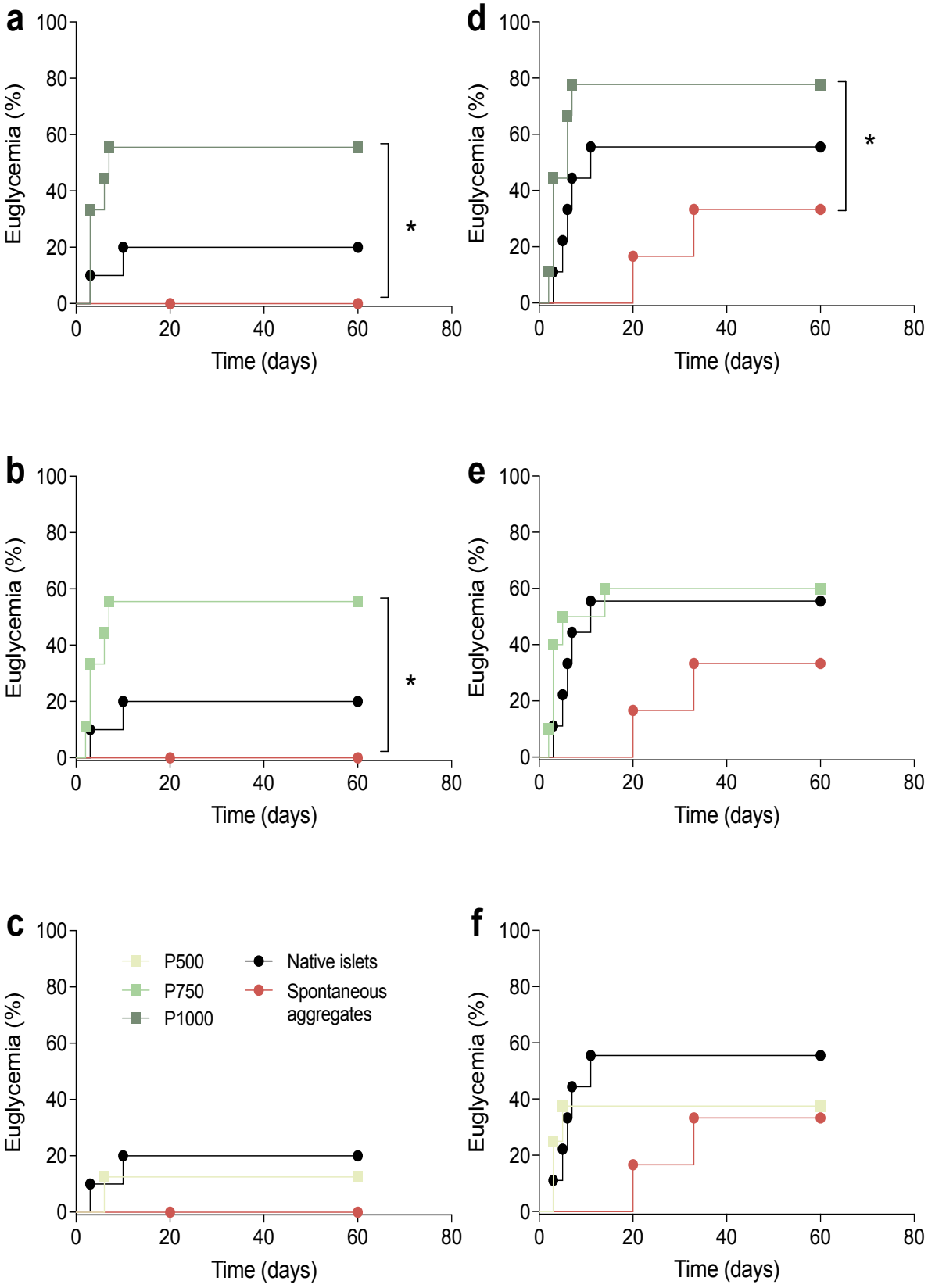
a
b
c
d
e
f
Euglycemia (%)
Time (days)
0
20
40
60
80
100
*
P500
P750
P1000
Native islets
Spontaneous aggregates

Figure 3. 4: Efficacy of Engraftment

Efficacy of human CFA-PI transplanted into mice at marginal-mass doses. At 500 IEQ, significantly more animals reversed diabetes in both the P1000 (n=9) and P750 (n=9) CFA-PI groups when compared to the single-cell spontaneous aggregates transplanted (n=6, $*p<0.05$) with median reversal time at day 7 (**a, b**). They also showed improved diabetes reversal rates (55.6%) over native islets (20%, n=10) however this did not reach statistical significance. There was no significant difference between the P500 group (n=8) compared to native islets (n=10) and spontaneous aggregates (n=6) (**c**). At 1000 IEQ, displayed significant improvement in diabetes reversal in the P1000 group ($*p<0.05$, n=9) compared to the spontaneous aggregates but did not reach statistical significance (n=10) (**d**). The proportion of mice that reversed diabetes (non-fasting blood glucose <11.1 mmol/l) was similar between the P750 (n=10) and P500 (n=8) CFA-PI groups compared to the native islets (n=9), with P500 appearing somewhat lower (**e, f**). Animals transplanted with n=6 human islet/CFA-PI preparations. Comparison of euglycemia curves by Gehan-Breslow-Wilcoxon test.

One week prior to endpoint, mice were administered an intraperitoneal glucose tolerance test (IPGTT). Glucose clearance was significantly improved in the P1000 CFA-PI group compared to the native islet groups transplanted with 500 IEQ (**Fig. 3.5a**), although differences only reached statistical significance at the 60-minute time-point. There was significant impairment in the glucose clearance in the mice transplanted with the native islets when compared to the naïve non-diabetic control mice; this impairment however was not evident in the P1000 CFA-PI group (**Fig. 3.5a**). The P750 and P500 CFA-PI groups did not show statistically significant improvement over the native islet controls at any timepoint (**Fig. 3.5a**). At a higher transplant dose of 1,000 IEQ, glucose clearance was significantly improved in the P1000 group at 60 minutes when compared to the native islet group (**Fig. 3.5b**). There was no significant difference between the P750 and P500 groups when compared to animals transplanted with native islets at any time during the IPGTT with this higher transplant mass (**Fig. 3.5b**). Area under the curve (AUC) analysis was used to assess overall glucose clearance. While the CFA-PI appeared to show improved overall glucose clearance as compared to native islets, due to the high variability associated with human donor material these differences did not reach statistical significance. There was no significant difference in the overall glucose clearance in the mice transplanted with native islets versus those transplanted with CFA-PI regardless of islet mass transplanted, 500 IEQ or 1000 IEQ (**Fig. 3.5c,d**). As a negative control, animals were also transplanted with spontaneous aggregates (**Fig. 3.7.3.8**). At 500 IEQ, glucose clearance was significantly improved in the P1000 group compared to the spontaneous aggregate group at several time-points. At 1,000 IEQ, glucose clearance was similarly significantly improved in both the P1000 and P750 groups when compared to the spontaneous aggregate group. There was significant reduction in AUC in the P1000 group compared to spontaneous aggregate group. As expected, glucose clearance was significantly impaired in the spontaneous aggregate group as compared with the non-STZ-treated non-transplanted euglycemic control mice at 60, 90 and 120 minutes at both 500 IEQ and 1000 IEQ.

A subset of harvested grafts was homogenized for analysis of total insulin content. No significant differences in insulin content were observed between CFA-PI and native islet transplanted groups transplanted with the same islet mass (data not shown).

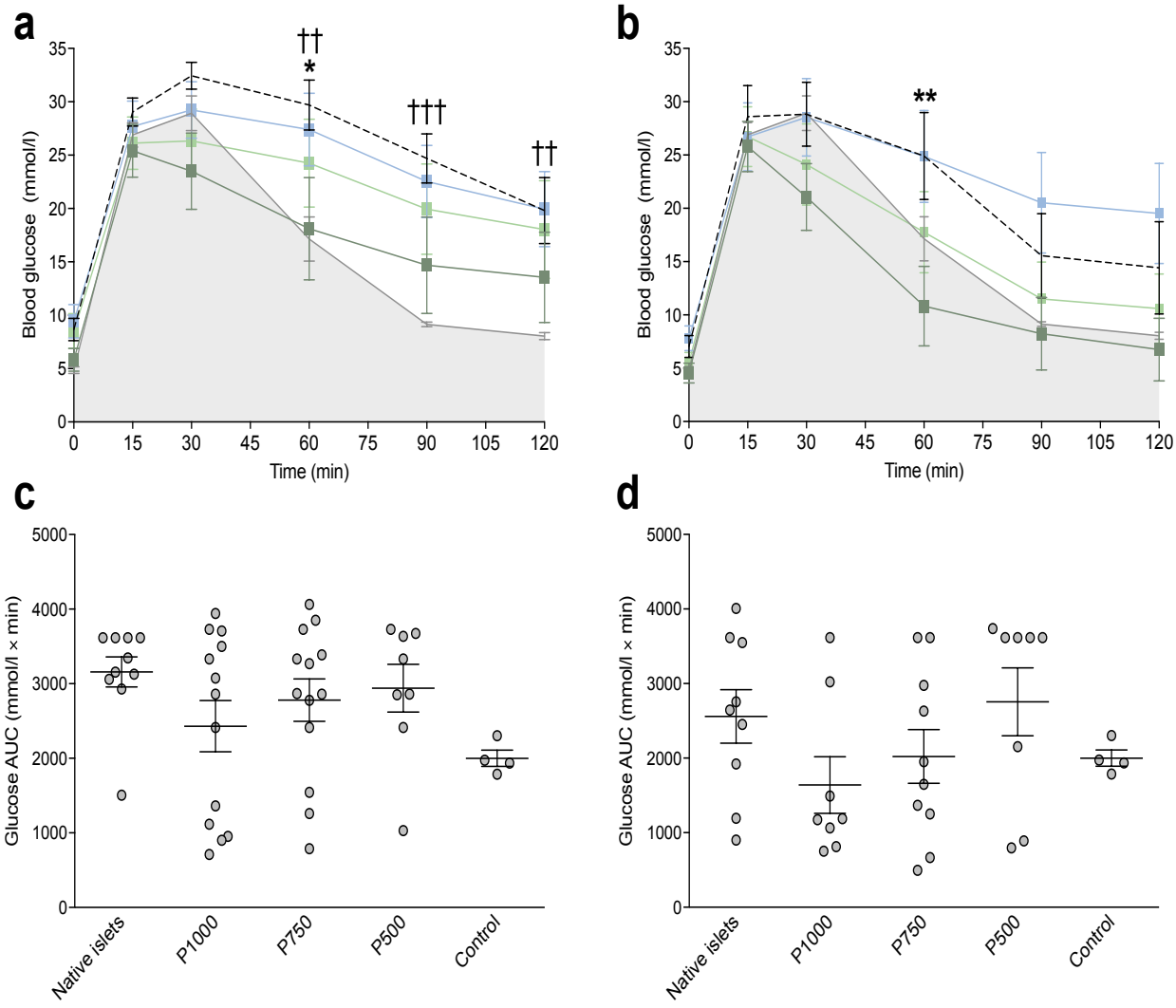

Figure 3. 5: Intraperitoneal Glucose Tolerance Test

At 500 IEQ, glucose clearance was significantly improved in the P1000 group (dark green) (n=9) compared to the (dotted black) native islet groups (*$p<0.05$ at 60 minutes, n=10) (**a**). There was significant impairment in the glucose clearance in the mice transplanted with the native islets (dotted black) when compared to the naïve non-diabetic (grey) control mice (††$p<0.01$ at 60 and 120 minutes, †††$p<0.001$ at 90 minutes, n=4); this impairment however was not mirrored in the P1000 group (dark green). There was no significant difference between the P750 (light green) (n=9), and P500 (blue) (n=8), groups at any time during the IPGTT. At 1000 IEQ, glucose clearance was significantly improved in the P1000 group (dark green) at 60 minutes

(**$p<0.01$, n=9) when compared to the native islet (dotted black) transplant group (n=9) (**b**). There was no significant difference between the P750 (light green) (n=10) and P500 (blue) (n=8) groups when compared to animals transplanted with native islets at any time during the IPGTT. Area Under the Curve, differences in overall glucose clearance in the mice transplanted with native islets versus those transplanted with CFA-PI did not reach statistical significance (**c, d**). Data measured by student's *t* test between groups at each time point corrected for multiple comparison using the Holm-Sidak method; analysis of AUC by one-way ANOVA with Tukey's multiple comparisons.

3.3.5 Analysis of immunohistochemistry of human native islet graft and human pseudoislets grafts post-transplant

Immunohistochemistry was performed on a subset of tissue grafts removed 60 days post-transplant. All transplant bearing kidneys were removed, but no tissue graft could be located on some kidneys transplanted with native islets and spontaneous aggregates at 500 IEQ. Fluorescent staining for major endocrine cell components included insulin, glucagon, somatostatin and PP (**Fig. 3.6**). There was no obvious difference in overall morphology when comparing the CFA-PI grafts to the native islet groups (**Fig. 3.6c**). However, the CFA-PI grafts present a higher area of PP immunoreactivity and a slightly lower area of insulin immunoreactivity (**Fig. 3.6a,b**). Two tissue grafts were analyzed for the spontaneous aggregate group, and though smaller, the grafts were consistent in composition with both the CFA-PI and native islet grafts (data not shown).

CD31 and Von Willebrand factor(vWF) combined staining for blood vessels was also performed in the same subset of tissue grafts (**Fig. 3.7a**). Quantification of CD31 and vWF immunoreactivity within the grafts suggested a significantly higher vessel density of CFA-PI grafts compared to native islet grafts (**Fig. 3.7d**). In each image we examined the mean (**Fig. 3.7b**) and median (**Fig. 3.7c**) distances to the nearest vascular element from randomly selected starting points (N = 10,000). CFA-PI grafts showed a significant reduction in both distances, suggesting improved vascular distribution when compared to native islet grafts.

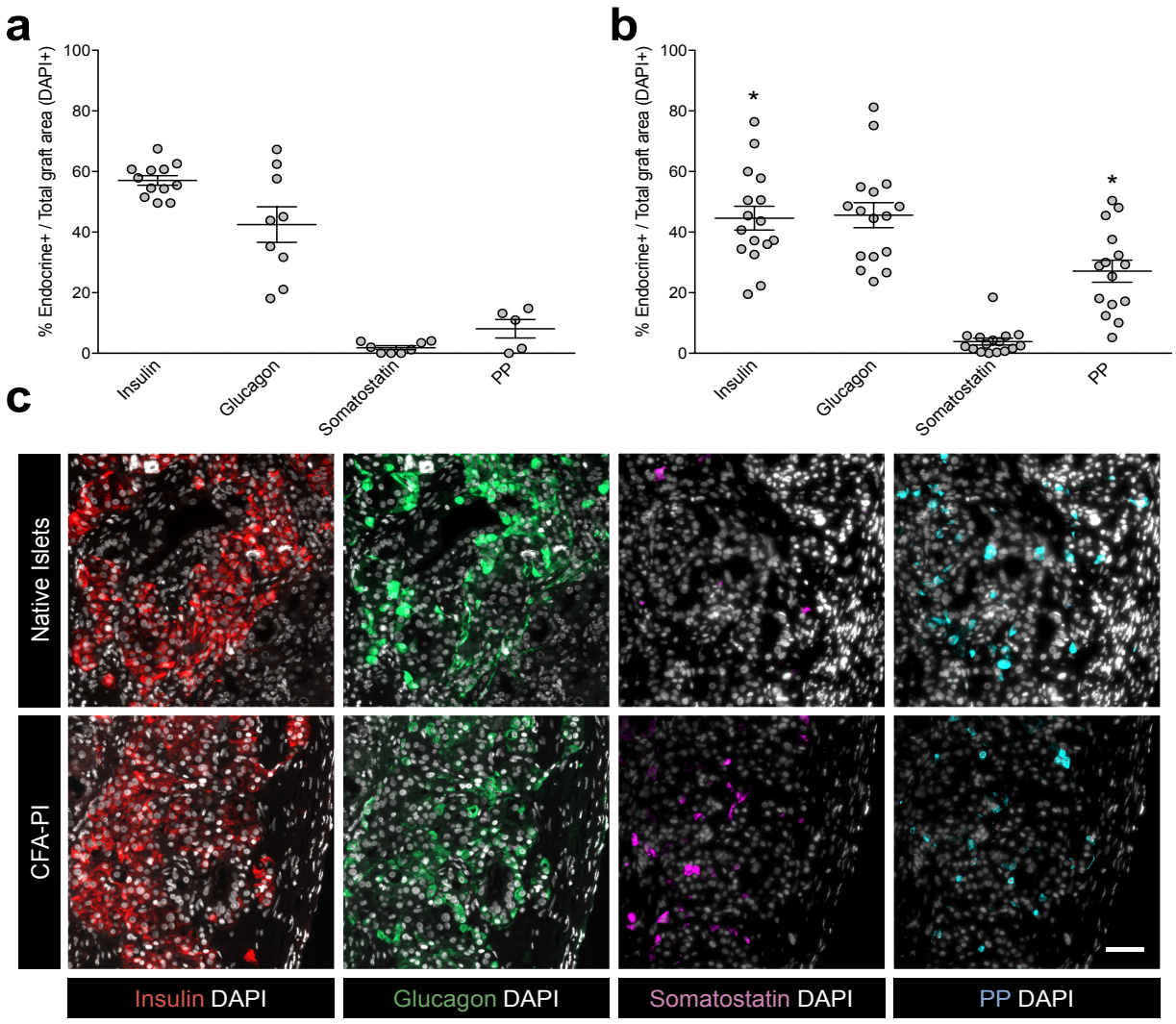

Figure 3. 6: Immunohistochemistry Quantification

Quantification of the total area of insulin, glucagon, somatostatin and pancreatic peptide (PP) immunoreactivity within native islet (**a**) and CFA-PI (**b**) grafts recovered 60 days post-transplant (DAPI+ within the graft area, each symbol = one transplant). CFA-PI grafts showed increased total PP+ area, and decreased total insulin+ area (*$p<0.05$, *both*). Fluorescent images of human native islet grafts and human CFA-PI grafts stained positive for insulin (red), glucagon (green), somatostatin (magenta), PP (cyan) and nuclei (DAPI, grey) (**c**). Data from animals transplanted with n=6 independent human islet/CFA-PI preparations. Differences compared by unpaired *t*-test.

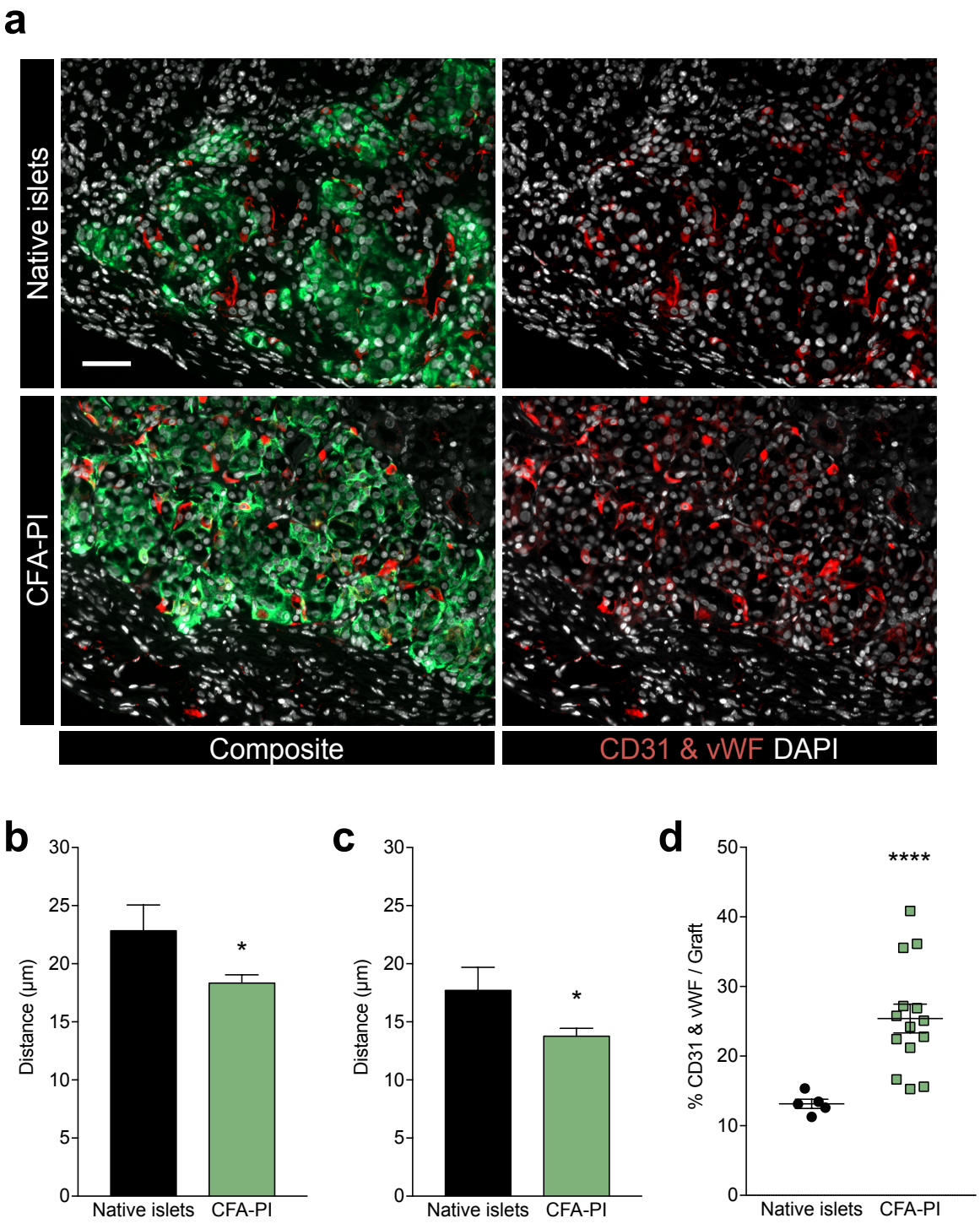

Figure 3.7: Blood Vessel Quantification

Fluorescent images of human native islet and CFA-PI grafts recovered after 60 days and stained for blood vessels (CD31 and vWF, red), insulin(green), and nuclei (DAPI, grey) (**a**). F-function (distance from 10,000 randomly generated points to the nearest vascular element) results

show a 19.7% reduction in mean (**b**) and a 22.3% reduction in median (**c**) distances for CFA-PI grafts when compared with native islet grafts, and increase in overall vascular staining (**d**). Animals transplanted with n=6 independent human islet/CFA-PI preparations. *$p<0.05$, ***$p<0.001$, unpaired *t*-test.

3.4 Discussion

In this study we were able to rapidly, efficiently and reproducibly generate size-controlled CFA-PI from donated human islet material from 21 different donors, obtained from 3 different distribution programs across North America. Spontaneous aggregation and hanging-drop methods have been the standard for generating pseudoislets for over a decade, [22,24,28-30] and recently more complex strategies (generally employing gravity-mediated settling) have been reported. Where efficiency has been reported, it has generally been low due to high cell loss during the process, which would nullify any potentially benefit brought about by re-aggregation. [42,43] Our CFA-PI approach enables rapid pseudoislet assembly, minimizing the time spent as a single-cell suspension, and efficiently generating uniform, spherical, size controlled structures whose in vitro survival and functionality was significantly improved over the native islets from which they were derived – even after taking into account cell loss during CFA-PI production. Importantly, we are able to complete pseudoislet assembly with minimal loss of islet mass. We attribute this achievement to the rapid sedimentation of islet cells under centrifugation in our process (as opposed to the relatively slow processes inherent in spontaneous-aggregation or hanging-drop approaches) – thus they may experience only a few minutes as single cells from the moment trituration breaks up the islets to the time they are brought back into contact in the microwells.

Consistent with the predictions of previous modeling studies, [17] we observed substantial improvements in both viability and apoptosis in CFA-PI on long-term culture under ambient oxygen levels, which were exaggerated as oxygen levels were further reduced. Likely due to a combination of enhanced survival, improved function of individual cells due to better oxygen and nutrient access, and improved mass transport of glucose and insulin, we also observed a dramatic enhancement of static glucose stimulated insulin secretion over unmodified native islets. The nine- to eleven-fold improvement in ER (which accounts for cell loss during CFA-PI generation) we observed for CFA-PI therefore represents a significant and substantial improvement in the

efficient use of limited donor material. CFA-PI capacity to retain function for at least two weeks in vitro confirms that the constituent cells do not suffer from de-differentiation, and this is reinforced by the lack of change in gene expression patterns between CFA-PI and native islets. When dealing with three-dimensional structures with a broad size distribution such as islets, it is important to recognize that consideration of diameter alone tends to exaggerate the contribution of smaller units to the overall population, as the volume of (and hence proportion of material found within) larger structures increases with the cube of the diameter. While representing only an approximation due to the non-spherical nature of native islets, this is visible in comparing the estimated mass distributions in Figure 1f with the diameter distributions from which they are derived. This is consistent with previous reports that showed better performance from smaller islets, [18] while the larger islets - which are inherently less hypoxia tolerant - generally make up a large proportion of the total islet mass transplanted. [44]

In vivo, CFA-PI were examined in terms of ability to rescue hyperglycemia as well as short-term glucose clearance, where they performed at least as well as native islets, with indications of even better performance (albeit not all statistically significant). In part, this may be simply a reflection of the large variability inherent in both the donor material and the marginal-mass transplant model. We also hypothesize that transplantation under the highly-vascularized kidney capsule, [45] chosen for the ability to recover the graft, likely provides a conservative model of the improvements CFA-PI will show in human portal-vein delivery. As clinically transplanted islets are believed to undergo a sustained period of hypoxia while trapped within thrombi inside vessels in the liver, [46] we expect that the greater hypoxia tolerance exhibited by CFA-PI (Fig. 2d) may provide an added advantage over native islets in this context. Intriguingly, the density of vascular elements (as assessed by dual-staining for CD31 and von Willebrand Factor) is significantly higher in CFA-PI (Fig. 7) grafts, with both the mean and median distances from randomly chosen points to the nearest vascular element (Fig. 7b, c) significantly reduced in

CFA-PI. We hypothesize that this is due to a combination of the relatively smaller size of the CFA-PI, and also that their assembly from single cells shortly prior to transplantation meant that their extracellular matrix was likely less mature and therefore more easily penetrated by newly-forming capillaries. This difference (in the order of 20%) is particularly interesting as it represents a shorter path for the transport of essential metabolites (oxygen, nutrients, etc) required for cell survival, a smaller number of cells competing for those metabolites along that path, and a reduced barrier to both glucose stimulation and insulin secretion. We speculate that this phenomenon may explain the observed changes in glucose clearance (Fig. 5). This phenotype would also be expected to further enhance performance of CFA-PI over native islets in clinical portal-vein delivery, where revascularization of transplanted islets is slow [8], and likely incomplete. [11-13] Much of the performance advantage of CFA-PI is likely attributable to a combination of the fact that in a preparation of native islets, much of the material will be located in larger islets (to a degree that is not always fully appreciated, see Fig. 1f) with the previously reported observations that preparations of smaller islets outperform larger ones in the clinic. [17-19]

3.5 Conclusion

In conclusion, we have demonstrated the ability to dissociate and re-aggregate human islet material into engineered CFA-PI efficiently and effectively, with a high level of consistency, well controlled size, greatly enhanced hypoxia tolerance, and strong *in vitro* function. CFA-PI performance compares favourably to native human islets (and is substantially better than conventional spontaneously-aggregated pseudoislets) in the mouse kidney capsule, and there are reasons to believe they would perform even better in human portal vein delivery. While our present efforts have targeted the use of donated human islet material to align with our long-standing experience using this material in the clinic, our CFA-PI process has been designed as a source-agnostic packaging approach that will also be applicable to material derived from e.g. stem cells, [47] cell lines [48,49] or xenogeneic sources [50] which promise to greatly increase practical impact. In addition, the ability to generate large numbers of uniform pseudoislets provides a powerful platform to enhance our understanding of islet biology and further optimize performance by assessing the impact of varying cellular composition, and formation and culture conditions. The ability to genetically modify and manipulate the proportions of different cell types used to form CFA-PI will also provide new opportunities for both basic research and clinical interventions. CFA-PI were formed and transplanted within 72 hours, congruent with current clinical islet transplant protocols, cell loss is not significantly greater than that seen with native islets, and production is not labour intensive, and is linearly scalable with microwell surface area. We are in the process of developing microwell bioreactors that will deliver the quantities of CFA-PI required for clinical applications

3.6 References

1. You W-P, Henneberg M (2016) Type 1 diabetes prevalence increasing globally and regionally: the role of natural selection and life expectancy at birth. BMJ Open Diabetes Res Care 4:e000161

2. Patterson CC, Dahlquist GG, Gyürüs E, et al (2009) Incidence trends for childhood type 1 diabetes in Europe during 1989–2003 and predicted new cases 2005–20: a multicentre prospective registration study. The Lancet 373:2027–2033

3. Hex N, Bartlett C, Wright D, et al (2012) Estimating the current and future costs of Type 1 and Type 2 diabetes in the UK, including direct health costs and indirect societal and productivity costs. Diabet Med 29:855–862

4. American Diabetes Association (2013) Economic Costs of Diabetes in the U.S. in 2012. Diabetes Care 36:1033–1046

5. Shapiro AM, Lakey JR, Ryan EA, et al (2000) Islet transplantation in seven patients with type 1 diabetes mellitus using a glucocorticoid-free immunosuppressive regimen. N Engl J Med 343:230–238

6. Ryan EA, Lakey JR, Rajotte RV, et al (2001) Clinical outcomes and insulin secretion after islet transplantation with the Edmonton protocol. Diabetes 50:710–719

7. Ryan EA, Lakey JRT, Paty BW, et al (2002) Successful islet transplantation: continued insulin reserve provides long-term glycemic control. Diabetes 51:2148–2157

8. Korsgren O, Lundgren T, Felldin M, et al (2008) Optimising islet engraftment is critical for successful clinical islet transplantation. Diabetologia 51:227–232

9. Sherar MD, Noss MB, Foster FS (1987) Ultrasound backscatter microscopy images the internal structure of living tumour spheroids. Nature 330:493–495

10. Johnson AS, Fisher RJ, Weir GC, Colton CK (2009) Oxygen consumption and diffusion in assemblages of respiring spheres: Performance enhancement of a bioartificial pancreas. Chem Eng Sci 64:4470–4487

11. Mattsson G, Jansson L, Carlsson P-O (2002) Decreased vascular density in mouse pancreatic islets after transplantation. Diabetes 51:1362–1366

12. Carlsson P-O, Palm F, Mattsson G (2002) Low revascularization of experimentally transplanted human pancreatic islets. J Clin Endocrinol Metab 87:5418–5423

13. Lau J, Carlsson P-O (2009) Low revascularization of human islets when experimentally transplanted into the liver. Transplantation 87:322–325

14. Toso C, Isse K, Demetris AJ, et al (2009) Histologic Graft Assessment After Clinical Islet Transplantation. Transplantation 88:1286–1293

15. Dionne KE, Colton CK, Yarmush ML (1993) Effect of hypoxia on insulin secretion by isolated rat and canine islets of Langerhans. Diabetes 42:12–21

16. Li R (1998) Materials for immunoisolated cell transplantation. Adv Drug Deliv Rev 33:87–109

17. Buchwald P (2009) FEM-based oxygen consumption and cell viability models for avascular pancreatic islets. Theor Biol Med Model 6:5

18. Lehmann R, Zuellig RA, Kugelmeier P, et al (2007) Superiority of small islets in human islet transplantation. Diabetes 56:594–603

19. Kin T, Senior P, O'Gorman D, et al (2008) Risk factors for islet loss during culture prior to transplantation. Transpl Int 21:1029–1035

20. Persaud SJ, Arden C, Bergsten P, et al (2010) Pseudoislets as primary islet replacements for research: Report on a symposium at King's College London, London UK. Islets 2:236–239

21. Halban PA, Wollheim CB, Blondel B, et al (1982) The Possible Importance of Contact between Pancreatic Islet Cells for the Control of Insulin Release*. Endocrinology 111:86–94

22. Guo-Parke H, McCluskey JT, Kelly C, et al (2012) Configuration of electrofusion-derived human insulin-secreting cell line as pseudoislets enhances functionality and therapeutic utility. J Endocrinol 214:257–265

23. Green AD, Vasu S, Flatt PR (2016) Functionality and antidiabetic utility of β- and L-cell containing pseudoislets. Exp Cell Res 344:201–209

24. Green AD, Vasu S, McClenaghan NH, Flatt PR (2016) Implanting 1.1B4 human β-cell pseudoislets improves glycaemic control in diabetic severe combined immune deficient mice. World J Diabetes 7:523

25. Hauge-Evans AC, Squires PE, Persaud SJ, Jones PM (1999) Pancreatic beta-cell-to-beta-cell interactions are required for integrated responses to nutrient stimuli: enhanced Ca2+ and insulin secretory responses of MIN6 pseudoislets. Diabetes 48:1402–1408

26. Green AD, Vasu S, McClenaghan NH, Flatt PR (2015) Pseudoislet formation enhances gene expression, insulin secretion and cytoprotective mechanisms of clonal human insulin-secreting 1.1B4 cells. Pflüg Arch - Eur J Physiol 467:2219–2228

27. O'Sullivan ES, Johnson AS, Omer A, et al (2010) Rat islet cell aggregates are superior to islets for transplantation in microcapsules. Diabetologia 53:937–945

28. Westenfelder C, Gooch A, Hu Z, et al (2017) Durable Control of Autoimmune Diabetes in Mice Achieved by Intraperitoneal Transplantation of "Neo-Islets," Three-Dimensional Aggregates of Allogeneic Islet and "Mesenchymal Stem Cells." STEM CELLS Transl Med 6:1631–1643

29. Cavallari G, Zuellig RA, Lehmann R, et al (2007) Rat pancreatic islet size standardization by the "hanging drop" technique. Transplant Proc 39:2018–2020

30. Zuellig RA, Cavallari G, Gerber P, et al (2017) Improved physiological properties of gravity-enforced reassembled rat and human pancreatic pseudo-islets. J Tissue Eng Regen Med 11:109–120

31. Mendelsohn AD, Nyitray C, Sena M, Desai TA (2012) Size-controlled insulin-secreting cell clusters. Acta Biomater 8:4278–4284

32. Lee GH, Lee JS, Wang X, Lee SH (2016) Bottom-Up Engineering of Well-Defined 3D Microtissues Using Microplatforms and Biomedical Applications. Adv Healthc Mater 5:56–74

33. Pathak S, Regmi S, Gupta B, et al (2017) Engineered islet cell clusters transplanted into subcutaneous space are superior to pancreatic islets in diabetes. FASEB J Off Publ Fed Am Soc Exp Biol 31:5111–5121

34. Ungrin MD, Joshi C, Nica A, et al (2008) Reproducible, Ultra High-Throughput Formation of Multicellular Organization from Single Cell Suspension-Derived Human Embryonic Stem Cell Aggregates. PLoS ONE 3:e1565

35. Integrated Islet Distribution Program | IIDP | Human Islets for Research > Investigators > Policies & Standard Operating Procedures. https://iidp.coh.org/Investigators/Policies-Standard-Operating-Procedures. Accessed 5 Dec 2017

36. Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods San Diego Calif 25:402–408

37. Vandesompele J, De Preter K, Pattyn F, et al (2002) Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol 3:RESEARCH0034

38. Andrey P, Kiêu K, Kress C, et al (2010) Statistical Analysis of 3D Images Detects Regular Spatial Distributions of Centromeres and Chromocenters in Animal and Plant Nuclei. PLOS Comput Biol 6:e1000853

39. Ollion J, Cochennec J, Loll F, et al (2013) TANGO: a generic tool for high-throughput 3D image analysis for studying nuclear organization. Bioinformatics 29:1840–1841

40. Steinberg MS (1975) Adhesion-guided multicellular assembly: a commentary upon the postulates, real and imagined, of the differential adhesion hypothesis, with special attention to computer simulations of cell sorting. J Theor Biol 55:431–443

41. Foty RA, Pfleger CM, Forgacs G, Steinberg MS (1996) Surface tensions of embryonic tissues predict their mutual envelopment behavior. Dev Camb Engl 122:1611–1620

42. Callewaert H, Gysemans C, Cardozo AK, et al (2007) Cell Loss during Pseudoislet Formation Hampers Profound Improvements in Islet Lentiviral Transduction Efficacy for Transplantation Purposes. Cell Transplant 16:527–537

43. Lione L, Puddu A, Pedemonte A, Viviani GL (2001) Rat serum improves rat pseudoislet formation and insulin gene expression. Boll Della Soc Ital Biol Sper 77:13–19

44. Kilimnik G, Jo J, Periwal V, et al (2012) Quantification of islet size and architecture. Islets 4:167–172

45. Cunha GR, Baskin L (2016) Use of sub-renal capsule transplantation in developmental biology. Differ Res Biol Divers 91:4–9

46. Olsson R, Olerud J, Pettersson U, Carlsson P-O (2011) Increased Numbers of Low-Oxygenated Pancreatic Islets After Intraportal Islet Transplantation. Diabetes 60:2350–2353

47. Rezania A, Bruin JE, Arora P, et al (2014) Reversal of diabetes with insulin-producing cells derived in vitro from human pluripotent stem cells. Nat Biotechnol 32:1121–1133

48. Scharfmann R, Didiesheim M, Richards P, et al (2016) Mass production of functional human pancreatic β-cells: why and how? Diabetes Obes Metab 18:128–136

49. Green AD, Vasu S, Flatt PR (2018) Cellular models for beta-cell function and diabetes gene therapy. Acta Physiol 222:e13012

50. Mihalicz D, Rajotte RV, Rayat GR (2011) Porcine Islet Xenotransplantation for the Treatment of Type 1 Diabetes. Type 1 Diabetes - Pathog Genet Immunother. doi: 10.5772/22206

3.7 Supplementary Information

3.7.1 Supplemental Methods

3.7.1.2 Human islet isolation

Human islet preparations were provided by the Alberta Diabetes Institute IsletCore and the Clinical Islet Transplant Program at the University of Alberta in Edmonton with the assistance of the Human Organ Procurement and Exchange (HOPE) program, Trillium Gift of Life Network (TGLN) and Canadian Organ Procurement Organizations (OPO), as well as the NIDDK-funded Integrated Islet Distribution Program (IIDP) at City of Hope, NIH Grant #2UC4DK098085-02, United States. While we saw no evidence of source- specific or purity-specific effects on our findings, *in vivo* experiments were carried out entirely with islets sourced from the ADI IsletCore to maximize consistency and minimize transport effects. *In vitro* experiments were performed in both Calgary (elevation 1,045 m) and Edmonton (elevation 645 m) under approvals for the use of human tissue from the respective Health Research Ethics Boards.

3.7.1.3 Human islet dissociation and re-aggregation

Islets were suspended in TrypLE Select (Thermo Fisher Scientific, Waltham, MA, USA) and incubated at 37°C in a shaking water bath for 8-10 minutes, followed by trituration to break up remaining clumps. After centrifugation (200g, 2 minutes) the cells were re-suspended in culture medium pre-warmed in a 37oC water bath. Culture media consisted of CMRL-1066 (Corning, Manassas, VA, USA) supplemented with 1.2 g nicotinamide, 10 ml ITS, 2 mmol/l $ZnSO_4$, 50 ml sodium pyruvate, 10 ml Glutamax, 10 ml penicillin/streptomycin, 25 ml HEPES and 10% FBS (Sigma-Aldrich, Oakville, ON, Canada). CFA-PI were formed in 24-well or 6-well AggreWell 400 plates (STEMCELL Technologies, Vancouver, BC, Canada) as P1000, P750 and P500

(where P1000 represents CFA-PI formed from 1000 cells apiece, P750 from 750 cells, etc.), with centrifugation (200g, 5 minutes) and cultured at density equivalent to 750, 563, 375 IEQ/ml respectively, with one IEQ defined as 1,600 cells. [1] Native islet controls were cultured in 24-well or 6-well ultra-low attachment plates (Corning, Manassas, VA, USA), as were dissociated single cells for spontaneous-aggregation controls, both plated at 500 IEQ/ml and cultured at 37^{o}C and 5% CO_2 for 3-5 days prior to transplant and further assay. Example comparisons with pseudoislet formation in hanging drops is shown in Figure 3.7.3.9. Medium volumes used were 1ml/well for 24-well plates and 2.5ml/well for 6-well plates.

3.7.1.4 Static glucose-stimulated insulin secretion (s-GSIS)

An s-GSIS assay was performed for pre-cultured native islets and dissociated single cells, and post 3-5 days of culture for all groups at 200 IEQ/group. The islets were washed of residual glucose three times in glucose free medium, incubated in RPMI-1640 containing low (2.8 mmol/l) glucose for one hour, followed by high (16.7 mmol/l) glucose for an additional hour at 37^{o}C and 5% CO_2. The supernatant was harvested post glucose incubation and insulin levels measured by ELISA (Cat#10-1113-01, Mercodia, Uppsala, Sweden).

3.7.1.5 Hypoxic culture and viability analysis

CFA-PI and islets were cultured as described above at 37°C, 5% CO_2 for 7 days in both ambient and hypoxic (5% O_2 incubator) conditions. Islet and CFA-PI viability was then assessed using the inclusion and exclusion dyes fluorescein diacetate (FDA) and propidium iodide (PI) [2]. Islets were aliquoted into a 10 x 35 mm culture dish containing 460 µl Dulbecco's phosphate buffered saline (DPBS), 10 µl of PI (750 µmol/l in DPBS) and 10 µl of FDA (24 µmol/l in acetone). Imaging by Zeiss AxioObserver Z1 inverted microscope.

3.7.1.6 Apoptosis TUNEL staining

Apoptosis of islets and pseudoislets was assessed by TUNEL assay (Promega, Madison, WI, USA). Tissue sections were co-stained with anti-insulin antibody at 1:200 concentration (Dako, Mississauga, ON, Canada) and DAPI to identify nuclei. Apoptosis was determined by analyzing the number of positive TUNEL-stained cells as a percentage nuclei within the insulin-positive.

3.7.1.7 Real-time qPCR

RNA was isolated using a Total RNA Purification Kit (Norgen Biotek, Thorold, Canada) and quantified on a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific) by absorbance at 260 nm. Reverse transcription (RT) was carried out with 1 µg input RNA using the iScript Reverse Transcription Supermix (BioRad, Hercules, USA) per the manufacturer's instructions. Quantitative PCR (qPCR) was carried out on the resulting cDNA diluted 40x with nuclease-free water. Reactions for qPCR were composed of: 4 µl diluted cDNA; 1 µl specific primer pairs (SM Table 1) at a concentration of 3 µM each; and 5 µl PowerUp SYBR Green Master Mix (Thermo Fisher Scientific). Reactions were carried out on an Applied Biosystems Cycler (Thermo Fisher Scientific) as specified in the manuals provided with the SYBR Green Master Mix. Analysis of RT-qPCR results was performed using the ΔΔCT method [3] with normalization against two stable internal reference genes (*POLR2A* and *EIF2B1)*. [4]

3.7.1.8 Islet transplantation

To induce diabetes, female and male adult (8-10 weeks, 20-30 gm) immunodeficient mice (B6.129S7- Rag1$^{tm1Mom/J}$; Jackson Laboratories, Canada) were administered an intraperitoneal injection of streptozotocin (175 mg/kg, Sigma-Aldrich) in acetate phosphate buffer, pH 4.5. Blood glucose levels ≥18 mmol/l for two consecutive readings, were considered diabetic. Exogenous insulin was administered (Linbit® sustained release insulin implant pellet, 0.1U/day, Linshin

Canada, Inc.) prior to islet transplantation. Transplantation was within 30 days of diabetes induction. At the time of transplant the insulin pellets were removed and no subsequent insulin therapy was supplied. Animals were bright, alert and active prior to transplant and body weight loss was <10%. Animals that lost >10% of their body weight after transplant and remained hyperglycemic, ≥18 mmol/l, or were not transplanted within 30 days of diabetes induction were electively euthanized.

Native islets, CFA-PI (P500, P750 and P1000), and spontaneous aggregates were transplanted under the capsule of the left kidney [5] at an islet equivalent mass of 500 or 1000 IEQ ± 10% at 90% purity. The islet graft-function was assessed through non-fasting blood glucose measurements, three times per week for 60 days. Blood glucose monitoring was conducted using a portable glucometer (OneTouch Ultra 2, LifeScan, Burnaby, BC, Canada). Two consecutive readings <11.1 mmol/l [5] were considered to confirm graft function and reversal of diabetes. At day 60, a recovery nephrectomy of the left kidney was performed, and considered to confirm the graft as responsible for diabetes reversal once blood glucose returned to a hyperglycemic state (≥18 mmol/l). Representative blood glucose sampling data is shown in Figure 10. Two mice were excluded from the data, one died at day 3 post-transplant, and one did not become hyperglycemic after recovery nephrectomy – data from this mouse was excluded from the analysis due the potential for regeneration of endogenous beta cells. [6]

To confirm the islet mass transplanted, islet and CFA-PI DNA content were measured by Quant-iT PicoGreen dsDNA Assay (Invitrogen, Eugene, OR, USA). Islet and CFA-PI aliquots were washed in citrate buffer (150 mmol/l NaCl, 15 mmol/l citrate, 3 mmol/l EDTA, pH=7.4) and stored as cell pellets at -20oC. Cell pellets were placed in 200 ml of lysis buffer (10 mmol/l Tris, 1 mmol/l EDTA, 0.5% Triton X-100, 48oC, pH 7.5). Aliquots of 25 and 50 µl were assayed in duplicate with dilution in 1 ml of DNA buffer (10 mmol/l Tris, 1 mmol/l EDTA, pH 7.5).

Fluorescence was measured at 490 excitation / 515 emission nm after the addition of 1 ml of Pico Green reagent (1/200 dilution with DNA buffer).

3.7.1.9 Intraperitoneal glucose tolerance test (IPGTT)

In vivo glucose tolerance and islet function was assessed by IPGTT 60 days post-transplant. All mice within each transplant group was tested regardless of euglycemia. For those mice that were hyperglycemic an IPGTT was performed before they were electively euthanized (≤60 days). The mice were fasted overnight, and 25% dextrose was administered intraperitoneally at 3 g/kg body weight (Hospira, Lake Forest, IL, USA). Blood glucose measurements were monitored at baseline (t=0), 15, 30, 60, 90 and 120 minutes. In order to comply with ethics requirements, it was necessary to euthanize some animals that did not reverse diabetes and were identified as in extremely poor health (at the 500 IEQ dose: 4 animals in the native islet group, and two animals in the spontaneous aggregation group; at the 1000 IEQ dose: 1 animal in the native islet group, 3 animals in the spontaneous aggregation group, 4 animals in the P500 group, 2 animals in the P750 group, and 1 animal from the P1000 group) without performing IPGTT. To avoid biasing the data due to the selective elimination of these animals, the missing values were filled in as an average of the results from 30 diabetic animals not otherwise involved in this study.

3.7.1.10 Immunohistochemistry

Islet and CFA-PI transplant grafts were removed from mice. Immediately after explantation, the kidney bearing the islet graft was fixed in 10% formalin. The tissue was dissected, embedded in paraffin, and sectioned. Following de-paraffinization and antigen heat retrieval, sections were blocked with 20% goat serum (Sigma-Aldrich) in DPBS for 1.5 hours at room temperature. For vessel staining an additional enzymatic antigen retrieval step was performed prior to blocking (Proteinase K at 20 μg/ml for 20 minutes at 37°C). Sections were incubated with primary antibodies overnight at 4°C: guinea pig anti-insulin (Dako, A0564, 1:200), rabbit

anti-glucagon (Abcam, ab43837, Cambridge, MA, USA, 1:100), rabbit anti-human pancreatic polypeptide (Abcam, ab14985, 1:100), rat anti-human somatostatin (Abcam, ab30788, 1:100), or rabbit anti-CD31 and anti-von Willebrand factor (vWF) (Abcam, ab124432, 1:50). The following day sections were washed with Tween buffer followed by incubation with secondary antibodies 1:200 for 1 hour at room temperature utilizing goat anti-guinea pig Rhodamine, goat anti-rat fluorescein (Jackson ImmunoResearch, 106-025-003, West Grove, PA, USA), or goat anti-rabbit Alexa Fluro 568 (Abcam). Samples were counterstained with DAPI (Invitrogen, P36931).

Data are expressed as mean ± SEM unless otherwise specified. Statistical analyses were performed using GraphPad Prism 7. Imaging was carried out on a Zeiss COLIBRI inverted fluorescence microscope unless otherwise specified and analysis was via ImageJ software (http://rsb.info.nih.gov/ij). Spatial statistical analysis of vascular element distribution was performed using the "Spatial statistics 2D/3D" plugin, implementing the previously published F-function, [7,8] with independent evaluation points set to 10,000, hardcore distance set to 0, pattern samples set to 10.

3.7.1.11 Supplemental References:

1. Pisania A, Weir GC, O'Neil JJ, et al (2010) Quantitative analysis of cell composition and purity of human pancreatic islet preparations. Lab Invest 90:1661–1675

2. Integrated Islet Distribution Program | IIDP | Human Islets for Research > Investigators > Policies & Standard Operating Procedures. https://iidp.coh.org/Investigators/Policies-Standard-Operating- Procedures. Accessed 5 Dec 2017

3. Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods San Diego Calif 25:402–408

4. Vandesompele J, De Preter K, Pattyn F, et al (2002) Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol 3:RESEARCH0034

5. Pepper AR, Gala-Lopez B, Pawlick R, et al (2015) A prevascularized subcutaneous device-less site for islet and cellular transplantation. Nat Biotechnol 33:518

6. King AJ (2012) The use of animal models in diabetes research. Br J Pharmacol 166:877–894

7. Andrey P, Kiêu K, Kress C, et al (2010) Statistical Analysis of 3D Images Detects Regular Spatial Distributions of Centromeres and Chromocenters in Animal and Plant Nuclei. PLOS Comput Biol 6:e1000853

8. Ollion J, Cochennec J, Loll F, et al (2013) TANGO: a generic tool for high-throughput 3D image analysis for studying nuclear organization. Bioinformatics 29:1840–1841

3.7.2 Supplemental Tables

Gene symbol	Alias/common name	Primer sequence (5'-3')
Referencing genes		
POLR2A	RNA Polymerase II Subunit A	F-TCACAGCAGTGCGCAAATTC R-CCACGTCGACAGGAACATCA
EIF2B1	Eukaryotic Translation Initiation Factor 2B Subunit Alpha	F-CGGACGTTGCTGGAGTTCTT R-CCACACCACACAGGGTTTCT
Secretory function		
INS	Insulin	F-GGCCTTTGCGTCAGATCACTG R-GTTCCCCGCACACTAGGTAGA
PDX1	Pancreatic and duodenal homeobox 1, insulin promoter factor 1	F-GGGAAAACCCGCTCTCTCAG R-CCAAGGTGGAGTGCTGTAGG
GLP1R	Glucagon Like Peptide 1 Receptor	F-TTGTGAAACCACAGGCCCTT R-CTTGCAAGCCCCAGTTTCAC
PCSK1	Proprotein Convertase Subtilisin/Kexin Type 1	F-GCCGAACTGACTATGGGGAA R-AAAGGCACTCCTTCGAGACC
PCSK2	Proprotein Convertase Subtilisin/Kexin Type 2	F-GTGTGTTTGCACTGGCTCTG R-TTAAATTCCAGGCCGACCCC
Cell communication		
GJA1	Connexin 43, gap junction protein alpha 1	F-CAATCTCTCATGTGCGCTTCT R-GGCAACCTTGAGTTCTTCCTCT
CDH1	E-Cadherin	F-GCTGGACCGAGAGAGTTTCC R-CGACGTTAGCCTCGTTCTCA
LAMB1	Laminin Subunit Beta 1	F-AAAAGACATCCTGGCGCAGA R-TTCTTTGGCTGTGCTGTTGC
ITGB1	Integrin Subunit Beta 1	F-GCCGCGCGGAAAAGATGAAT R-ACATCGTGCAGAAGTAGGCA
ITGB7	Integrin Subunit Beta 7	F-AGAATGGCGGAATCCTCACCT R-TGAAGTTCAGTTGCTTGCACC
Apoptosis		
NFKB1	Nuclear Factor of Kappa Light Polypeptide Gene Enhancer In B-Cells 1	F-AACAGAGAGGATTTCGTTTCCG R-TTTGACCTGAGGGTAAGACTTCT
NOS2	Inducible Nitric Oxide Synthase	F-CCCACCAGACAGTGCGCCTG R-GGAGCAGCAGCTGGGTTGGG
NOS3	Endothelial Nitric Oxide Synthase	F-GTGGCTGGTACATGAGCACT R-GTGGTCCACGATGGTGACTT
MAPK8	Mitogen-Activated Protein Kinase 8	F-TGTGTGGAATCAAGCACCTTC R-AGGCGTCATCATAAAACTCGTTC
MAPK10	Mitogen-Activated Protein Kinase 10	F-CTGGTATGACCCAGCCGAAG R-GCACCTGTGCTGAAGGAGAA
APAF1	Apoptotic Peptidase Activating Factor 1	F-TCCAGTCCAGGTTTCAGCAC R-CTGTTTCCTGATGGCCTCGT

Table 3.7.2. 1: Human gene primers for real-time qPCR

Table 3.7.2. 2: Summary of in vitro parameters and in vivo outcomes of native islets and corresponding CFA-PIs by donor islets preparations used both *in vitro* and *in vivo*.

Due to the amount of data presented, Table 3.7.2.2 is provided as an Excel file and can be acquired by request.

R200	Stimulation Index	Basal (fg/input cell)	Stimulated (fg/input cell)	ER (fg/input cell)	GSIS (fg/incorporated cell)	GSIS basal (fg /incorporated cell)	GSIS stimulated (fg /incorporated cell)
Native Islets	1.78	90.83	161.83	71.00	95.99	122.79	218.78
	3.05	28.10	85.64	57.54	77.78	37.99	115.78
	1.35	200.77	271.72	70.95	95.92	271.42	367.33
Spontaneous aggregates	0.69	90.83	62.83	-27.99	-69.91	226.82	156.91
	2.10	66.22	139.06	72.84	181.91	165.37	347.28
	0.86	87.83	75.34	-12.49	-31.20	219.34	188.14
P1000	2.92	82.04	239.92	157.88	189.09	98.26	287.35
	1.13	119.67	134.63	14.96	17.92	143.33	161.25
	1.39	142.09	197.56	55.47	66.43	170.18	236.61
P750	2.79	58.36	162.79	104.43	147.15	82.24	229.39
	2.51	91.36	229.27	137.90	194.32	128.74	323.05
P500	0.91	134.87	122.27	-12.60	-18.99	203.22	184.23
	2.80	75.74	212.05	136.31	205.38	114.12	319.51

Table 3.7.2. 3: *In vitro* parameters of individual samples tested in donor preparation R200

R201	Stimulation Index	Basal (fg/input cell)	Stimulated (fg/input cell)	ER (fg/input cell)	GSIS (fg/incorporated cell)	GSIS basal (fg /incorporated cell)	GSIS stimulated (fg /incorporated cell)
Native Islets	0.53	279.98	148.26	-131.72	-151.39	321.79	170.40
	1.16	104.39	121.07	16.68	19.17	119.98	139.15
	0.66	182.33	119.97	-62.36	-71.67	209.56	137.89
Spontaneous aggregates	0.73	225.33	163.39	-61.94	-246.96	898.39	651.42
	0.79	211.62	166.24	-45.38	-180.93	843.72	662.78
	0.58	216.28	125.06	-91.22	-363.67	862.28	498.61
P1000	1.49	146.83	218.66	71.83	70.27	143.64	213.90
	1.22	110.59	134.94	24.36	23.83	108.18	132.01
	0.79	218.17	171.78	-46.39	-45.38	213.43	168.05
P750	2.76	104.27	287.83	183.55	174.22	98.97	273.19
	0.78	189.53	147.07	-42.46	-40.30	179.89	139.59
	1.85	139.55	258.32	118.78	112.73	132.45	245.18
P500	0.91	209.85	190.35	-19.50	-18.97	204.08	185.11
	0.58	292.66	170.98	-121.68	-118.33	284.60	166.28
	0.83	180.77	150.63	-30.14	-29.31	175.79	146.48

Table 3.7.2. 4: *In vitro* parameters of individual samples tested in donor preparation R201

R202	Stimulation Index	Basal (fg/input cell)	Stimulated (fg/input cell)	ER (fg/input cell)	GSIS (fg/incorporated cell)	GSIS basal (fg /incorporated cell)	GSIS stimulated (fg /incorporated cell)
Native Islets	1.44	79.00	114.01	35.01	39.47	89.06	128.53
	1.61	38.34	61.88	23.54	26.54	43.22	69.76
	1.24	95.38	117.88	22.50	25.36	107.53	132.89
Spontaneous aggregates	0.75	53.99	40.30	-13.69	-70.82	279.27	208.45
	1.72	36.38	62.46	26.08	134.90	188.18	323.08
	1.18	35.33	41.60	6.27	32.42	182.77	215.19
P1000	1.25	26.36	32.84	6.47	7.40	30.13	37.54
	2.88	23.30	67.14	43.83	50.11	26.64	76.75
	1.09	40.96	44.59	3.63	4.14	46.82	50.97
P750	1.34	41.45	55.39	13.94	17.00	50.56	67.55
	1.34	39.86	53.21	13.35	16.29	48.61	64.89
	2.19	28.75	63.00	34.25	41.77	35.06	76.83
P500	0.96	35.56	34.15	-1.41	-1.61	40.62	39.01
	2.23	20.90	46.53	25.62	29.27	23.87	53.14
	1.41	34.26	48.46	14.19	16.21	39.14	55.35

Table 3.7.2. 5*: In vitro* parameters of individual samples tested in donor preparation R202

R226	Stimulation Index	Basal (fg/input cell)	Stimulated (fg/input cell)	ER (fg/input cell)	GSIS (fg/incorporated cell)	GSIS basal (fg /incorporated cell)	GSIS stimulated (fg /incorporated cell)
Native Islets	0.65	350.64	229.30	-121.34	-131.07	378.77	247.70
	0.29	462.94	132.59	-330.35	-356.85	500.07	143.23
	0.65	228.66	148.11	-80.54	-87.01	247.00	159.99
Spontaneous aggregates	0.72	224.33	160.70	-63.63	-92.22	325.14	232.92
	1.72	107.22	184.62	77.39	112.17	155.41	267.58
	1.03	127.25	131.66	4.41	6.39	184.43	190.82
P1000	7.04	76.67	539.81	463.14	454.93	75.31	530.24
	12.07	64.24	775.51	711.27	698.66	63.11	761.77
	8.00	52.59	420.57	367.98	361.46	51.66	413.12
P750	10.30	74.77	770.24	695.46	647.54	69.62	717.16
	14.98	61.16	916.39	855.23	796.30	56.95	853.25
	7.94	58.71	465.86	407.15	379.09	54.66	433.76
P500	4.11	114.96	472.65	357.69	320.09	102.88	422.97
	6.06	123.87	751.01	627.15	561.23	110.85	672.08
	8.95	56.25	503.59	447.34	400.32	50.34	450.66

Table 3.7.2. 6: *In vitro* parameters of individual samples tested in donor preparation R226

R226	Stimulation Index	Basal (fg/input cell)	Stimulated (fg/input cell)	ER (fg/input cell)	GSIS (fg/incorporated cell)	GSIS basal (fg /incorporated cell)	GSIS stimulated (fg /incorporated cell)
Native Islets	0.96	89.2	85.6	-3.61	-6.72	166.09	159.37
	1.02	84.56	86.11	1.55	2.88	157.45	160.33
	3.69	76.31	281.6	205.29	382.23	142.09	524.32
Spontaneous aggregates	0.67	457.94	305.01	-152.93	-336.45	1007.45	671
	1.9	215.78	409.89	194.11	427.04	474.71	901.74
	2.71	152.77	414.72	261.95	576.29	336.1	912.38
P1000	7.12	175.72	1251.9	1076.18	1027.12	167.71	1194.83
	9.12	170.58	1556.01	1385.43	1322.27	162.8	1485.07
	11.3	111.83	1263.26	1151.43	1098.94	106.73	1205.67
P750	5.62	162.47	912.58	750.11	692.6	150.01	842.61
	7.37	146.16	1077.63	931.48	860.06	134.95	995.01
	8.9	194.8	1734.21	1539.41	1421.38	179.87	1601.25
P500	6.36	77.34	492.29	414.95	509.2	94.91	604.12
	3.97	126.69	503.35	376.66	462.22	155.47	617.69
	7.64	84.05	642	557.95	684.69	103.14	787.83

Table 3.7.2. 7: In vitro parameters of individual samples tested in donor preparation R227

	500 IEQ					1000 IEQ				
Cohort	Native islets (n=10)	Spontaneous aggregates (n=6)	P500 (n=8)	P750 (n=9)	P1000 (n=9)	Native islets (n=9)	Spontaneous aggregates (n=6)	P500 (n=8)	P750 (n=10)	P1000 (n=9)
R190	3			3		3			3	
R200	X	X	X	X	X					
R201	X, X	X, X	X, X	X, X, X	X, X	X	X		X	X
R202	10	X	6, X	3, 6	3, 6, X	6	20	3, X	3, X	6, 6
R226	X, X, X	X	X, X, X	7	3, 3	5, 7, 11	33	3, 5, X, X	3, 5	3, 3, 3
R227	X, X	X		2	7	X, X, 7	X, X, X	X, X	2, 14, X, X	2, 7, X

Table 3.7.2. 8: Diabetes reversal days and transplant distribution.

Human islet donor cohorts are assigned a unique R= research identification number prior to tissue release. IEQ = islet equivalents. Numbers shown in the table represent the number of days it took for each animal to reach euglycemia. X represents animals that did not reach euglycemia.

	Native islets		CFA-PI		Spontaneous aggregates	
	mean	SD	mean	SD	mean	SD
Prior to dissociation (µg)	10.77	1.23	12.08	1.74	12.08	1.74
Post dissociation (µg)	-	-	9.45	0.39	9.45	0.39
Post culture/pre transplantation (µg)	8.21	1.52	8.95	1.43	3.82	1.87
Dissociation recovery rate	-	-	78%	12%	78%	12%
Overall recovery rate	76%	17%	74%	16%	32%	16%

Table 3.7.2. 9: Assessment of genomic DNA per IEQ.

3.7.3 Supplemental Figures

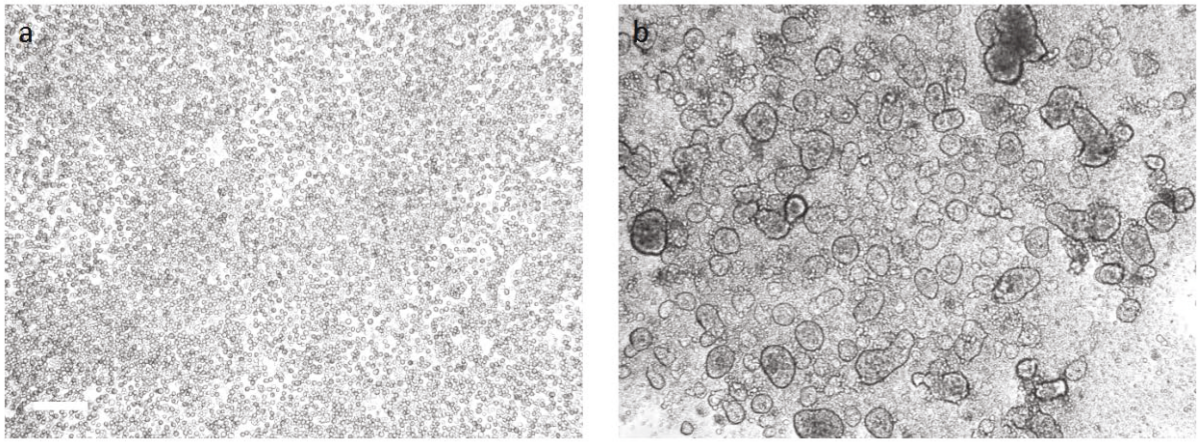

Figure 3.7.3. 1: Pancreatic Cell Suspension

Pancreatic cell suspensions immediately post-dissociation (**a**) appear uniformly as single cells. The single-cell suspensions were cultured on ultra-low attachment plates for conventional spontaneous aggregate formation, visible here after 96 hours in culture - note heterogeneous size distribution with a substantial proportion of single cells and debris (**b**). Scale bar represents 200 μm.

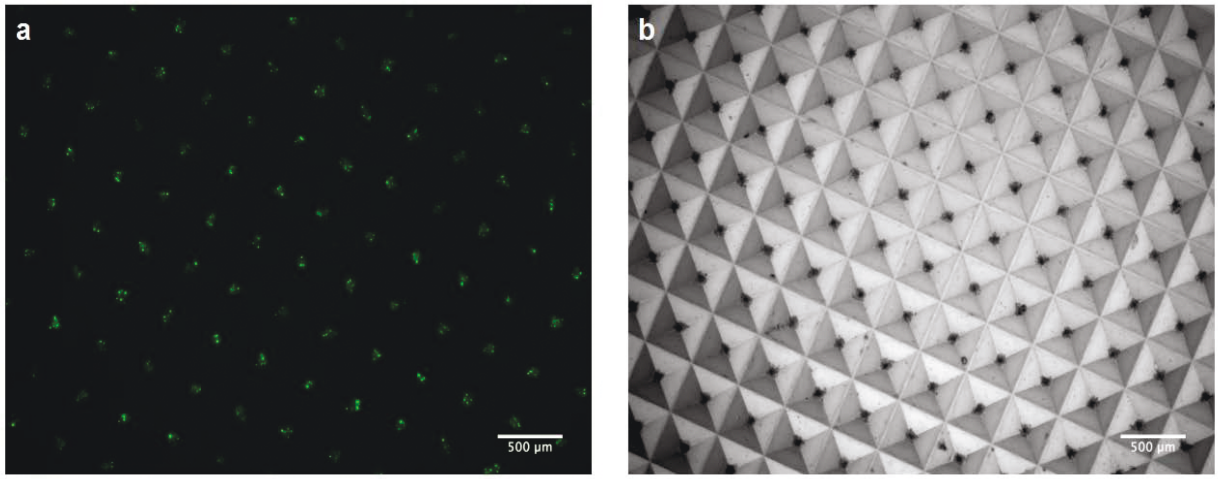

Figure 3.7.3. 2 : CFA-PI GFP Visualization

CFA-PI in Aggrewell 48 hours after GFP transfection at dissociated single cells stage prior to formation, showing GFP fluorescence retained in CFA-PI (**a**) and its bright phase (**b**). Scale bar represents 200 µm.

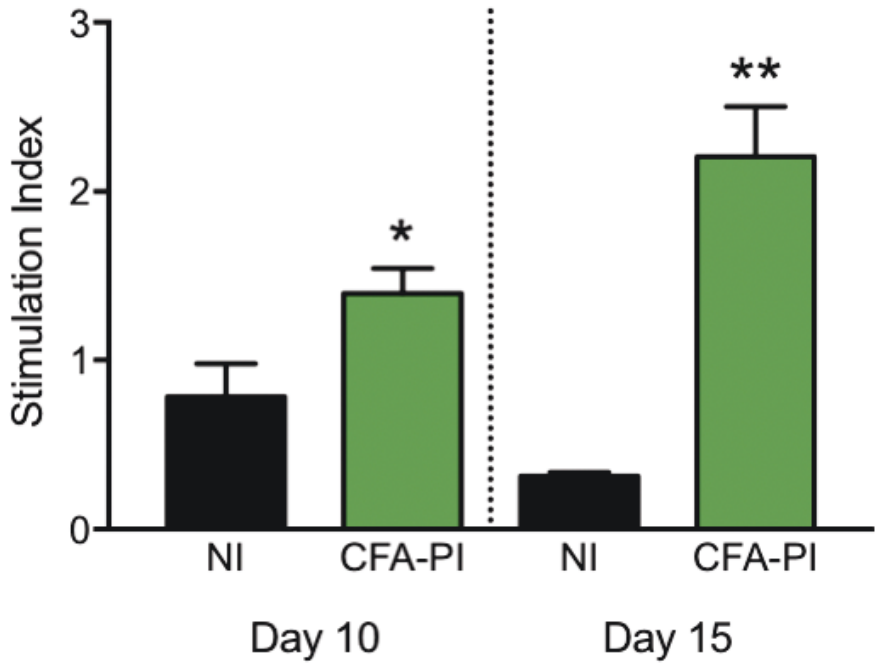

Figure 3.7.3. 3: Stimulation Index During Culture

The Stimulation Index shows increased tolerance for long term culture by CFA-PI (P1000, P750 and P500) as compared to native islets. At both day 10 (**a**) and day 15 (**b**) post-culture, CFA-PI retained a significantly higher simulation index compared to the native islets from which they were formed ($n \geq 10$ at day 10, $n \geq 3$ at day 15; $^{**}p<0.01$, $^{*}p<0.05$, Mann-Whitney test).

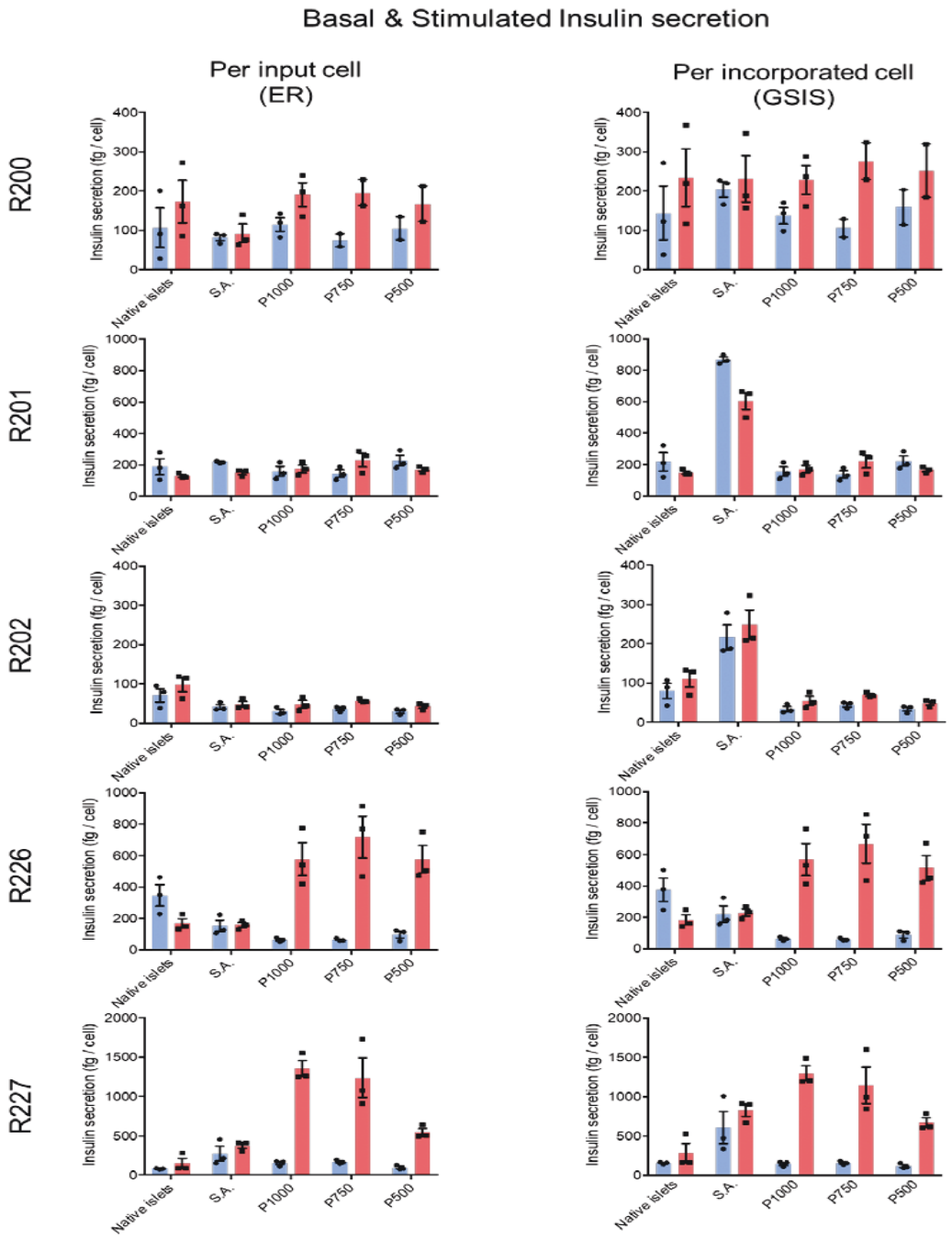

Figure 3.7.3. 4: **Donor Basal and Stimulated Insulin Secretion and Efficacy Ratio**

Basal and stimulated insulin secretion of native islets, spontaneous aggregates and CFA-PIs broken out by donor, normalized to the amount of material (left) present prior to dissociation and re- aggregation (Efficacy Ratio); and (right) present at time of assay (traditional normalization for Glucose Stimulated Insulin Secretion).

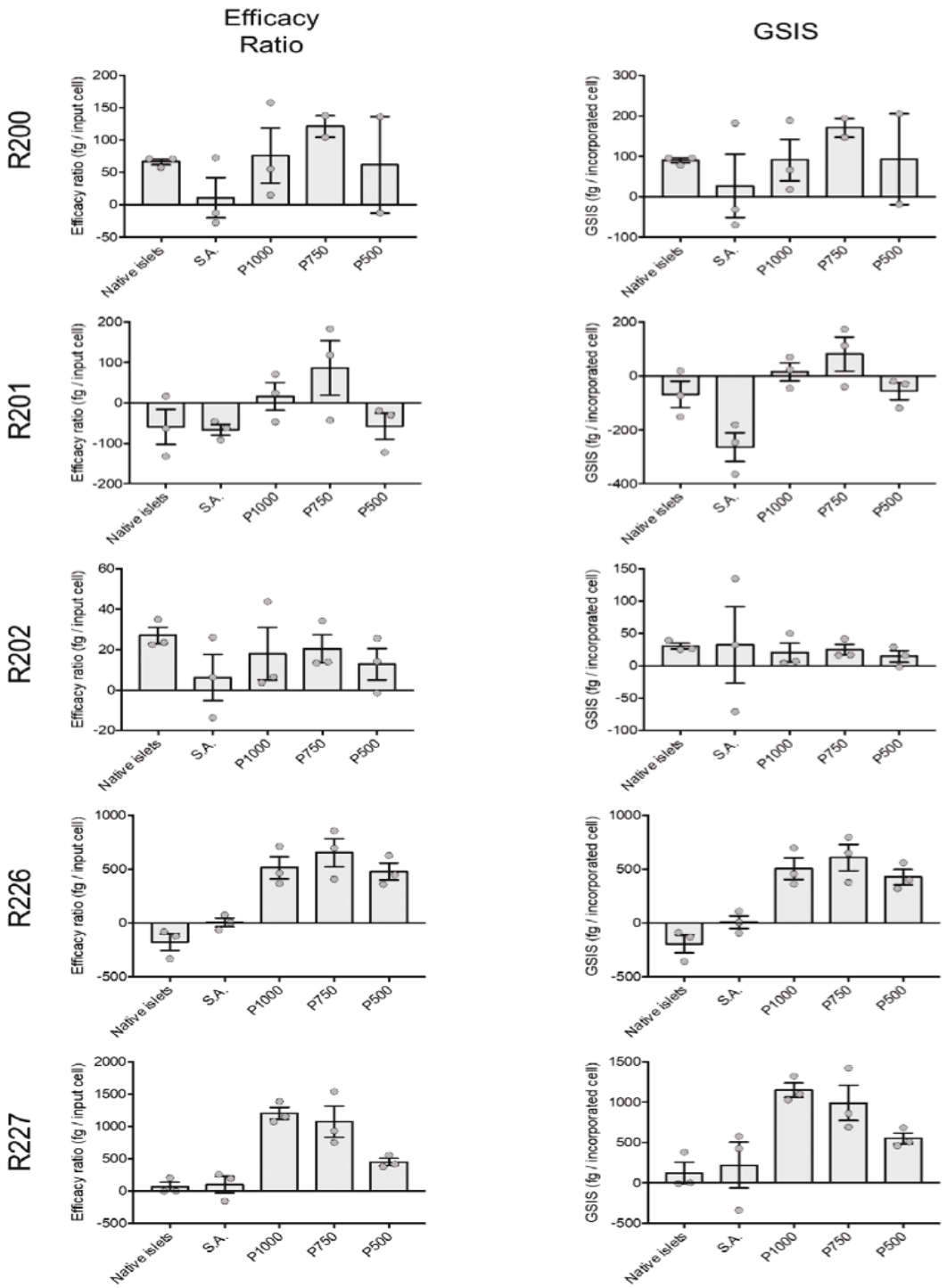

Figure 3.7.3. 5: Donor Basal and Stimulated Insulin Secretion

Basal and stimulated insulin secretion of native islets, spontaneous aggregates and CFA-PIs broken out by donor, calculated from the basal and stimulated insulin secretion data shown in Fig. 3.7.3.4.

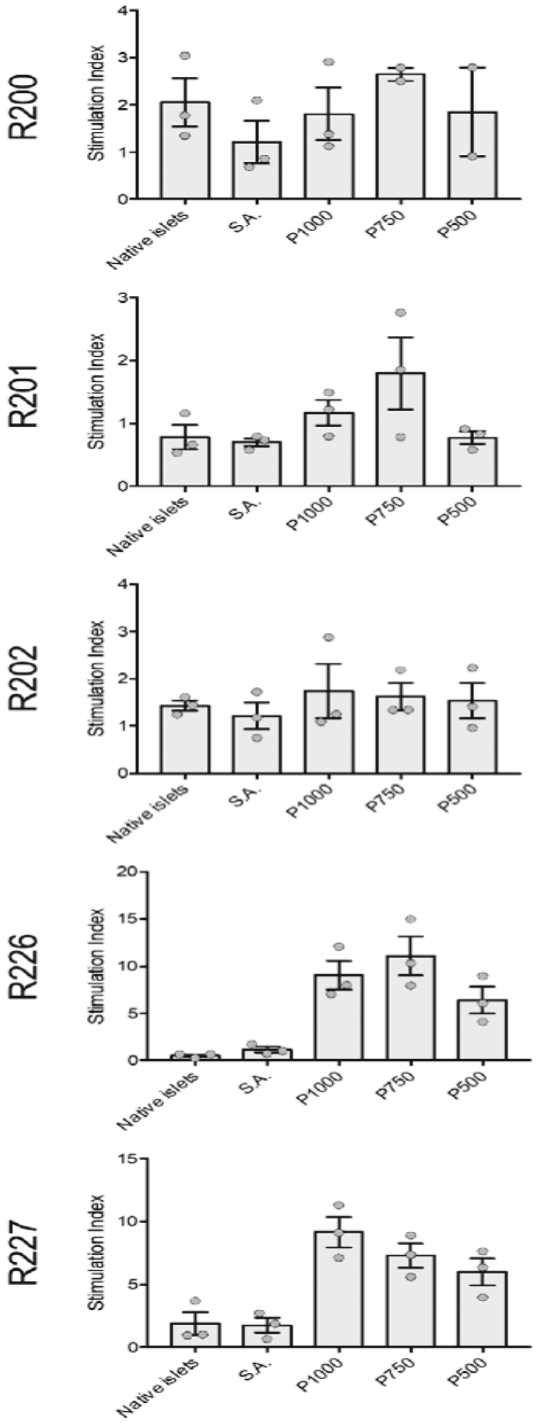

Figure 3.7.3. 6: **Stimulation Index**

Stimulation Index of native islets, spontaneous aggregates and CFA-PIs broken out by donor, calculated from the basal and stimulated insulin secretion data shown in Fig. 3.7.3.4

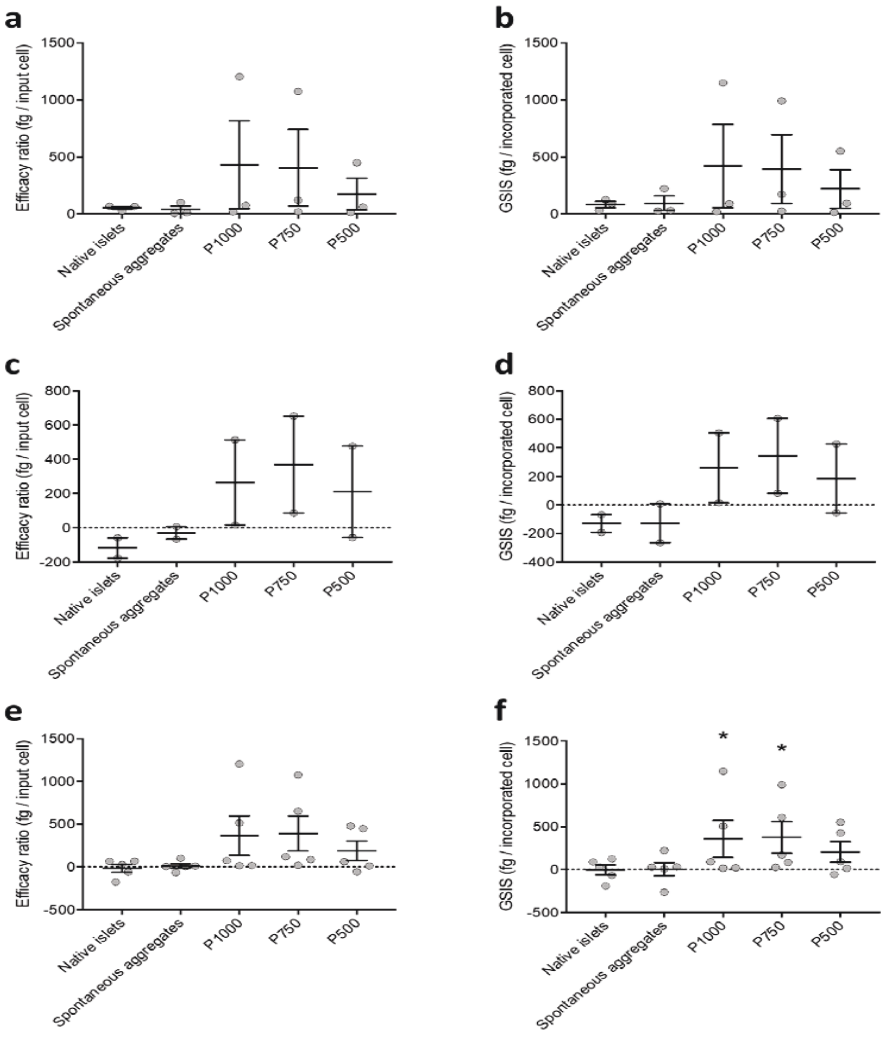

Figure 3.7.3. 7: Efficacy Ratio and Static Glucose Insulin Secretion

Overview of ER and GSIS results for native islets, spontaneous aggregates and CFA-PIs either divided into subgroups of "high-function" islet preparations (native islet controls exhibit Stimulation Index > 1) (**a**, **b** respectively) and "low-function" islet preparations (native islet controls exhibit Stimulation Index < 1) (**c**, **d** respectively); or pooled (**e**, **f** respectively). Paired and matched one-way ANOVA with Holm- Sidak's multiple comparison analysis has been performed for both e and f. GSIS for P1000 and P750 is significantly improved over native islets, with *$p<0.05$ (**f**). Corresponding ER values for P1000 and P750 also appear increased, although this difference does not reach statistical significance ($p=0.0746$, $p=0.0714$, respectively) (**e**).

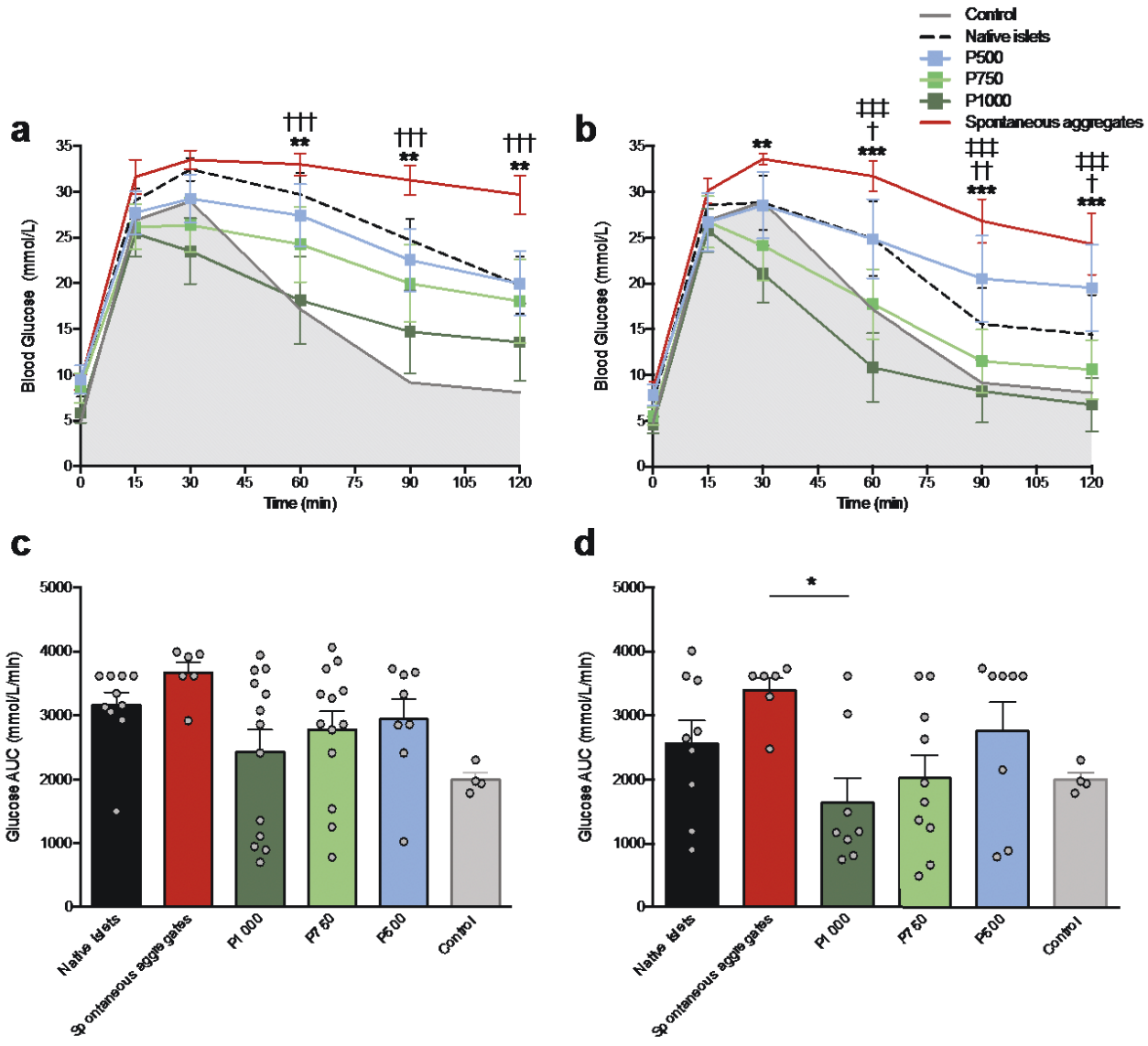

Figure 3.7.3. 8: Intraperitoneal Glucose Tolerance Test

As a negative control, animals were also transplanted with spontaneous aggregates. At 500 IEQ, glucose clearance was significantly improved in the P1000 group, n=8, compared to the spontaneous aggregates, n=6 (**p<0.01 at 60, 90 and 120 minutes) (**a**). There was no significant difference in the P750 group, n=9 and P500 group, n=8. At 1000 IEQ, glucose clearance was markedly improved within both the P1000, n=9, and P750 groups, n=10 (**p<0.01 at 30 minutes and ***p<0.001 at 60, 90 and 120 minutes; †p<0.05 at 60 and 120 minutes and ††p<0.01 at 90

minutes, respectively) (**b**). There were no significant differences between groups transplanted with 500 IEQ when comparing areas under the curve (**c**). There was significantly improved overall clearance in the P1000 group transplanted with 1000 IEQ when comparing the areas under the curve (*$p<0.05$) (**d**). As expected, naïve non-transplanted euglycemic control were significantly different from the spontaneous aggregate transplanted mice at 60, 90 and 120 minutes for both 500 IEQ and 1000 IEQ islet mass' (†††$p<0.001$, ‡‡‡ $p<0.001$) (Student's t test between groups at each time point corrected for multiple comparison using the Holm-Sidak method; analysis of AUC by one-way ANOVA with Tukey's multiple comparisons).

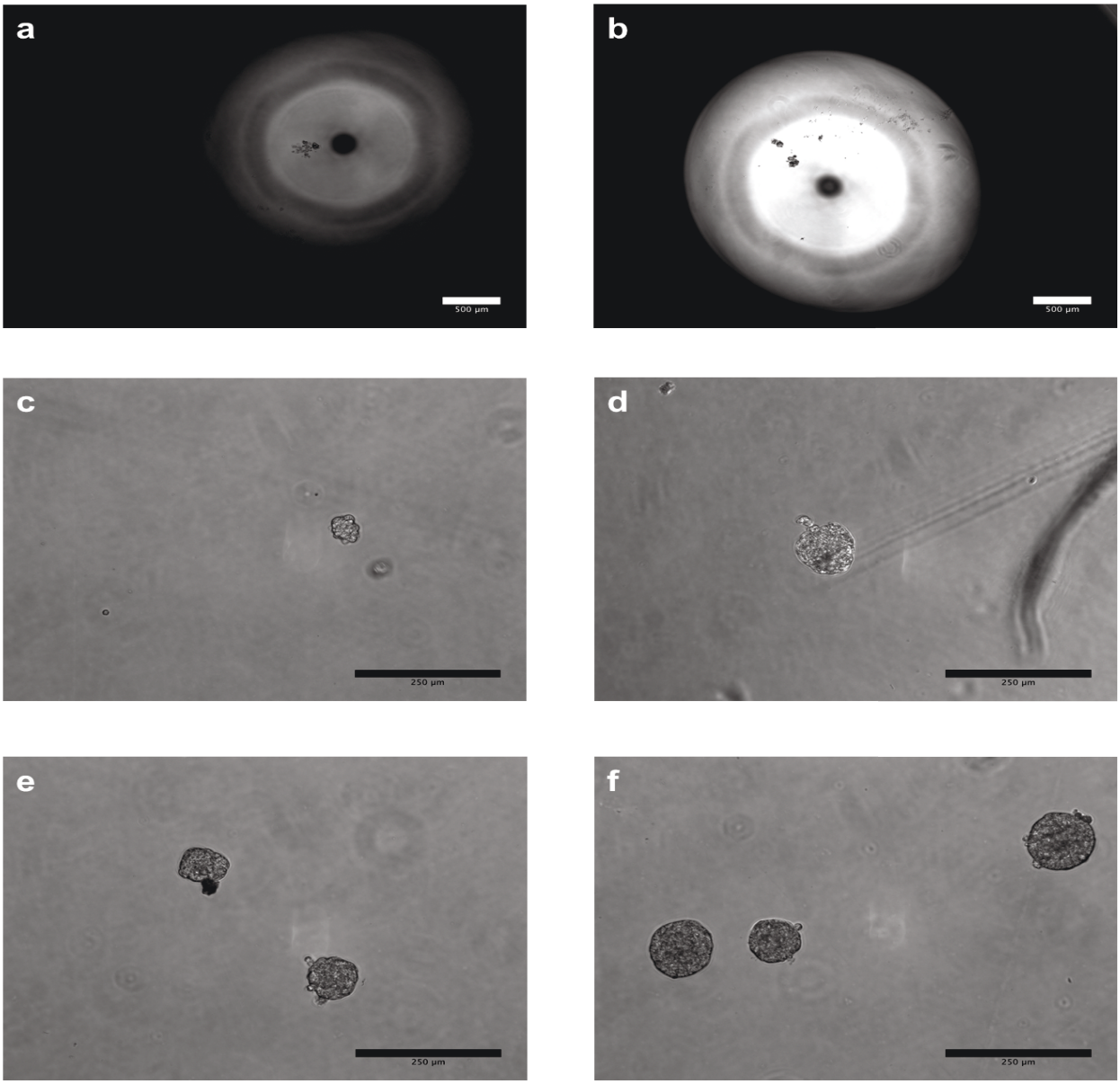

Figure 3.7.3. 9: Comparison of Hanging Drop and CFA-PI

Comparison of pseudoislets formed via hanging drop and CFA-PI approaches, 5 days post initiation. Pseudoislets were initiated from 250 (**a**, **c**, **e**) or 750 (**b**, **d**, **f**) cells apiece, and representative images are shown inside (**a**, **b**), or after extraction from the hanging drop (**c**, **d**), alongside CFA-PI controls formed from the same donor islet preparation (**e**, **f**). CFA-PI were larger and more cohesive, likely as a result of accelerated clustering (and therefore reduced time as isolated single cells).

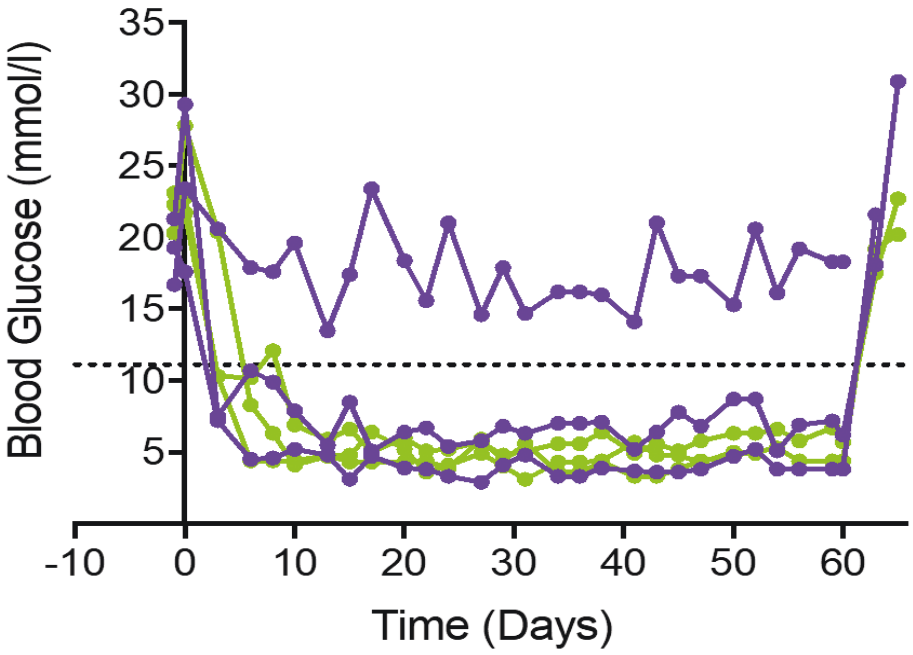

Figure 3.7.3. 10: Blood Glucose Profiles

Representative data showing blood glucose changes over 60 days of mice transplanted with CFA-PIs at 1000 IEQ dose (purple line) and 500 IEQ dose (green line), dotted line showing 11.1mmol/l blood glucose threshold. At day 60, a recovery nephrectomy of the left kidney was performed, and considered to confirm the graft as responsible for diabetes reversal once blood glucose returned to a hyperglycemic state. While the donor-to-donor, recipient-to-recipient and day-to-day variability characteristic of work with human islet material was present, the impact of the transplanted material on blood glucose levels is evident both at both introduction and removal.

Chapter 4

Discussion and Concluding Remarks

CHAPTER 4: DISCUSSION AND CONCLUDING REMARKS

SEGMENTS OF THESE CONCLUDING REMARKS HAVE BEEN PUBLISHED AS A BOOK CHAPTER AFTER PEER REVIEW IN THE ENCYCLOPEDIA OF ENDOCRINOLOGY DISEASES UNDER THE TITLE "TRANSPLANTATION: PANCREATIC AND ISLET CELLS"

Transplantation: Pancreatic and Islet Cells

Anissa Gamble, Antonio Bruni, and AM James Shapiro, University of Alberta, Edmonton, AB, Canada

4.1 General Discussion

Globally, the prevalence of diabetes is estimated to rise from 2.8% in 2000 to 4.4% by 2030 across countries apart of the United Nations population. [1] The WHO projects that diabetes will be the seventh leading cause of mortality by 2030. [2] Patients with Type 1 Diabetes Mellitus (T1DM) must undergo a lifelong regimen of exogenous insulin injections and daily glucose monitoring. Failure to control T1DM can lead to long-term end-organ microvascular complications including: nephropathy, neuropathy, retinopathy, peripheral vascular disease, and coronary artery disease. [3] Improved insulin formulation of short and long-acting forms and continuous glucose monitoring (CGMs) and closed loop pumps systems with or without low glucose suspend may improve monitoring and control, but cannot guarantee avoidance of secondary and acute complications. A study done by The Diabetes Control and Complications Trials (DCCT) revealed tight glycemic control can mitigate secondary complications and end-organ failure, but substantially increases the risk of severe and recurrent hypoglycemic reactions. [4-6]

An alternate means to restore euglycemia in patients suffering from T1DM is to replace the β cell mass through a pancreas or islet cell transplantation. Whole pancreas transplantation is an invasive surgical procedure that carries the risk of complications and potential death but is currently the most reliable means to restore insulin independence with glycemic reserve. Islets of Langerhans are clusters of cells composed of four main cellular components: glucagon-producing α cells, insulin-producing β cells, somatostatin-producing δ cells, and pancreatic polypeptide-producing cells. [7] Islets range in size from <50um to ~800um of diameter and only constitute ~1-2% of the total pancreatic tissue. [7-9] Furthermore, as highly vascularized cells, islets rely on complex cell-cell interactions between different cell subsets to maintain glucose

homeostasis. Islet transplantation is generally considered a safer approach as it does not require invasive surgery, and only the cells are transplanted without the challenges associated with exocrine drainage. Islet cells are obtained from deceased organ donor pancreatectomy, ABO compatible with the recipient, and infused into the recipient portal vein under the cover of anticoagulation, anti-inflammatory medications and inductive and maintenance immunosuppression. While insulin independence is often achieved at early time points, maintaining a completely insulin free state is challenging due to early and late islet cell loss. Islet loss may occur due to IBMIR triggered by tissue factors expressed on the islet surface including inflammation, alloimmune rejection, recurrence of autoimmunity, or toxicity to β cells from immunosuppressive medications. To alleviate islet cell loss, alternative transplantation sites, stem cell-derived cellular products, co-transplantation with alternative cells or immune protective agents are the focus of active study. While islet transplantation is safer than pancreas transplantation from a procedural perspective, it is associated with lower insulin reserve and lower rates of sustained insulin independence over time. Both require similar potent immunosuppression to sustain allograft function.

4.2 Alternative and Bioengineered Cell Sources to Improve Islet Engraftment

4.2.1 T Regulatory Cells Infusion

T regulatory cells (Tregs) are immune subpopulations of $CD4^{+}CD25^{+}$ lymphocytes that can promote the tolerance of foreign particles and maintain immune homeostasis. [10] When tested in mouse models and most recently in new-onset human diabetes, Tregs can delay the progression of T1DM and improve islet engraftment. Bluestone and colleagues demonstrated that autologous *ex vivo* expanded Tregs infused into newly diagnosed T1DM patients

significantly prolonged the honeymoon period and attenuated ongoing autoimmune β cell destruction. [11, 12] After intravenous Treg infusion or co-transplantation, Tregs migrate and their highly selective receptors can mitigate alloimmune-rejection. [13-15] In animal models, co-transplantation of islets together with beneath the kidney capsule prolonged islet functional survival. [10] However, during simultaneous islet transplantation and peripheral Treg infusion, Tregs have yet to demonstrate effective immunosuppressive capability and ongoing trials are now underway in Edmonton and San Francisco to harness this potential. [16]

4.2.1 Xeno-derived Cell Sources

Xenotransplantation of porcine-derived islets offers an alternative more limitless source of cells for transplantation into patients, but this approach comes with its own unique challenges. Xenograft rejection is a far more aggressive reaction to control, and therefore requires far more potent multi-pronged immunosuppression and immunodepletion for persistent cell survival, with consequently far more potential risk than an allograft would carry. Carl Groth and colleagues in Sweden in 1984 transplanted fetal porcine islets beneath the kidney capsule of patients undergoing kidney transplantation, and found persistent urinary porcine C-peptide was detectable for several months, but was unable to restore euglycemia and insulin-independence. [17] Although no patient has yet achieved insulin independence after xenoislet transplantation, substantial progress has been made. The possibility for elimination of porcine immune targets using genetic engineering is opening up new possibilities. Knock-out of decay accelerating factor (DAF) and other drivers of hyperacute rejection has allowed pig islets to survive from minutes to days and months, provided potent immunodepletion is continued. Genetically-engineered porcine islets transplanted into an immunosuppressed diabetic primate model was able to sustain euglycemia for more than a year post transplant. [18, 19] The recent possibility for multiple gene knock-outs using technology called

CRISP-Cas9 could completely transform this field.[20-24] The alternative approach of microencapsulating pig islets within an impenetrable immune-barrier membrane has also been explored in patients recently. In New Zealand, microencapsulated neonatal porcine islets were transplanted into non-immunosuppressed patients with T1DM. No porcine C-peptide was detectable however, and no patient was rendered insulin free for any period, although it was claimed that some subjects had mild improvement in hypoglycemic awareness. [25] A further trial of encapsulated pig islets carried out in Argentina also failed to render any patient insulin free, and no porcine C-peptide was detectable, but again it was claimed that hypoglycemic unawareness was improved temporarily. [26]

A further theoretical concern with application of porcine islets in patients is the potential transmission of porcine endogenous retroviruses (PERV). [18, 26, 27] To circumvent PERV transmission, Nui and colleagues utilize CRISPR-Cas9 technology to inactivate 62 copies of the PERV *pol* gene in a porcine kidney cell line resulting in a >1000-fold reduction in PERV transmission to human cells. [28, 29] Ongoing studies will determine the potential of this tissue source in future patients.

4.2.3 Stem Cell Transplantation- Synopsis

Considerable efforts are underway to explore the potential of pancreatic progenitor stem cells to provide a renewable source for insulin-producing cells. Sequential administration of selected transcription factors can potentially drive β-cell lineage production and expansion when either human embryonic stem cells (hESCs) or inducible pluripotential stem cells (iPSCs) are tested. [30, 31] Cells derived from the hematopoietic lineage, including bone marrow or umbilical cord blood derived cells have demonstrated capacity to differentiate into functional insulin-producing cells. [32, 33] iPSCs offer the future potential for self-compatible transplantation without

need for immunosuppression when transplanted back in to the same patient with T1DM. [34] Yamanaka *et al.* demonstrated the ability to dedifferentiate skin fibroblasts into mature cardiomyocytes or self-compatible β-cells capable of regulated insulin production. [34] The major challenges to imminent future application of iPSC technology are the costs and complex regulation surrounding generation, expansion and testing of these cells, and the major disadvantage that we currently do not know how to effectively eliminate autoimmunity in patients with T1DM. Unless HLA and other targets of autoimmune destruction can be CRISPR-Cas9 gene edited out of the cells, recurrent autoimmunity is certain to hamper early clinical testing of these exciting regenerative medicine cure-based approaches.

Of more immediate application and currently undergoing active clinical trials including in Edmonton, the use of human embryonic stem cells (hESCs) is garnering considerable attention. In 2004 and 2006, the group from ViaCyte and others generated hESC-derived pancreatic endoderm cells (PEC) capable of regulated secretion of human insulin and C-peptide, after further *in vivo* maturation. [35, 36] PEC 'Stage 4' cells are glucose-responsive, insulin-producing β cells capable of restoring euglycemia in diabetic rodent models. [35, 37-41] Additionally, the US Food and Drug Administration and Health Canada-approved the first in-human pilot phase 1/2 clinical trial utilizing Viacyte Inc. CyT49 hESC-derived PEC cells. The 2014 trial combined CyT49 hESC-derived PECs contained within an immunoprotective, microencapsulation 'Encaptra™' device (NCT02239354). Further ongoing trials in Edmonton and elsewhere (NCT03162926) are now testing perforated microencapsulation devices that considerably improve neovascularization, oxygen delivery and thereby improved endocrine cell survival, but the perforations compromise the immunoisolating capacity of PEC-Direct VC-02 approach. Further studies with hESC-derived pancreatic progenitor cells are underway, and offer promise for an unlimited cell source.

4.2.4 Application of Pseudoislet Technologies to Islet Transplantation

The complex process of organ retrieval, islet isolation and engraftment, presents obstacles that limit islet survival. To survive and function post-transplantation, islets must undergo adequate neovascularization in a process that is initiated in the first days after transplant, and matures over several weeks. During this time, islets are vulnerable to hypoxia, and cannot participate in dynamic responsive insulin release and control as effectively. [42] During this time, islets depend on the diffusion of oxygen and nutrients from the surrounding environment for acute survival. [43] In addition, islets are subjected to considerable innate injurious immune events including the instant blood mediated inflammatory reaction (IBMIR) and other environmental stress that contributes to early death of at least 50–70% of the original islet implant mass. [42, 44, 45] Environmental factors relating to early islet cell death include but not limited to donor-related factors, IBMIR, immune rejection, immunosuppressant toxicity, and hypoxia. Hypoxia is detrimental for islet survival and is problematic throughout all stages of the isolation, culture and transplantation process. Islets have an intrinsic high oxygen demand and especially larger islets ($\geq$ 500μm) that contribute most to the total islet count are most susceptible to core necrosis due to poor oxygen and nutrient diffusion. [46] The size of islets contributes to hypoxia, where larger islets with a diameter greater than 150 μm have impaired viability and function relative to smaller islets whose diameter are under 125 μm. [47, 48] To overcome this challenge, artificial generation of size-controlled bioengineered islet cells known as "pseudoislets" offer the potential to reduce central necrosis. Pseudoislets are formed by dissociating native islets and re-aggregating them into desired sizes. Investigators demonstrated the ability to form pseudoislets by spontaneous aggregation, [49, 50] hanging-drop method [51, 52] or with a microwell technique. [53-55] Regardless of the technique, the manipulation of the islet architecture has been shown to improve

oxygen-consumption rates, insulin-to-DNA ratio, viability and increased glucose diffusion. [56-60] Enhanced islet function could be a result of enhanced oxygen and nutrient diffusion into small islets compared to larger islets. [48, 61] In theory, pseudoislet formation appears to be beneficial and the clinical application could decrease the nonimmune-mediated physiological stress during revascularization and optimize the diffusion of oxygen and nutrients for islets. While promising *in vitro* and in small animal models, it remains to be seen how feasible it might be to scale up this process to accommodate a full human islet preparation, and what costs would be involved. Islet loss during pseudoislet generation might not be acceptable. Further, most human islet preparations are only 30%-50% pure unlike mouse islets, and handling this level of impurity in the process of reaggregation may post an insurmountable challenge.

Chapter 3 focuses on the formation of pseudoislets from human tissue. Human islets were dissociated and re-aggregated into uniform, size-controlled CFA-PI pseudoislets by Ungrin *et al.'s* microwell system. [62] In collaboration with Mark Ungrin *et al.* from the University of Calgary, we demonstrated the ability to form effective and homogenous sizes of pseudoislets. We evaluated various sizes of pseudoislets and their performance in an effort to determine an optimal size for islet function. Our study revealed improved glucose-stimulated insulin secretion and hypoxia tolerance while maintaining exocrine composition when compared to unmodified native islets. *In vivo*, we revealed that pseudoislets' efficacy was similar to native islets for euglycemia function, and glucose clearance**.** Subsequently, we evaluated vessel density of the islet engraftments and demonstrated improved vascularization for pseudoislets compared to native islets grafts. Others have noted impaired islet function and poor islet engraftment when cells were dissociated and re-aggregated, [63] but herein we demonstrated similar function.

Overall, we clearly demonstrate the potential to dissociate and re-aggregate native islets into engineered pseudoislets with limited consequence on a small scale.

4.2.5 Co-Transplantation with Mesenchymal Stem Cells

Mesenchymal stem cells (MSCs) are non-hematopoietic precursor cells that can differentiate into mesoderm lineages: adipocytes, osteocytes, chondrocytes, and myocytes. [64, 65] MSCs can be isolated from a multiple tissues sources such as amniotic fluid [66], skeletal muscle, [67] adipose tissue, [68] or umbilical cord. [69] Currently, limited evidence of MCSs' ability to differentiate into insulin producing cells is available, but the utilization of co-transplanting islets with MSCs can support islet engraftment. [70] The employment of MSCs in the islet transplant field is an exciting endeavor because MSCs' can ameliorate islet engraftment by their secretion of immunoregulatory and proangiogenic trophic factors that assist the immune regulation.[71] Trophic factors secreted by MSCs can enhance islet engraftment through the secretion of vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), Angiopoetin-1 (Ang-1), and transforming growth factor-β (TGF-β). [72, 73] Trophic factors assist in the reduction of the migration and proliferation of immune cells, and promote vascularization in the islet engraftment. [74-76] MSCs have demonstrated the ability to downregulate the immune response by interfering with different immunological pathways, via direct and indirect cell-to-cell interactions both *in vitro* and *in vivo*. [77 73, 78, 79] MSCs immune regulatory effects can suppress T and B cell proliferation, interfere with dendritic cell maturation, modulate natural killer cells cytotoxicity, and decrease immunoglobulin production to a certain extent that promotes islet cell engraftment survival. [80-82] Co-transplantation of human islets with MSCs has the potential to facilitate islet engraftment in a future clinical setting. [83]

4.2.6 Improving Islet Function and Yield Post Culture By the Co-Culture of Mesenchymal Stem Cells

A recent report in the 2016 Collaborative Islet Transplant Registry (CITR) revealed 1,927 islet infusions have been completed for 1,011 recipients reporting 80% insulin independence following to infusion. [84] Despite improved induced T-cell depletion and TNF anti-inflammatory treatments, a five-year follow-up estimates 50% of patients remain insulin independent post-transplant. [85-87] In an effort to understand and circumvent this defeat is through the analysis of islet culture conditions prior to transplantation.

The minimal islet yield after isolation and culture should exceed a minimum of 4,000 – 5,000 IEQ/kg based on the recipient's weight for each transplant. [88] To predict islet isolation outcomes, a scoring system was developed based on donor characteristics. Cause of death and characteristics such as age (between 20-50 years), BMI (>30 kg m^{-2}) and normalized HbA_{1c} levels are critical. [89-98] A single donor pancreas typically yields an islet mass of 100,000 to 300,000 IEQ per pancreas, but on occasion may rarely exceed 1,000,000 IEQ. [97] The process of islet isolation is therefore inefficient as a typical human pancreas contains between 1-2 million islets. [99] Culture conditions may further impact islet yield. In the clinical setting, islets are cultured at 20^{o}C or 37^{o}C for periods up to 72 hours. [100] This culture period is seen as beneficial for the islets because it may help reduce immunogenicity and acute inflammation, and further provides time to conduct quality control testing and an adequate period to allow the recipient to be conditioned with anti-inflammatory and immunodepleting medications. [101]

In an effort to circumvent islet cell loss post culture, **Chapter 2** focuses on the implication of supplementing islets through co-culturing together with mesenchymal stem cells. In this study, we assessed the effects of co-culturing murine islets with human adipose-derived

mesenchymal stem cells and its effect on islet cell function and engraftment. We found that co-cultured islets with Ad-MSCs (1:300 and 1:2000 islet to MSC ratio) results in higher cell yield, decreased apoptosis, and higher insulin content when compared to islets cultured alone for 48-hours. In a mouse model, we found islets co-cultured and transplanted with Ad-MSCs (1:2000) had superior blood glycemia and glucose tolerance than control islet recipients. Considering the decline islet yield post culture in the control group, the number of pancreata were increased to subsidize post culture cell loss and supports further investigation for the clinical application. Overall, our study may be clinically relevant as it questions the possibility for the use of autologous Ad-MSCs during a culture period to possibly augment islet yield during culture.

4.2.7 Immunosuppression for Islet Transplantation

Islet transplant recipients require potent chronic maintenance immunosuppression to prevent graft rejection and attempt to control recurrence of diabetic autoimmunity. The most potent antirejection drugs (tacrolimus, a calcineurin inhibitor (CNi)) have direct toxicity to beta cells. [102-104] Further, such drugs inhibit insulin section from the human islets. [105] Alternative CNi-free immunosuppression regimens have been investigated for IT, but no durable protocol has yet prevailed despite many promises to date. [106] Notably, the anti-LFA-1 inhibitor efalizumab [107] combined with an anti-CTLA4Ig antibody Belatacept [108] showed early promise of persistent islet function in the absence of long term CNi medications. Unfortunately efalizumab was withdrawn from the market because of a small risk of progressive multifocal leukoencephalopathy, and no effective replacement has been found to date. While a CNi-free regimen is desirable for islet engraftment, all medications that suppress the immune surveillance system pose potential risk for cancer or opportunistic infections. [109] Immunological tolerance seeks to induce a stable, non-responsive state to an islet or other organ allograft without need for chronic immunosuppression,

and remains a much sought after goal in IT. [110] Moving forward, the ability to utilize immune regulatory cells such as Tregs or MSCs during co-transplantation or supplemented during culture could perhaps facilitate a more tolerant-like state.

4.3 Concluding remarks

The primary goal of islet transplantation is to stabilize glycemic control in patients at high risk of severe and recurrent hypoglycemia, that cannot be stabilized by other more standard means. Insulin independence is a desirable by-product of that process but is often not sustainable long-term. Islet transplantation has been shown to improve quality of life, and markedly reduce risk of hypoglycemia. Remarkable progress has occurred in this field, as documented in the recent reports of the CITR. Today, IT is an advantageous clinical procedure for patients with brittle T1DM and is covered by public or private health care services in countries like Canada, Australia, the UK, Switzerland, Italy, France, and other parts of Europe. [89] Recently, an NIH funded phase III multicenter trial in North America has confirmed IT as a safe and effective method that lead to the FDA approval for USA. Furthermore, a completed European randomized multicenter clinical trial (NCT01148680) TRIMECO supported islet transplantation as an effective procedure to improve glycemic control for patients with severe glycemic complications. [111, 112] Compared to whole pancreas transplantation, IT is a safer procedure to render insulin independence and glycemic control. Optimistically, IT has the future potential to serve as a feasible procedure for all patients with diabetes. In order to make this a reality, several challenges must be addressed and herein we suggest two potential alternatives. First, we reveal the ability to preserve murine islet yield and function during a 48-hour co-culture with human-derived Ad-MSCs. Secondly, we demonstrate the ability to dissociate and re-aggregate human islets into pseudoislets with similar function relative to intact native islets. The ability to collaborate research techniques and findings in the research field has the high potential to feasibly acquire islet transplantation for all patients with T1DM and maintain normoglycemia and insulin independence.

4.4 References

1. Wild S, Roglic G, Green A, Sicree R, King H. Global prevalence of diabetes: estimates for the year 2000 and projections for 2030. Diabetes Care 2004; 27:1047-53.

2. Mathers CD, Loncar D. Projections of global mortality and burden of disease from 2002 to 2030. PLoS Med 2006; 3:e442.

3. Nathan DM, Group DER. The diabetes control and complications trial/epidemiology of diabetes interventions and complications study at 30 years: overview. Diabetes Care 2014; 37:9-16.

4. Keen H. The Diabetes Control and Complications Trial (DCCT). Health Trends 1994; 26:41-3.

5. The effect of intensive treatment of diabetes on the development and progression of long-term complications in insulin-dependent diabetes mellitus. The Diabetes Control and Complications Trial Research Group. N Engl J Med 1993; 329:977-86.

6. Nathan DM, Lachin J, Cleary P, Orchard T, Brillon DJ, Backlund JY, et al. Intensive diabetes therapy and carotid intima-media thickness in type 1 diabetes mellitus. N Engl J Med 2003; 348:2294-303.

7. Halban PA. Cellular sources of new pancreatic beta cells and therapeutic implications for regenerative medicine. Nat Cell Biol 2004; 6:1021-5.

8. Bonner-Weir S, Baxter LA, Schuppin GT, Smith FE. A second pathway for regeneration of adult exocrine and endocrine pancreas. A possible recapitulation of embryonic development. Diabetes 1993; 42:1715-20.

9. Vanikar AV, Trivedi HL, Thakkar UG. Stem cell therapy emerging as the key player in treating type 1 diabetes mellitus. Cytotherapy 2016; 18:1077-86.

10. Krzystyniak A, Golab K, Witkowski P, Trzonkowski P. Islet cell transplant and the incorporation of Tregs. Curr Opin Organ Transplant 2014; 19:610-5.

11. Gitelman SE, Bluestone JA. Regulatory T cell therapy for type 1 diabetes: May the force be with you. J Autoimmun 2016; 71:78-87.

12. Bluestone JA, Buckner JH, Fitch M, Gitelman SE, Gupta S, Hellerstein MK, et al. Type 1 diabetes immunotherapy using polyclonal regulatory T cells. Sci Transl Med 2015; 7:315ra189.

13. Bluestone JA, Tang Q. Immunotherapy: making the case for precision medicine. Sci Transl Med 2015; 7:280ed3.

14. Marek N, Krzystyniak A, Ergenc I, Cochet O, Misawa R, Wang LJ, et al. Coating human pancreatic islets with CD4(+)CD25(high)CD127(-) regulatory T cells as a novel approach for the local immunoprotection. Ann Surg 2011; 254:512-8; discussion 8-9.

15. Wu DC, Hester J, Nadig SN, Zhang W, Trzonkowski P, Gray D, et al. Ex vivo expanded human regulatory T cells can prolong survival of a human islet allograft in a humanized mouse model. Transplantation 2013; 96:707-16.

16. Zhang N, Schroppel B, Lal G, Jakubzick C, Mao X, Chen D, et al. Regulatory T cells sequentially migrate from inflamed tissues to draining lymph nodes to suppress the alloimmune response. Immunity 2009; 30:458-69.

17. Groth CG, Korsgren O, Tibell A, Tollemar J, Moller E, Bolinder J, et al. Transplantation of porcine fetal pancreas to diabetic patients. Lancet 1994; 344:1402-4.

18. Ekser B, Bottino R, Cooper DK. Clinical Islet Xenotransplantation: A Step Forward. EBioMedicine 2016; 12:22-3.

19. Park CG, Bottino R, Hawthorne WJ. Current status of islet xenotransplantation. Int J Surg 2015; 23:261-6.

20. Cardona K, Korbutt GS, Milas Z, Lyon J, Cano J, Jiang W, et al. Long-term survival of neonatal porcine islets in nonhuman primates by targeting costimulation pathways. Nat Med 2006; 12:304-6.

21. Hering BJ, Walawalkar N. Pig-to-nonhuman primate islet xenotransplantation. Transpl Immunol 2009; 21:81-6.

22. Hering BJ, Wijkstrom M, Graham ML, Hardstedt M, Aasheim TC, Jie T, et al. Prolonged diabetes reversal after intraportal xenotransplantation of wild-type porcine islets in immunosuppressed nonhuman primates. Nat Med 2006; 12:301-3.

23. van der Windt DJ, Bottino R, Casu A, Campanile N, Smetanka C, He J, et al. Long-term controlled normoglycemia in diabetic non-human primates after transplantation with hCD46 transgenic porcine islets. Am J Transplant 2009; 9:2716-26.

24. van der Windt DJ, Bottino R, Kumar G, Wijkstrom M, Hara H, Ezzelarab M, et al. Clinical islet xenotransplantation: how close are we? Diabetes 2012; 61:3046-55.

25. Matsumoto S, Tan P, Baker J, Durbin K, Tomiya M, Azuma K, et al. Clinical porcine islet xenotransplantation under comprehensive regulation. Transplant Proc 2014; 46:1992-5.

26. Matsumoto S, Abalovich A, Wechsler C, Wynyard S, Elliott RB. Clinical Benefit of Islet Xenotransplantation for the Treatment of Type 1 Diabetes. EBioMedicine 2016; 12:255-62.

27. Morozov VA, Wynyard S, Matsumoto S, Abalovich A, Denner J, Elliott R. No PERV transmission during a clinical trial of pig islet cell transplantation. Virus Res 2017; 227:34-40.

28. Yang L, Guell M, Niu D, George H, Lesha E, Grishin D, et al. Genome-wide inactivation of porcine endogenous retroviruses (PERVs). Science 2015; 350:1101-4.

29. Niu D, Wei HJ, Lin L, George H, Wang T, Lee IH, et al. Inactivation of porcine endogenous retrovirus in pigs using CRISPR-Cas9. Science 2017; 357:1303-7.

30. Bouwens L, Houbracken I, Mfopou JK. The use of stem cells for pancreatic regeneration in diabetes mellitus. Nat Rev Endocrinol 2013; 9:598-606.

31. Zhou Q, Brown J, Kanarek A, Rajagopal J, Melton DA. In vivo reprogramming of adult pancreatic exocrine cells to beta-cells. Nature 2008; 455:627-32.

32. Ianus A, Holz GG, Theise ND, Hussain MA. In vivo derivation of glucose-competent pancreatic endocrine cells from bone marrow without evidence of cell fusion. J Clin Invest 2003; 111:843-50.

33. Suri A, Calderon B, Esparza TJ, Frederick K, Bittner P, Unanue ER. Immunological reversal of autoimmune diabetes without hematopoietic replacement of beta cells. Science 2006; 311:1778-80.

34. Takahashi K, Yamanaka S. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell 2006; 126:663-76.

35. D'Amour KA, Bang AG, Eliazer S, Kelly OG, Agulnick AD, Smart NG, et al. Production of pancreatic hormone-expressing endocrine cells from human embryonic stem cells. Nat Biotechnol 2006; 24:1392-401.

36. Kubo A, Shinozaki K, Shannon JM, Kouskoff V, Kennedy M, Woo S, et al. Development of definitive endoderm from embryonic stem cells in culture. Development 2004; 131:1651-62.

37. Kroon E, Martinson LA, Kadoya K, Bang AG, Kelly OG, Eliazer S, et al. Pancreatic endoderm derived from human embryonic stem cells generates glucose-responsive insulin-secreting cells in vivo. Nat Biotechnol 2008; 26:443-52.

38. Bruin JE, Rezania A, Xu J, Narayan K, Fox JK, O'Neil JJ, et al. Maturation and function of human embryonic stem cell-derived pancreatic progenitors in macroencapsulation devices following transplant into mice. Diabetologia 2013; 56:1987-98.

39. Kelly OG, Chan MY, Martinson LA, Kadoya K, Ostertag TM, Ross KG, et al. Cell-surface markers for the isolation of pancreatic cell types derived from human embryonic stem cells. Nat Biotechnol 2011; 29:750-6.

40. Rezania A, Bruin JE, Riedel MJ, Mojibian M, Asadi A, Xu J, et al. Maturation of human embryonic stem cell-derived pancreatic progenitors into functional islets capable of treating pre-existing diabetes in mice. Diabetes 2012; 61:2016-29.

41. Schulz TC, Young HY, Agulnick AD, Babin MJ, Baetge EE, Bang AG, et al. A scalable system for production of functional pancreatic progenitors from human embryonic stem cells. PLoS One 2012; 7:e37004.

42. Emamaullee JA, Shapiro AM. Factors influencing the loss of beta-cell mass in islet transplantation. Cell transplantation 2007; 16:1-8.

43. Korsgren O, Lundgren T, Felldin M, Foss A, Isaksson B, Permert J, et al. Optimising islet engraftment is critical for successful clinical islet transplantation. Diabetologia 2008; 51:227-32.

44. Merani S, Toso C, Emamaullee J, Shapiro AM. Optimal implantation site for pancreatic islet transplantation. Br J Surg 2008; 95:1449-61.

45. Wang X, Meloche M, Verchere CB, Ou D, Mui A, Warnock GL. Improving islet engraftment by gene therapy. Journal of transplantation 2011; 2011:594851.

46. Komatsu H, Cook C, Wang CH, Medrano L, Lin H, Kandeel F, et al. Oxygen environment and islet size are the primary limiting factors of isolated pancreatic islet survival. PLoS One 2017; 12:e0183780.

47. MacGregor RR, Williams SJ, Tong PY, Kover K, Moore WV, Stehno-Bittel L. Small rat islets are superior to large islets in in vitro function and in transplantation outcomes. Am J Physiol Endocrinol Metab 2006; 290:E771-9.

48. Lehmann R, Zuellig RA, Kugelmeier P, Baenninger PB, Moritz W, Perren A, et al. Superiority of small islets in human islet transplantation. Diabetes 2007; 56:594-603.

49. Green AD, Vasu S, McClenaghan NH, Flatt PR. Pseudoislet formation enhances gene expression, insulin secretion and cytoprotective mechanisms of clonal human insulin-secreting 1.1B4 cells. Pflugers Arch 2015; 467:2219-28.

50. Westenfelder C, Gooch A, Hu Z, Ahlstrom J, Zhang P. Durable Control of Autoimmune Diabetes in Mice Achieved by Intraperitoneal Transplantation of "Neo-Islets," Three-Dimensional Aggregates of Allogeneic Islet and "Mesenchymal Stem Cells". Stem Cells Transl Med 2017; 6:1631-43.

51. Cavallari G, Zuellig RA, Lehmann R, Weber M, Moritz W. Rat pancreatic islet size standardization by the "hanging drop" technique. Transplant Proc 2007; 39:2018-20.

52. Zuellig RA, Cavallari G, Gerber P, Tschopp O, Spinas GA, Moritz W, et al. Improved physiological properties of gravity-enforced reassembled rat and human pancreatic pseudo-islets. J Tissue Eng Regen Med 2017; 11:109-20.

53. Mendelsohn AD, Nyitray C, Sena M, Desai TA. Size-controlled insulin-secreting cell clusters. Acta Biomater 2012; 8:4278-84.

54. Lee GH, Lee JS, Wang X, Lee SH. Bottom-Up Engineering of Well-Defined 3D Microtissues Using Microplatforms and Biomedical Applications. Adv Healthc Mater 2016; 5:56-74.

55. Pathak S, Regmi S, Gupta B, Pham TT, Yong CS, Kim JO, et al. Engineered islet cell clusters transplanted into subcutaneous space are superior to pancreatic islets in diabetes. FASEB J 2017; 31:5111-21.

56. O'Sullivan ES, Johnson AS, Omer A, Hollister-Lock J, Bonner-Weir S, Colton CK, et al. Rat islet cell aggregates are superior to islets for transplantation in microcapsules. Diabetologia 2010; 53:937-45.

57. Ramachandran K, Williams SJ, Huang HH, Novikova L, Stehno-Bittel L. Engineering islets for improved performance by optimized reaggregation in a micromold. Tissue Eng Part A 2013; 19:604-12.

58. Hauge-Evans AC, Squires PE, Persaud SJ, Jones PM. Pancreatic beta-cell-to-beta-cell interactions are required for integrated responses to nutrient stimuli: enhanced Ca2+ and insulin secretory responses of MIN6 pseudoislets. Diabetes 1999; 48:1402-8.

59. Tze WJ, Tai J. Preparation of pseudoislets for morphological and functional studies. Transplantation 1982; 34:228-31.

60. Tze WJ, Tai J. Allotransplantation and xenotransplantation of pseudoislets in diabetic rats and mice. Transplantation 1984; 38:438-40.

61. Kin T, Senior P, O'Gorman D, Richer B, Salam A, Shapiro AM. Risk factors for islet loss during culture prior to transplantation. Transpl Int 2008; 21:1029-35.

62. Ungrin MD, Joshi C, Nica A, Bauwens C, Zandstra PW. Reproducible, ultra high-throughput formation of multicellular organization from single cell suspension-derived human embryonic stem cell aggregates. PLoS One 2008; 3:e1565.

63. Kojima N. In vitro reconstitution of pancreatic islets. Organogenesis 2014; 10:225-30.

64. Brusko TM. Mesenchymal stem cells: a potential border patrol for transplanted islets? Diabetes 2009; 58:1728-9.

65. Majumdar MK, Thiede MA, Haynesworth SE, Bruder SP, Gerson SL. Human marrow-derived mesenchymal stem cells (MSCs) express hematopoietic cytokines and support long-term hematopoiesis when differentiated toward stromal and osteogenic lineages. J Hematother Stem Cell Res 2000; 9:841-8.

66. Loukogeorgakis SP, De Coppi P. Stem cells from amniotic fluid--Potential for regenerative medicine. Best Pract Res Clin Obstet Gynaecol 2016; 31:45-57.

67. Williams JT, Southerland SS, Souza J, Calcutt AF, Cartledge RG. Cells isolated from adult human skeletal muscle capable of differentiating into multiple mesodermal phenotypes. Am Surg 1999; 65:22-6.

68. Mahmoudifar N, Doran PM. Mesenchymal Stem Cells Derived from Human Adipose Tissue. Methods Mol Biol 2015; 1340:53-64.

69. Lazarus HM, Koc ON, Devine SM, Curtin P, Maziarz RT, Holland HK, et al. Cotransplantation of HLA-identical sibling culture-expanded mesenchymal stem cells and hematopoietic stem cells in hematologic malignancy patients. Biol Blood Marrow Transplant 2005; 11:389-98.

70. English K, Wood KJ. Mesenchymal stromal cells in transplantation rejection and tolerance. Cold Spring Harb Perspect Med 2013; 3:a015560.

71. Zhang Y, Li C, Jiang X, Zhang S, Wu Y, Liu B, et al. Human placenta-derived mesenchymal progenitor cells support culture expansion of long-term culture-initiating cells from cord blood CD34+ cells. Exp Hematol 2004; 32:657-64.

72. Kinnaird T, Stabile E, Burnett MS, Lee CW, Barr S, Fuchs S, et al. Marrow-derived stromal cells express genes encoding a broad spectrum of arteriogenic cytokines and promote in vitro and in vivo arteriogenesis through paracrine mechanisms. Circ Res 2004; 94:678-85.

73. Liu M, Han ZC. Mesenchymal stem cells: biology and clinical potential in type 1 diabetes therapy. J Cell Mol Med 2008; 12:1155-68.

74. Sordi V, Malosio ML, Marchesi F, Mercalli A, Melzi R, Giordano T, et al. Bone marrow mesenchymal stem cells express a restricted set of functionally active chemokine receptors capable of promoting migration to pancreatic islets. Blood 2005; 106:419-27.

75. Caplan AI, Dennis JE. Mesenchymal stem cells as trophic mediators. J Cell Biochem 2006; 98:1076-84.

76. Chen L, Tredget EE, Wu PY, Wu Y. Paracrine factors of mesenchymal stem cells recruit macrophages and endothelial lineage cells and enhance wound healing. PLoS One 2008; 3:e1886.

77. Vija L, Farge D, Gautier JF, Vexiau P, Dumitrache C, Bourgarit A, et al. Mesenchymal stem cells: Stem cell therapy perspectives for type 1 diabetes. Diabetes Metab 2009; 35:85-93.

78. Le Blanc K, Tammik L, Sundberg B, Haynesworth SE, Ringden O. Mesenchymal stem cells inhibit and stimulate mixed lymphocyte cultures and mitogenic responses independently of the major histocompatibility complex. Scand J Immunol 2003; 57:11-20.

79. Ramasamy R, Fazekasova H, Lam EW, Soeiro I, Lombardi G, Dazzi F. Mesenchymal stem cells inhibit dendritic cell differentiation and function by preventing entry into the cell cycle. Transplantation 2007; 83:71-6.

80. Healy ME, Bergin R, Mahon BP, English K. Mesenchymal stromal cells protect against caspase 3-mediated apoptosis of CD19(+) peripheral B cells through contact-dependent upregulation of VEGF. Stem Cells Dev 2015; 24:2391-402.

81. Kim YH, Wee YM, Choi MY, Lim DG, Kim SC, Han DJ. Interleukin (IL)-10 induced by CD11b(+) cells and IL-10-activated regulatory T cells play a role in immune modulation of mesenchymal stem cells in rat islet allografts. Mol Med 2011; 17:697-708.

82. English K. Mesenchymal stem cells to promote islet transplant survival. Curr Opin Organ Transplant 2016; 21:568-73.

83. Yeung TY, Seeberger KL, Kin T, Adesida A, Jomha N, Shapiro AM, et al. Human mesenchymal stem cells protect human islets from pro-inflammatory cytokines. PLoS One 2012; 7:e38189.

84. Investigators TCCCa. 9th Collaborative Islet Transplant Registry 2014 Annual Report. CITR 2016.

85. Bellin MD, Barton FB, Heitman A, Harmon JV, Kandaswamy R, Balamurugan AN, et al. Potent induction immunotherapy promotes long-term insulin independence after islet transplantation in type 1 diabetes. Am J Transplant 2012; 12:1576-83.

86. Ryan EA, Paty BW, Senior PA, Bigam D, Alfadhli E, Kneteman NM, et al. Five-year follow-up after clinical islet transplantation. Diabetes 2005; 54:2060-9.

87. Ryan EA, Paty BW, Senior PA, Lakey JR, Bigam D, Shapiro AM. Beta-score: an assessment of beta-cell function after islet transplantation. Diabetes Care 2005; 28:343-7.

88. Al-Adra DP, Gill RS, Imes S, O'Gorman D, Kin T, Axford SJ, et al. Single-donor islet transplantation and long-term insulin independence in select patients with type 1 diabetes mellitus. Transplantation 2014; 98:1007-12.

89. Shapiro AM, Pokrywczynska M, Ricordi C. Clinical pancreatic islet transplantation. Nat Rev Endocrinol 2017; 13:268-77.

90. O'Gorman D, Kin T, Murdoch T, Richer B, McGhee-Wilson D, Ryan EA, et al. The standardization of pancreatic donors for islet isolations. Transplantation 2005; 80:801-6.

91. Benhamou PY, Watt PC, Mullen Y, Ingles S, Watanabe Y, Nomura Y, et al. Human islet isolation in 104 consecutive cases. Factors affecting isolation success. Transplantation 1994; 57:1804-10.

92. Goto M, Eich TM, Felldin M, Foss A, Kallen R, Salmela K, et al. Refinement of the automated method for human islet isolation and presentation of a closed system for in vitro islet culture. Transplantation 2004; 78:1367-75.

93. Ihm SH, Matsumoto I, Sawada T, Nakano M, Zhang HJ, Ansite JD, et al. Effect of donor age on function of isolated human islets. Diabetes 2006; 55:1361-8.

94. Lakey JR, Rajotte RV, Warnock GL, Kneteman NM. Human pancreas preservation prior to islet isolation. Cold ischemic tolerance. Transplantation 1995; 59:689-94.

95. Lakey JR, Warnock GL, Rajotte RV, Suarez-Alamazor ME, Ao Z, Shapiro AM, et al. Variables in organ donors that affect the recovery of human islets of Langerhans. Transplantation 1996; 61:1047-53.

96. Hering BJ, Kandaswamy R, Harmon JV, Ansite JD, Clemmings SM, Sakai T, et al. Transplantation of cultured islets from two-layer preserved pancreases in type 1 diabetes with anti-CD3 antibody. Am J Transplant 2004; 4:390-401.

97. Nano R, Clissi B, Melzi R, Calori G, Maffi P, Antonioli B, et al. Islet isolation for allotransplantation: variables associated with successful islet yield and graft function. Diabetologia 2005; 48:906-12.

98. Zeng Y, Torre MA, Karrison T, Thistlethwaite JR. The correlation between donor characteristics and the success of human islet isolation. Transplantation 1994; 57:954-8.

99. Ionescu-Tirgoviste C, Gagniuc PA, Gubceac E, Mardare L, Popescu I, Dima S, et al. A 3D map of the islet routes throughout the healthy human pancreas. Sci Rep 2015; 5:14634.

100. Yamamoto T, Horiguchi A, Ito M, Nagata H, Ichii H, Ricordi C, et al. Quality control for clinical islet transplantation: organ procurement and preservation, the islet processing facility, isolation, and potency tests. J Hepatobiliary Pancreat Surg 2009; 16:131-6.

101. Ichii H PA, Khan A, Fraker C, Ricordi C. Culture and transportation of human islets between centers. Islet Transplantation and beta cell replacement therapy New York: Informa healthcare 2007:251

.

102. Nir T, Melton DA, Dor Y. Recovery from diabetes in mice by beta cell regeneration. J Clin Invest 2007; 117:2553-61.

103. Chand DH, Southerland SM, Cunningham RJ, 3rd. Tacrolimus: the good, the bad, and the ugly. Pediatr Transplant 2001; 5:32-6.

104. Ekberg H, Bernasconi C, Tedesco-Silva H, Vitko S, Hugo C, Demirbas A, et al. Calcineurin inhibitor minimization in the Symphony study: observational results 3 years after transplantation. Am J Transplant 2009; 9:1876-85.

105. Rangel EB. Tacrolimus in pancreas transplant: a focus on toxicity, diabetogenic effect and drug-drug interactions. Expert Opin Drug Metab Toxicol 2014; 10:1585-605.

106. Posselt AM, Szot GL, Frassetto LA, Masharani U, Tavakol M, Amin R, et al. Islet transplantation in type 1 diabetic patients using calcineurin inhibitor-free immunosuppressive

protocols based on T-cell adhesion or costimulation blockade. Transplantation 2010; 90:1595-601.

107. Turgeon NA, Avila JG, Cano JA, Hutchinson JJ, Badell IR, Page AJ, et al. Experience with a novel efalizumab-based immunosuppressive regimen to facilitate single donor islet cell transplantation. Am J Transplant 2010; 10:2082-91.

108. Vincenti F, Charpentier B, Vanrenterghem Y, Rostaing L, Bresnahan B, Darji P, et al. A phase III study of belatacept-based immunosuppression regimens versus cyclosporine in renal transplant recipients (BENEFIT study). Am J Transplant 2010; 10:535-46.

109. Penn I. Post-transplant malignancy: the role of immunosuppression. Drug Saf 2000; 23:101-13.

110. Shapiro AM, Pokrywczynska M, Ricordi C. Clinical pancreatic islet transplantation. Nat Rev Endocrinol 2016.

111. Forbes S, Senior PA, Shapiro AMJ. Islet transplantation in type 1 diabetes: moving forward. Lancet Diabetes Endocrinol 2018.

112. Lablanche S, Vantyghem MC, Kessler L, Wojtusciszyn A, Borot S, Thivolet C, et al. Islet transplantation versus insulin therapy in patients with type 1 diabetes with severe hypoglycaemia or poorly controlled glycaemia after kidney transplantation (TRIMECO): a multicentre, randomised controlled trial. Lancet Diabetes Endocrinol 2018.

GENERAL BIBLIOGRAPHY

1. The effect of intensive treatment of diabetes on the development and progression of long-term complications in insulin-dependent diabetes mellitus. The Diabetes Control and Complications Trial Research Group. N Engl J Med 1993; 329:977-86.

2. Collaborative Islet Transplant Registry. Rockville, MD, 2016.

3. Adeghate E, Donath T. Morphological findings in long-term pancreatic tissue transplants in the anterior eye chamber of rats. Pancreas 1990; 5:298-305.

4. al-Abdullah IH, Anil Kumar MS, Kelly-Sullivan D, Abouna GM. Site for unpurified islet transplantation is an important parameter for determination of the outcome of graft survival and function. Cell transplantation 1995; 4:297-305.

5. Al-Adra DP, Gill RS, Imes S, O'Gorman D, Kin T, Axford SJ, et al. Single-donor islet transplantation and long-term insulin independence in select patients with type 1 diabetes mellitus. Transplantation 2014; 98:1007-12.

6. Al-Jazaeri A, Xu BY, Yang H, Macneil D, Leventhal JR, Wright JR, Jr. Effect of glucose toxicity on intraportal tilapia islet xenotransplantation in nude mice. Xenotransplantation 2005; 12:189-96.

7. Algire GH, Weaver JM, Prehn RT. Growth of cells in vivo in diffusion chambers. I. Survival of homografts in immunized mice. J Natl Cancer Inst 1954; 15:493-507.

8. American Diabetes A. Economic costs of diabetes in the U.S. in 2012. Diabetes Care 2013; 36:1033-46.

9. Andrey P, Kieu K, Kress C, Lehmann G, Tirichine L, Liu Z, et al. Statistical analysis of 3D images detects regular spatial distributions of centromeres and chromocenters in animal and plant nuclei. PLoS Comput Biol 2010; 6:e1000853.

10. Baidal DA, Ricordi C, Berman DM, Alvarez A, Padilla N, Ciancio G, et al. Bioengineering of an Intraabdominal Endocrine Pancreas. N Engl J Med 2017; 376:1887-9.

11. Ballinger WF, Lacy PE. Transplantation of intact pancreatic islets in rats. Surgery 1972; 72:175-86.

12. Banting FG, Best CH, Collip JB, Campbell WR, Fletcher AA. Pancreatic Extracts in the Treatment of Diabetes Mellitus. Can Med Assoc J 1922; 12:141-6.

13. Barkai U, Weir GC, Colton CK, Ludwig B, Bornstein SR, Brendel MD, et al. Enhanced oxygen supply improves islet viability in a new bioartificial pancreas. Cell transplantation 2013; 22:1463-76.

14. Bellin MD, Barton FB, Heitman A, Harmon JV, Kandaswamy R, Balamurugan AN, et al. Potent induction immunotherapy promotes long-term insulin independence after islet transplantation in type 1 diabetes. Am J Transplant 2012; 12:1576-83.

15. Benhamou PY, Watt PC, Mullen Y, Ingles S, Watanabe Y, Nomura Y, et al. Human islet isolation in 104 consecutive cases. Factors affecting isolation success. Transplantation 1994; 57:1804-10.

16. Berman DM, Willman MA, Han D, Kleiner G, Kenyon NM, Cabrera O, et al. Mesenchymal stem cells enhance allogeneic islet engraftment in nonhuman primates. Diabetes 2010; 59:2558-68.

17. Berney T. Islet culture and counter-culture. Commentary on: Effect of short-term culture on functional and stress-related parameters in isolated human islets by Ihm et al. Transpl Int 2009; 22:531-3.

18. Biarnes M, Montolio M, Nacher V, Raurell M, Soler J, Montanya E. Beta-cell death and mass in syngeneically transplanted islets exposed to short- and long-term hyperglycemia. Diabetes 2002; 51:66-72.

19. Bluestone JA, Buckner JH, Fitch M, Gitelman SE, Gupta S, Hellerstein MK, et al. Type 1 diabetes immunotherapy using polyclonal regulatory T cells. Sci Transl Med 2015; 7:315ra189.

20. Bluestone JA, Tang Q. Immunotherapy: making the case for precision medicine. Sci Transl Med 2015; 7:280ed3.

21. Bonner-Weir S, Baxter LA, Schuppin GT, Smith FE. A second pathway for regeneration of adult exocrine and endocrine pancreas. A possible recapitulation of embryonic development. Diabetes 1993; 42:1715-20.

22. Bouwens L, Houbracken I, Mfopou JK. The use of stem cells for pancreatic regeneration in diabetes mellitus. Nat Rev Endocrinol 2013; 9:598-606.

23. Brauker J, Martinson LA, Young SK, Johnson RC. Local inflammatory response around diffusion chambers containing xenografts. Nonspecific destruction of tissues and decreased local vascularization. Transplantation 1996; 61:1671-7.

24. Brendel M HB, Shulz A, Bretzel R. . International Islet Transplant Registry Report. University of Giessen 1999:1–20.

25. Brissova M, Powers AC. Revascularization of transplanted islets: can it be improved? Diabetes 2008; 57:2269-71.

26. Bruin JE, Rezania A, Xu J, Narayan K, Fox JK, O'Neil JJ, et al. Maturation and function of human embryonic stem cell-derived pancreatic progenitors in macroencapsulation devices following transplant into mice. Diabetologia 2013; 56:1987-98.

27. Bruni A, Pepper AR, Gala-Lopez B, Pawlick R, Abualhassan N, Crapo JD, et al. A novel redox-active metalloporphyrin reduces reactive oxygen species and inflammatory markers but does not improve marginal mass engraftment in a murine donation after circulatory death islet transplantation model. Islets 2016; 8:e1190058.

28. Brusko TM. Mesenchymal stem cells: a potential border patrol for transplanted islets? Diabetes 2009; 58:1728-9.

29. Buchwald P. FEM-based oxygen consumption and cell viability models for avascular pancreatic islets. Theor Biol Med Model 2009; 6:5.

30. Cabrera O, Jacques-Silva MC, Berman DM, Fachado A, Echeverri F, Poo R, et al. Automated, high-throughput assays for evaluation of human pancreatic islet function. Cell transplantation 2008; 16:1039-48.

31. Cabrera SM, Rigby MR, Mirmira RG. Targeting regulatory T cells in the treatment of type 1 diabetes mellitus. Curr Mol Med 2012; 12:1261-72.

32. Calafiore R, Basta G, Luca G, Boselli C, Bufalari A, Bufalari A, et al. Transplantation of pancreatic islets contained in minimal volume microcapsules in diabetic high mammalians. Ann N Y Acad Sci 1999; 875:219-32.

33. Callewaert H, Gysemans C, Cardozo AK, Elsner M, Tiedge M, Eizirik DL, et al. Cell loss during pseudoislet formation hampers profound improvements in islet lentiviral transduction efficacy for transplantation purposes. Cell transplantation 2007; 16:527-37.

34. Caplan AI, Dennis JE. Mesenchymal stem cells as trophic mediators. J Cell Biochem 2006; 98:1076-84.

35. Cardona K, Korbutt GS, Milas Z, Lyon J, Cano J, Jiang W, et al. Long-term survival of neonatal porcine islets in nonhuman primates by targeting costimulation pathways. Nat Med 2006; 12:304-6.

36. Carlsson PO, Palm F, Mattsson G. Low revascularization of experimentally transplanted human pancreatic islets. J Clin Endocrinol Metab 2002; 87:5418-23.

37. Cavallari G, Olivi E, Bianchi F, Neri F, Foroni L, Valente S, et al. Mesenchymal stem cells and islet cotransplantation in diabetic rats: improved islet graft revascularization and function by human adipose tissue-derived stem cells preconditioned with natural molecules. Cell transplantation 2012; 21:2771-81.

38. Cavallari G, Zuellig RA, Lehmann R, Weber M, Moritz W. Rat pancreatic islet size standardization by the "hanging drop" technique. Transplant Proc 2007; 39:2018-20.

39. Chand DH, Southerland SM, Cunningham RJ, 3rd. Tacrolimus: the good, the bad, and the ugly. Pediatr Transplant 2001; 5:32-6.

40. Chang TM. Semipermeable Microcapsules. Science 1964; 146:524-5.

41. Chen L, Tredget EE, Wu PY, Wu Y. Paracrine factors of mesenchymal stem cells recruit macrophages and endothelial lineage cells and enhance wound healing. PLoS One 2008; 3:e1886.

42. Cheng K, Fraga D, Zhang C, Kotb M, Gaber AO, Guntaka RV, et al. Adenovirus-based vascular endothelial growth factor gene delivery to human pancreatic islets. Gene Ther 2004; 11:1105-16.

43. Christoffersson G, Henriksnas J, Johansson L, Rolny C, Ahlstrom H, Caballero-Corbalan J, et al. Clinical and experimental pancreatic islet transplantation to striated muscle: establishment of a vascular system similar to that in native islets. Diabetes 2010; 59:2569-78.

44. Corcione A, Benvenuto F, Ferretti E, Giunti D, Cappiello V, Cazzanti F, et al. Human mesenchymal stem cells modulate B-cell functions. Blood 2006; 107:367-72.

45. Couri CE, Oliveira MC, Stracieri AB, Moraes DA, Pieroni F, Barros GM, et al. C-peptide levels and insulin independence following autologous nonmyeloablative hematopoietic stem cell transplantation in newly diagnosed type 1 diabetes mellitus. JAMA 2009; 301:1573-9.

46. Cunha GR, Baskin L. Use of sub-renal capsule transplantation in developmental biology. Differentiation 2016; 91:4-9.

47. D'Amour KA, Bang AG, Eliazer S, Kelly OG, Agulnick AD, Smart NG, et al. Production of pancreatic hormone-expressing endocrine cells from human embryonic stem cells. Nat Biotechnol 2006; 24:1392-401.

48. De Miguel MP, Fuentes-Julian S, Blazquez-Martinez A, Pascual CY, Aller MA, Arias J, et al. Immunosuppressive properties of mesenchymal stem cells: advances and applications. Curr Mol Med 2012; 12:574-91.

49. de Souza BM, Boucas AP, Oliveira FD, Reis KP, Ziegelmann P, Bauer AC, et al. Effect of co-culture of mesenchymal stem/stromal cells with pancreatic islets on viability and function outcomes: a systematic review and meta-analysis. Islets 2017; 9:30-42.

50. Desai T, Shea LD. Advances in islet encapsulation technologies. Nat Rev Drug Discov 2016.

51. Desai T, Shea LD. Advances in islet encapsulation technologies. Nat Rev Drug Discov 2017; 16:367.

52. Ding Y, Xu D, Feng G, Bushell A, Muschel RJ, Wood KJ. Mesenchymal stem cells prevent the rejection of fully allogenic islet grafts by the immunosuppressive activity of matrix metalloproteinase-2 and -9. Diabetes 2009; 58:1797-806.

53. Dionne KE, Colton CK, Yarmush ML. Effect of hypoxia on insulin secretion by isolated rat and canine islets of Langerhans. Diabetes 1993; 42:12-21.

54. do Amaral AS, Pawlick RL, Rodrigues E, Costal F, Pepper A, Galvao FH, et al. Glutathione ethyl ester supplementation during pancreatic islet isolation improves viability and transplant outcomes in a murine marginal islet mass model. PLoS One 2013; 8:e55288.

55. Dubois S, Madec AM, Mesnier A, Armanet M, Chikh K, Berney T, et al. Glucose inhibits angiogenesis of isolated human pancreatic islets. J Mol Endocrinol 2010; 45:99-105.

56. Eich T, Eriksson O, Lundgren T, Nordic Network for Clinical Islet T. Visualization of early engraftment in clinical islet transplantation by positron-emission tomography. N Engl J Med 2007; 356:2754-5.

57. Ekberg H, Bernasconi C, Tedesco-Silva H, Vitko S, Hugo C, Demirbas A, et al. Calcineurin inhibitor minimization in the Symphony study: observational results 3 years after transplantation. Am J Transplant 2009; 9:1876-85.

58. Ekser B, Bottino R, Cooper DK. Clinical Islet Xenotransplantation: A Step Forward. EBioMedicine 2016; 12:22-3.

59. Emamaullee JA, Shapiro AM. Factors influencing the loss of beta-cell mass in islet transplantation. Cell transplantation 2007; 16:1-8.

60. English K. Mesenchymal stem cells to promote islet transplant survival. Curr Opin Organ Transplant 2016; 21:568-73.

61. English K, Wood KJ. Mesenchymal stromal cells in transplantation rejection and tolerance. Cold Spring Harb Perspect Med 2013; 3:a015560.

62. Ferguson J, Scothorne RJ. Extended survival of pancreatic islet allografts in the testis of guinea-pigs. J Anat 1977; 124:1-8.

63. Figliuzzi M, Bonandrini B, Silvani S, Remuzzi A. Mesenchymal stem cells help pancreatic islet transplantation to control type 1 diabetes. World J Stem Cells 2014; 6:163-72.

64. Fiorina P, Shapiro AM, Ricordi C, Secchi A. The clinical impact of islet transplantation. Am J Transplant 2008; 8:1990-7.

65. Forbes S, Senior PA, Shapiro AMJ. Islet transplantation in type 1 diabetes: moving forward. Lancet Diabetes Endocrinol 2018.

66. Foty RA, Pfleger CM, Forgacs G, Steinberg MS. Surface tensions of embryonic tissues predict their mutual envelopment behavior. Development 1996; 122:1611-20.

67. Fraker C, Montelongo J, Szust J, Khan A, Ricordi C. The use of multiparametric monitoring during islet cell isolation and culture: a potential tool for in-process corrections of critical physiological factors. Cell transplantation 2004; 13:497-502.

68. Friedenstein AJ, Piatetzky S, II, Petrakova KV. Osteogenesis in transplants of bone marrow cells. J Embryol Exp Morphol 1966; 16:381-90.

69. Fu L, Wei N, Wang JS, Wu L, Wang YN, Huang DY, et al. [The clinical characteristics of adult hemophagocytic lymphohistiocytosis treated with haploidentical donor hematopoietic stem cell transplantation]. Zhonghua Nei Ke Za Zhi 2017; 56:273-8.

70. Fung J, Wong T, Chok K, Chan A, Cheung TT, Dai J, et al. Long Term Outcomes of Entecavir Monotherapy for Chronic Hepatitis B after Liver Transplantation: Results up to 8 years. Hepatology 2017.

71. Gala-Lopez B. L. PAR, Dinyari P., Malcolm A. J., Kin T., Pawlick L. R., Senior P. A., and Shapiro A.M. J. Subcutaneous clinical islet transplantation in a prevascularized subcutaneous pouch – preliminary experience. CellR4 2016; 4:e2132.

72. Gala-Lopez BL, Pepper AR, Pawlick RL, O'Gorman D, Kin T, Bruni A, et al. Antiaging Glycopeptide Protects Human Islets Against Tacrolimus-Related Injury and Facilitates Engraftment in Mice. Diabetes 2016; 65:451-62.

73. Gan SU, Notaridou M, Fu ZY, Lee KO, Sia KC, Nathwani AC, et al. Correction of Murine Diabetic Hyperglycaemia With A Single Systemic Administration of An AAV2/8 Vector Containing A Novel Codon Optimized Human Insulin Gene. Curr Gene Ther 2016; 16:65-72.

74. Gitelman SE, Bluestone JA. Regulatory T cell therapy for type 1 diabetes: May the force be with you. J Autoimmun 2016; 71:78-87.

75. Golocheikine A, Tiriveedhi V, Angaswamy N, Benshoff N, Sabarinathan R, Mohanakumar T. Cooperative signaling for angiogenesis and neovascularization by VEGF and HGF following islet transplantation. Transplantation 2010; 90:725-31.

76. Goto M, Eich TM, Felldin M, Foss A, Kallen R, Salmela K, et al. Refinement of the automated method for human islet isolation and presentation of a closed system for in vitro islet culture. Transplantation 2004; 78:1367-75.

77. Green AD, Vasu S, Flatt PR. Functionality and antidiabetic utility of beta- and L-cell containing pseudoislets. Exp Cell Res 2016; 344:201-9.

78. Green AD, Vasu S, Flatt PR. Cellular models for beta-cell function and diabetes gene therapy. Acta Physiol (Oxf) 2018; 222.

79. Green AD, Vasu S, McClenaghan NH, Flatt PR. Pseudoislet formation enhances gene expression, insulin secretion and cytoprotective mechanisms of clonal human insulin-secreting 1.1B4 cells. Pflugers Arch 2015; 467:2219-28.

80. Green AD, Vasu S, McClenaghan NH, Flatt PR. Implanting 1.1B4 human beta-cell pseudoislets improves glycaemic control in diabetic severe combined immune deficient mice. World J Diabetes 2016; 7:523-33.

81. Groth CG, Korsgren O, Tibell A, Tollemar J, Moller E, Bolinder J, et al. Transplantation of porcine fetal pancreas to diabetic patients. Lancet 1994; 344:1402-4.

82. Gruber R, Kandler B, Holzmann P, Vogele-Kadletz M, Losert U, Fischer MB, et al. Bone marrow stromal cells can provide a local environment that favors migration and formation of tubular structures of endothelial cells. Tissue Eng 2005; 11:896-903.

83. Grzenkowicz-Wydra J, Cisowski J, Nakonieczna J, Zarebski A, Udilova N, Nohl H, et al. Gene transfer of CuZn superoxide dismutase enhances the synthesis of vascular endothelial growth factor. Mol Cell Biochem 2004; 264:169-81.

84. Guariguata L, Whiting DR, Hambleton I, Beagley J, Linnenkamp U, Shaw JE. Global estimates of diabetes prevalence for 2013 and projections for 2035. Diabetes Res Clin Pract 2014; 103:137-49.

85. Guo-Parke H, McCluskey JT, Kelly C, Hamid M, McClenaghan NH, Flatt PR. Configuration of electrofusion-derived human insulin-secreting cell line as pseudoislets enhances functionality and therapeutic utility. J Endocrinol 2012; 214:257-65.

86. Halban PA. Cellular sources of new pancreatic beta cells and therapeutic implications for regenerative medicine. Nat Cell Biol 2004; 6:1021-5.

87. Halban PA, Wollheim CB, Blondel B, Meda P, Niesor EN, Mintz DH. The possible importance of contact between pancreatic islet cells for the control of insulin release. Endocrinology 1982; 111:86-94.

88. Haller MJ, Viener HL, Wasserfall C, Brusko T, Atkinson MA, Schatz DA. Autologous umbilical cord blood infusion for type 1 diabetes. Exp Hematol 2008; 36:710-5.

89. Harjutsalo V, Sjoberg L, Tuomilehto J. Time trends in the incidence of type 1 diabetes in Finnish children: a cohort study. Lancet 2008; 371:1777-82.

90. Hauge-Evans AC, Squires PE, Persaud SJ, Jones PM. Pancreatic beta-cell-to-beta-cell interactions are required for integrated responses to nutrient stimuli: enhanced Ca2+ and insulin secretory responses of MIN6 pseudoislets. Diabetes 1999; 48:1402-8.

91. Haynesworth SE, Baber MA, Caplan AI. Cytokine expression by human marrow-derived mesenchymal progenitor cells in vitro: effects of dexamethasone and IL-1 alpha. J Cell Physiol 1996; 166:585-92.

92. Hayward JA, Ellis CE, Seeberger K, Lee T, Salama B, Mulet-Sierra A, et al. Cotransplantation of Mesenchymal Stem Cells With Neonatal Porcine Islets Improve Graft Function in Diabetic Mice. Diabetes 2017; 66:1312-21.

93. He Y, Zhang D, Zeng Y, Ma J, Wang J, Guo H, et al. Bone Marrow-Derived Mesenchymal Stem Cells Protect Islet Grafts Against Endoplasmic Reticulum Stress-Induced Apoptosis During the Early Stage After Transplantation. Stem Cells 2018.

94. Healy ME, Bergin R, Mahon BP, English K. Mesenchymal stromal cells protect against caspase 3-mediated apoptosis of CD19(+) peripheral B cells through contact-dependent upregulation of VEGF. Stem Cells Dev 2015; 24:2391-402.

95. Hematti P, Kim J, Stein AP, Kaufman D. Potential role of mesenchymal stromal cells in pancreatic islet transplantation. Transplant Rev (Orlando) 2013; 27:21-9.

96. Hering BJ, Clarke WR, Bridges ND, Eggerman TL, Alejandro R, Bellin MD, et al. Phase 3 Trial of Transplantation of Human Islets in Type 1 Diabetes Complicated by Severe Hypoglycemia. Diabetes Care 2016; 39:1230-40.

97. Hering BJ, Kandaswamy R, Ansite JD, Eckman PM, Nakano M, Sawada T, et al. Single-donor, marginal-dose islet transplantation in patients with type 1 diabetes. JAMA 2005; 293:830-5.

98. Hering BJ, Kandaswamy R, Harmon JV, Ansite JD, Clemmings SM, Sakai T, et al. Transplantation of cultured islets from two-layer preserved pancreases in type 1 diabetes with anti-CD3 antibody. Am J Transplant 2004; 4:390-401.

99. Hering BJ, Walawalkar N. Pig-to-nonhuman primate islet xenotransplantation. Transpl Immunol 2009; 21:81-6.

100. Hering BJ, Wijkstrom M, Graham ML, Hardstedt M, Aasheim TC, Jie T, et al. Prolonged diabetes reversal after intraportal xenotransplantation of wild-type porcine islets in immunosuppressed nonhuman primates. Nat Med 2006; 12:301-3.

101. Hex N, Bartlett C, Wright D, Taylor M, Varley D. Estimating the current and future costs of Type 1 and Type 2 diabetes in the UK, including direct health costs and indirect societal and productivity costs. Diabet Med 2012; 29:855-62.

102. Hori Y, Rulifson IC, Tsai BC, Heit JJ, Cahoy JD, Kim SK. Growth inhibitors promote differentiation of insulin-producing tissue from embryonic stem cells. Proc Natl Acad Sci U S A 2002; 99:16105-10.

103. Ianus A, Holz GG, Theise ND, Hussain MA. In vivo derivation of glucose-competent pancreatic endocrine cells from bone marrow without evidence of cell fusion. J Clin Invest 2003; 111:843-50.

104. Ichii H PA, Khan A, Fraker C, Ricordi C. Culture and transportation of human islets between centers. Islet Transplantation and beta cell replacement therapy New York: Informa healthcare 2007:251

.

105. IDP IIDP. Human Islets for Research > Investigators > Policies & Standard Operating Procedures. https://iidpcohorg/Investigators/Policies-Standard-Operating-Procedures; Accessed 5 Dec 2017.

106. Ihm SH, Matsumoto I, Sawada T, Nakano M, Zhang HJ, Ansite JD, et al. Effect of donor age on function of isolated human islets. Diabetes 2006; 55:1361-8.

107. Imamura H, Adachi T, Kin T, Ono S, Sakai Y, Adachi T, et al. An engineered cell sheet composed of human islets and human fibroblast, bone marrow-derived mesenchymal stem cells, or adipose-derived mesenchymal stem cells: An in vitro comparison study. Islets 2018:1-11.

108. Investigators TCCCa. 9th Collaborative Islet Transplant Registry 2014 Annual Report. CITR 2016.

109. Ionescu-Tirgoviste C, Gagniuc PA, Gubceac E, Mardare L, Popescu I, Dima S, et al. A 3D map of the islet routes throughout the healthy human pancreas. Sci Rep 2015; 5:14634.

110. Ito T, Itakura S, Todorov I, Rawson J, Asari S, Shintaku J, et al. Mesenchymal stem cell and islet co-transplantation promotes graft revascularization and function. Transplantation 2010; 89:1438-45.

111. J C. Mesenchymal Stem Cells Promote Islet Survival In Vitro and Function In Vivo. CellR4 2013; 1:382.

112. Johansson H, Goto M, Dufrane D, Siegbahn A, Elgue G, Gianello P, et al. Low molecular weight dextran sulfate: a strong candidate drug to block IBMIR in clinical islet transplantation. Am J Transplant 2006; 6:305-12.

113. Johansson U, Rasmusson I, Niclou SP, Forslund N, Gustavsson L, Nilsson B, et al. Formation of composite endothelial cell-mesenchymal stem cell islets: a novel approach to promote islet revascularization. Diabetes 2008; 57:2393-401.

114. Johnson AS FR, Weir GC, Colton CK. Oxygen consumption and diffusion in assemblages of respiring spheres: Performance enhancement of a bioartificial pancreas. . Chem Eng Sci 2009; 64.

115. Jung EJ, Kim SC, Wee YM, Kim YH, Choi MY, Jeong SH, et al. Bone marrow-derived mesenchymal stromal cells support rat pancreatic islet survival and insulin secretory function in vitro. Cytotherapy 2011; 13:19-29.

116. Kanak MA, Takita M, Kunnathodi F, Lawrence MC, Levy MF, Naziruddin B. Inflammatory response in islet transplantation. Int J Endocrinol 2014; 2014:451035.

117. Karaoz E, Genc ZS, Demircan PC, Aksoy A, Duruksu G. Protection of rat pancreatic islet function and viability by coculture with rat bone marrow-derived mesenchymal stem cells. Cell Death Dis 2010; 1:e36.

118. Kawahara T, Kin T, Kashkoush S, Gala-Lopez B, Bigam DL, Kneteman NM, et al. Portal vein thrombosis is a potentially preventable complication in clinical islet transplantation. Am J Transplant 2011; 11:2700-7.

119. Keen H. The Diabetes Control and Complications Trial (DCCT). Health Trends 1994; 26:41-3.

120. Kelly OG, Chan MY, Martinson LA, Kadoya K, Ostertag TM, Ross KG, et al. Cell-surface markers for the isolation of pancreatic cell types derived from human embryonic stem cells. Nat Biotechnol 2011; 29:750-6.

121. Kilimnik G, Jo J, Periwal V, Zielinski MC, Hara M. Quantification of islet size and architecture. Islets 2012; 4:167-72.

122. Kim YH, Wee YM, Choi MY, Lim DG, Kim SC, Han DJ. Interleukin (IL)-10 induced by CD11b(+) cells and IL-10-activated regulatory T cells play a role in immune modulation of mesenchymal stem cells in rat islet allografts. Mol Med 2011; 17:697-708.

123. Kin T, Korbutt GS, Rajotte RV. Survival and metabolic function of syngeneic rat islet grafts transplanted in the omental pouch. Am J Transplant 2003; 3:281-5.

124. Kin T, Senior P, O'Gorman D, Richer B, Salam A, Shapiro AM. Risk factors for islet loss during culture prior to transplantation. Transpl Int 2008; 21:1029-35.

125. Kinnaird T, Stabile E, Burnett MS, Lee CW, Barr S, Fuchs S, et al. Marrow-derived stromal cells express genes encoding a broad spectrum of arteriogenic cytokines and promote in vitro and in vivo arteriogenesis through paracrine mechanisms. Circ Res 2004; 94:678-85.

126. Koh A, Senior P, Salam A, Kin T, Imes S, Dinyari P, et al. Insulin-heparin infusions peritransplant substantially improve single-donor clinical islet transplant success. Transplantation 2010; 89:465-71.

127. Kojima N. In vitro reconstitution of pancreatic islets. Organogenesis 2014; 10:225-30.

128. Komatsu H, Cook C, Wang CH, Medrano L, Lin H, Kandeel F, et al. Oxygen environment and islet size are the primary limiting factors of isolated pancreatic islet survival. PLoS One 2017; 12:e0183780.

129. Korsgren O, Lundgren T, Felldin M, Foss A, Isaksson B, Permert J, et al. Optimising islet engraftment is critical for successful clinical islet transplantation. Diabetologia 2008; 51:227-32.

130. Kroon E, Martinson LA, Kadoya K, Bang AG, Kelly OG, Eliazer S, et al. Pancreatic endoderm derived from human embryonic stem cells generates glucose-responsive insulin-secreting cells in vivo. Nat Biotechnol 2008; 26:443-52.

131. Krzystyniak A, Golab K, Witkowski P, Trzonkowski P. Islet cell transplant and the incorporation of Tregs. Curr Opin Organ Transplant 2014; 19:610-5.

132. Kubo A, Shinozaki K, Shannon JM, Kouskoff V, Kennedy M, Woo S, et al. Development of definitive endoderm from embryonic stem cells in culture. Development 2004; 131:1651-62.

133. Kumagai-Braesch M, Jacobson S, Mori H, Jia X, Takahashi T, Wernerson A, et al. The TheraCyte device protects against islet allograft rejection in immunized hosts. Cell transplantation 2013; 22:1137-46.

134. L. G-LB. The metalloporphyrin BMX-010 in human islet isolation and clinical transplantation. CellR4 2016; 4:2066.

135. Lablanche S, Vantyghem MC, Kessler L, Wojtusciszyn A, Borot S, Thivolet C, et al. Islet transplantation versus insulin therapy in patients with type 1 diabetes with severe hypoglycaemia or poorly controlled glycaemia after kidney transplantation (TRIMECO): a multicentre, randomised controlled trial. Lancet Diabetes Endocrinol 2018.

136. Lakey JR, Rajotte RV, Warnock GL, Kneteman NM. Human pancreas preservation prior to islet isolation. Cold ischemic tolerance. Transplantation 1995; 59:689-94.

137. Lakey JR, Warnock GL, Rajotte RV, Suarez-Alamazor ME, Ao Z, Shapiro AM, et al. Variables in organ donors that affect the recovery of human islets of Langerhans. Transplantation 1996; 61:1047-53.

138. Lau J, Carlsson PO. Low revascularization of human islets when experimentally transplanted into the liver. Transplantation 2009; 87:322-5.

139. Lazarus HM, Koc ON, Devine SM, Curtin P, Maziarz RT, Holland HK, et al. Cotransplantation of HLA-identical sibling culture-expanded mesenchymal stem cells and hematopoietic stem cells in hematologic malignancy patients. Biol Blood Marrow Transplant 2005; 11:389-98.

140. Le Blanc K, Tammik L, Sundberg B, Haynesworth SE, Ringden O. Mesenchymal stem cells inhibit and stimulate mixed lymphocyte cultures and mitogenic responses independently of the major histocompatibility complex. Scand J Immunol 2003; 57:11-20.

141. Lee GH, Lee JS, Wang X, Lee SH. Bottom-Up Engineering of Well-Defined 3D Microtissues Using Microplatforms and Biomedical Applications. Adv Healthc Mater 2016; 5:56-74.

142. Lehmann R, Zuellig RA, Kugelmeier P, Baenninger PB, Moritz W, Perren A, et al. Superiority of small islets in human islet transplantation. Diabetes 2007; 56:594-603.

143. Li FR, Wang XG, Deng CY, Qi H, Ren LL, Zhou HX. Immune modulation of co-transplantation mesenchymal stem cells with islet on T and dendritic cells. Clin Exp Immunol 2010; 161:357-63.

144. Li RH. Materials for immunoisolated cell transplantation. Adv Drug Deliv Rev 1998; 33:87-109.

145. Lim FS, A. M. . Microencapsulated islets as bioartificial endocrine pancreas. Science 1980; 210:908–10.

146. Lin G, Wang G, Liu G, Yang LJ, Chang LJ, Lue TF, et al. Treatment of type 1 diabetes with adipose tissue-derived stem cells expressing pancreatic duodenal homeobox 1. Stem Cells Dev 2009; 18:1399-406.

147. Linn T, Schmitz J, Hauck-Schmalenberger I, Lai Y, Bretzel RG, Brandhorst H, et al. Ischaemia is linked to inflammation and induction of angiogenesis in pancreatic islets. Clin Exp Immunol 2006; 144:179-87.

148. Lione L, Puddu A, Pedemonte A, Viviani GL. Rat serum improves rat pseudoislet formation and insulin gene expression. Boll Soc Ital Biol Sper 2001; 77:13-9.

149. Liu M, Han ZC. Mesenchymal stem cells: biology and clinical potential in type 1 diabetes therapy. J Cell Mol Med 2008; 12:1155-68.

150. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 2001; 25:402-8.

151. Loukogeorgakis SP, De Coppi P. Stem cells from amniotic fluid--Potential for regenerative medicine. Best Pract Res Clin Obstet Gynaecol 2016; 31:45-57.

152. Ludwig B, Reichel A, Steffen A, Zimerman B, Schally AV, Block NL, et al. Transplantation of human islets without immunosuppression. Proc Natl Acad Sci U S A 2013; 110:19054-8.

153. Lumelsky N, Blondel O, Laeng P, Velasco I, Ravin R, McKay R. Differentiation of embryonic stem cells to insulin-secreting structures similar to pancreatic islets. Science 2001; 292:1389-94.

154. MacGregor RR, Williams SJ, Tong PY, Kover K, Moore WV, Stehno-Bittel L. Small rat islets are superior to large islets in in vitro function and in transplantation outcomes. Am J Physiol Endocrinol Metab 2006; 290:E771-9.

155. Mahmoudifar N, Doran PM. Mesenchymal Stem Cells Derived from Human Adipose Tissue. Methods Mol Biol 2015; 1340:53-64.

156. Majumdar MK, Thiede MA, Haynesworth SE, Bruder SP, Gerson SL. Human marrow-derived mesenchymal stem cells (MSCs) express hematopoietic cytokines and support long-term hematopoiesis when differentiated toward stromal and osteogenic lineages. J Hematother Stem Cell Res 2000; 9:841-8.

157. Malmegrim KC, de Azevedo JT, Arruda LC, Abreu JR, Couri CE, de Oliveira GL, et al. Immunological Balance Is Associated with Clinical Outcome after Autologous Hematopoietic Stem Cell Transplantation in Type 1 Diabetes. Front Immunol 2017; 8:167.

158. Marek N, Krzystyniak A, Ergenc I, Cochet O, Misawa R, Wang LJ, et al. Coating human pancreatic islets with CD4(+)CD25(high)CD127(-) regulatory T cells as a novel approach for the local immunoprotection. Ann Surg 2011; 254:512-8; discussion 8-9.

159. Mathers CD, Loncar D. Projections of global mortality and burden of disease from 2002 to 2030. PLoS Med 2006; 3:e442.

160. Matsumoto S, Abalovich A, Wechsler C, Wynyard S, Elliott RB. Clinical Benefit of Islet Xenotransplantation for the Treatment of Type 1 Diabetes. EBioMedicine 2016; 12:255-62.

161. Matsumoto S, Tan P, Baker J, Durbin K, Tomiya M, Azuma K, et al. Clinical porcine islet xenotransplantation under comprehensive regulation. Transplant Proc 2014; 46:1992-5.

162. Mattsson G, Jansson L, Carlsson PO. Decreased vascular density in mouse pancreatic islets after transplantation. Diabetes 2002; 51:1362-6.

163. Mauda-Havakuk M, Litichever N, Chernichovski E, Nakar O, Winkler E, Mazkereth R, et al. Ectopic PDX-1 expression directly reprograms human keratinocytes along pancreatic insulin-producing cells fate. PLoS One 2011; 6:e26298.

164. McCall M, Shapiro AM. Update on islet transplantation. Cold Spring Harb Perspect Med 2012; 2:a007823.

165. Mendelsohn AD, Nyitray C, Sena M, Desai TA. Size-controlled insulin-secreting cell clusters. Acta Biomater 2012; 8:4278-84.

166. Merani S, Toso C, Emamaullee J, Shapiro AM. Optimal implantation site for pancreatic islet transplantation. Br J Surg 2008; 95:1449-61.

167. Miller RG, Secrest AM, Sharma RK, Songer TJ, Orchard TJ. Improvements in the life expectancy of type 1 diabetes: the Pittsburgh Epidemiology of Diabetes Complications study cohort. Diabetes 2012; 61:2987-92.

168. Morozov VA, Wynyard S, Matsumoto S, Abalovich A, Denner J, Elliott R. No PERV transmission during a clinical trial of pig islet cell transplantation. Virus Res 2017; 227:34-40.

169. Najarian JS, Sutherland DE, Baumgartner D, Burke B, Rynasiewicz JJ, Matas AJ, et al. Total or near total pancreatectomy and islet autotransplantation for treatment of chronic pancreatitis. Ann Surg 1980; 192:526-42.

170. Nano R, Clissi B, Melzi R, Calori G, Maffi P, Antonioli B, et al. Islet isolation for allotransplantation: variables associated with successful islet yield and graft function. Diabetologia 2005; 48:906-12.

171. Nathan DM, Group DER. The diabetes control and complications trial/epidemiology of diabetes interventions and complications study at 30 years: overview. Diabetes Care 2014; 37:9-16.

172. Nathan DM, Lachin J, Cleary P, Orchard T, Brillon DJ, Backlund JY, et al. Intensive diabetes therapy and carotid intima-media thickness in type 1 diabetes mellitus. N Engl J Med 2003; 348:2294-303.

173. Nilsson B, Ekdahl KN, Korsgren O. Control of instant blood-mediated inflammatory reaction to improve islets of Langerhans engraftment. Curr Opin Organ Transplant 2011; 16:620-6.

174. Nir T, Melton DA, Dor Y. Recovery from diabetes in mice by beta cell regeneration. J Clin Invest 2007; 117:2553-61.

175. Niu D, Wei HJ, Lin L, George H, Wang T, Lee IH, et al. Inactivation of porcine endogenous retrovirus in pigs using CRISPR-Cas9. Science 2017; 357:1303-7.

176. Noguchi H, Miyagi-Shiohira C, Kurima K, Kobayashi N, Saitoh I, Watanabe M, et al. Islet Culture/Preservation Before Islet Transplantation. Cell Med 2015; 8:25-9.

177. Nyqvist D, Kohler M, Wahlstedt H, Berggren PO. Donor islet endothelial cells participate in formation of functional vessels within pancreatic islet grafts. Diabetes 2005; 54:2287-93.

178. O'Gorman D, Kin T, Murdoch T, Richer B, McGhee-Wilson D, Ryan EA, et al. The standardization of pancreatic donors for islet isolations. Transplantation 2005; 80:801-6.

179. O'Sullivan ES, Johnson AS, Omer A, Hollister-Lock J, Bonner-Weir S, Colton CK, et al. Rat islet cell aggregates are superior to islets for transplantation in microcapsules. Diabetologia 2010; 53:937-45.

180. Oetjen E, Baun D, Beimesche S, Krause D, Cierny I, Blume R, et al. Inhibition of human insulin gene transcription by the immunosuppressive drugs cyclosporin A and tacrolimus in primary, mature islets of transgenic mice. Mol Pharmacol 2003; 63:1289-95.

181. Ollion J, Cochennec J, Loll F, Escude C, Boudier T. TANGO: a generic tool for high-throughput 3D image analysis for studying nuclear organization. Bioinformatics 2013; 29:1840-1.

182. Olsson R, Olerud J, Pettersson U, Carlsson PO. Increased numbers of low-oxygenated pancreatic islets after intraportal islet transplantation. Diabetes 2011; 60:2350-3.

183. Pagliuca FW, Millman JR, Gurtler M, Segel M, Van Dervort A, Ryu JH, et al. Generation of functional human pancreatic beta cells in vitro. Cell 2014; 159:428-39.

184. Parekkadan B, Tilles AW, Yarmush ML. Bone marrow-derived mesenchymal stem cells ameliorate autoimmune enteropathy independently of regulatory T cells. Stem Cells 2008; 26:1913-9.

185. Park CG, Bottino R, Hawthorne WJ. Current status of islet xenotransplantation. Int J Surg 2015; 23:261-6.

186. Park KS, Kim YS, Kim JH, Choi B, Kim SH, Tan AH, et al. Trophic molecules derived from human mesenchymal stem cells enhance survival, function, and angiogenesis of isolated islets after transplantation. Transplantation 2010; 89:509-17.

187. Park KS, Shim EY, Choi BK, Moon C, Kim SH, Kim YS, et al. Cartilage oligomeric matrix protein-angiopoientin-1 enhances angiogenesis of isolated islet and maintains normoglycemia following transplantation. Transplant Proc 2010; 42:2653-7.

188. Pathak S, Regmi S, Gupta B, Pham TT, Yong CS, Kim JO, et al. Engineered islet cell clusters transplanted into subcutaneous space are superior to pancreatic islets in diabetes. FASEB J 2017; 31:5111-21.

189. Patterson CC, Dahlquist GG, Gyurus E, Green A, Soltesz G, Group ES. Incidence trends for childhood type 1 diabetes in Europe during 1989-2003 and predicted new cases 2005-20: a multicentre prospective registration study. Lancet 2009; 373:2027-33.

190. Penn I. Post-transplant malignancy: the role of immunosuppression. Drug Saf 2000; 23:101-13.

191. Pepper AR, Gala-Lopez B, Pawlick R, Merani S, Kin T, Shapiro AM. A prevascularized subcutaneous device-less site for islet and cellular transplantation. Nat Biotechnol 2015; 33:518-23.

192. Pepper AR, Pawlick R, Bruni A, Gala-Lopez B, Wink J, Rafiei Y, et al. Harnessing the Foreign Body Reaction in Marginal Mass Device-less Subcutaneous Islet Transplantation in Mice. Transplantation 2016; 100:1474-9.

193. Pepper AR, Pawlick R, Bruni A, Wink J, Rafiei Y, O'Gorman D, et al. Transplantation of Human Pancreatic Endoderm Cells Reverses Diabetes Post Transplantation in a Prevascularized Subcutaneous Site. Stem Cell Reports 2017; 8:1689-700.

194. Pepper AR, Pawlick R, Gala-Lopez B, MacGillivary A, Mazzuca DM, White DJ, et al. Diabetes Is Reversed in a Murine Model by Marginal Mass Syngeneic Islet Transplantation Using a Subcutaneous Cell Pouch Device. Transplantation 2015; 99:2294-300.

195. Perez-Basterrechea M, Esteban MM, Alvarez-Viejo M, Fontanil T, Cal S, Sanchez Pitiot M, et al. Fibroblasts accelerate islet revascularization and improve long-term graft survival in a mouse model of subcutaneous islet transplantation. PLoS One 2017; 12:e0180695.

196. Persaud SJ, Arden C, Bergsten P, Bone AJ, Brown J, Dunmore S, et al. Pseudoislets as primary islet replacements for research: report on a symposium at King's College London, London UK. Islets 2010; 2:236-9.

197. Phelps CJ, Koike C, Vaught TD, Boone J, Wells KD, Chen SH, et al. Production of alpha 1,3-galactosyltransferase-deficient pigs. Science 2003; 299:411-4.

198. Phinney DG, Prockop DJ. Concise review: mesenchymal stem/multipotent stromal cells: the state of transdifferentiation and modes of tissue repair--current views. Stem Cells 2007; 25:2896-902.

199. Posselt AM, Szot GL, Frassetto LA, Masharani U, Tavakol M, Amin R, et al. Islet transplantation in type 1 diabetic patients using calcineurin inhibitor-free immunosuppressive protocols based on T-cell adhesion or costimulation blockade. Transplantation 2010; 90:1595-601.

200. Rackham CL, Dhadda PK, Chagastelles PC, Simpson SJ, Dattani AA, Bowe JE, et al. Pre-culturing islets with mesenchymal stromal cells using a direct contact configuration is beneficial for transplantation outcome in diabetic mice. Cytotherapy 2013; 15:449-59.

201. Rackham CL, Dhadda PK, Le Lay AM, King AJ, Jones PM. Preculturing Islets With Adipose-Derived Mesenchymal Stromal Cells Is an Effective Strategy for Improving Transplantation Efficiency at the Clinically Preferred Intraportal Site. Cell Med 2014; 7:37-47.

202. Rackham CL, Dhadda PK, Simpson SJS, Godazgar M, King AJF, Jones PM. Composite Mesenchymal Stromal Cell Islets: Implications for Transplantation via the Clinically Preferred Intraportal Route. Transplant Direct 2018; 4:e354.

203. Rackham CL, Vargas AE, Hawkes RG, Amisten S, Persaud SJ, Austin AL, et al. Annexin A1 Is a Key Modulator of Mesenchymal Stromal Cell-Mediated Improvements in Islet Function. Diabetes 2016; 65:129-39.

204. Rafael E, Tibell A, Ryden M, Lundgren T, Savendahl L, Borgstrom B, et al. Intramuscular autotransplantation of pancreatic islets in a 7-year-old child: a 2-year follow-up. Am J Transplant 2008; 8:458-62.

205. Ramachandran K, Williams SJ, Huang HH, Novikova L, Stehno-Bittel L. Engineering islets for improved performance by optimized reaggregation in a micromold. Tissue Eng Part A 2013; 19:604-12.

206. Ramasamy R, Fazekasova H, Lam EW, Soeiro I, Lombardi G, Dazzi F. Mesenchymal stem cells inhibit dendritic cell differentiation and function by preventing entry into the cell cycle. Transplantation 2007; 83:71-6.

207. Ramkumar KM, Sekar TV, Bhakkiyalakshmi E, Foygel K, Rajaguru P, Berger F, et al. The impact of oxidative stress on islet transplantation and monitoring the graft survival by non-invasive imaging. Curr Med Chem 2013; 20:1127-46.

208. Rangel EB. Tacrolimus in pancreas transplant: a focus on toxicity, diabetogenic effect and drug-drug interactions. Expert Opin Drug Metab Toxicol 2014; 10:1585-605.

209. Reichart B, Niemann H, Chavakis T, Denner J, Jaeckel E, Ludwig B, et al. Xenotransplantation of porcine islet cells as a potential option for the treatment of type 1 diabetes in the future. Horm Metab Res 2015; 47:31-5.

210. Rezania A, Bruin JE, Arora P, Rubin A, Batushansky I, Asadi A, et al. Reversal of diabetes with insulin-producing cells derived in vitro from human pluripotent stem cells. Nat Biotechnol 2014; 32:1121-33.

211. Rezania A, Bruin JE, Riedel MJ, Mojibian M, Asadi A, Xu J, et al. Maturation of human embryonic stem cell-derived pancreatic progenitors into functional islets capable of treating pre-existing diabetes in mice. Diabetes 2012; 61:2016-29.

212. Ricordi C, Lacy PE, Scharp DW. Automated islet isolation from human pancreas. Diabetes 1989; 38 Suppl 1:140-2.

213. Rostambeigi N, Lanza IR, Dzeja PP, Deeds MC, Irving BA, Reddi HV, et al. Unique cellular and mitochondrial defects mediate FK506-induced islet beta-cell dysfunction. Transplantation 2011; 91:615-23.

214. Russ HA, Parent AV, Ringler JJ, Hennings TG, Nair GG, Shveygert M, et al. Controlled induction of human pancreatic progenitors produces functional beta-like cells in vitro. EMBO J 2015; 34:1759-72.

215. Ryan EA, Lakey JR, Paty BW, Imes S, Korbutt GS, Kneteman NM, et al. Successful islet transplantation: continued insulin reserve provides long-term glycemic control. Diabetes 2002; 51:2148-57.

216. Ryan EA, Lakey JR, Rajotte RV, Korbutt GS, Kin T, Imes S, et al. Clinical outcomes and insulin secretion after islet transplantation with the Edmonton protocol. Diabetes 2001; 50:710-9.

217. Ryan EA, Paty BW, Senior PA, Bigam D, Alfadhli E, Kneteman NM, et al. Five-year follow-up after clinical islet transplantation. Diabetes 2005; 54:2060-9.

218. Ryan EA, Paty BW, Senior PA, Lakey JR, Bigam D, Shapiro AM. Beta-score: an assessment of beta-cell function after islet transplantation. Diabetes Care 2005; 28:343-7.

219. Ryan JM, Barry FP, Murphy JM, Mahon BP. Mesenchymal stem cells avoid allogeneic rejection. J Inflamm (Lond) 2005; 2:8.

220. Sakata N, Tan A, Chan N, Obenaus A, Mace J, Peverini R, et al. Efficacy comparison between intraportal and subcapsular islet transplants in a murine diabetic model. Transplant Proc 2009; 41:346-9.

221. Samy KP, Martin BM, Turgeon NA, Kirk AD. Islet cell xenotransplantation: a serious look toward the clinic. Xenotransplantation 2014; 21:221-9.

222. Sandler S, Bendtzen K, Eizirik DL, Welsh M. Interleukin-6 affects insulin secretion and glucose metabolism of rat pancreatic islets in vitro. Endocrinology 1990; 126:1288-94.

223. Sarugaser R, Lickorish D, Baksh D, Hosseini MM, Davies JE. Human umbilical cord perivascular (HUCPV) cells: a source of mesenchymal progenitors. Stem Cells 2005; 23:220-9.

224. Scharfmann R, Didiesheim M, Richards P, Chandra V, Oshima M, Albagli O. Mass production of functional human pancreatic beta-cells: why and how? Diabetes Obes Metab 2016; 18 Suppl 1:128-36.

225. Scharp DW, Lacy PE, Santiago JV, McCullough CS, Weide LG, Falqui L, et al. Insulin independence after islet transplantation into type I diabetic patient. Diabetes 1990; 39:515-8.

226. Scharp DW, Marchetti P. Encapsulated islets for diabetes therapy: history, current progress, and critical issues requiring solution. Adv Drug Deliv Rev 2014; 67-68:35-73.

227. Schive SW, Mirlashari MR, Hasvold G, Wang M, Josefsen D, Gullestad HP, et al. Human Adipose-Derived Mesenchymal Stem Cells Respond to Short-Term Hypoxia by Secreting Factors Beneficial for Human Islets In Vitro and Potentiate Antidiabetic Effect In Vivo. Cell Med 2017; 9:103-16.

228. Schulz TC, Young HY, Agulnick AD, Babin MJ, Baetge EE, Bang AG, et al. A scalable system for production of functional pancreatic progenitors from human embryonic stem cells. PLoS One 2012; 7:e37004.

229. Shapiro AM, Lakey JR, Ryan EA, Korbutt GS, Toth E, Warnock GL, et al. Islet transplantation in seven patients with type 1 diabetes mellitus using a glucocorticoid-free immunosuppressive regimen. N Engl J Med 2000; 343:230-8.

230. Shapiro AM, Pokrywczynska M, Ricordi C. Clinical pancreatic islet transplantation. Nat Rev Endocrinol 2016.

231. Shapiro AM, Pokrywczynska M, Ricordi C. Clinical pancreatic islet transplantation. Nat Rev Endocrinol 2017; 13:268-77.

232. Shapiro AM, Ryan EA, Lakey JR. Diabetes. Islet cell transplantation. Lancet 2001; 358 Suppl:S21.

233. Sharon M, Klausner RD, Cullen BR, Chizzonite R, Leonard WJ. Novel interleukin-2 receptor subunit detected by cross-linking under high-affinity conditions. Science 1986; 234:859-63.

234. Sherar MD, Noss MB, Foster FS. Ultrasound backscatter microscopy images the internal structure of living tumour spheroids. Nature 1987; 330:493-5.

235. Siegel JP, Sharon M, Smith PL, Leonard WJ. The IL-2 receptor beta chain (p70): role in mediating signals for LAK, NK, and proliferative activities. Science 1987; 238:75-8.

236. Skyler JS, Greenbaum CJ, Lachin JM, Leschek E, Rafkin-Mervis L, Savage P, et al. Type 1 Diabetes TrialNet--an international collaborative clinical trials network. Ann N Y Acad Sci 2008; 1150:14-24.

237. Sordi V, Malosio ML, Marchesi F, Mercalli A, Melzi R, Giordano T, et al. Bone marrow mesenchymal stem cells express a restricted set of functionally active chemokine receptors capable of promoting migration to pancreatic islets. Blood 2005; 106:419-27.

238. Spaggiari GM, Capobianco A, Abdelrazik H, Becchetti F, Mingari MC, Moretta L. Mesenchymal stem cells inhibit natural killer-cell proliferation, cytotoxicity, and cytokine production: role of indoleamine 2,3-dioxygenase and prostaglandin E2. Blood 2008; 111:1327-33.

239. Spaggiari GM, Capobianco A, Becchetti S, Mingari MC, Moretta L. Mesenchymal stem cell-natural killer cell interactions: evidence that activated NK cells are capable of killing MSCs, whereas MSCs can inhibit IL-2-induced NK-cell proliferation. Blood 2006; 107:1484-90.

240. Stagner JI, Rilo HL, White KK. The pancreas as an islet transplantation site. Confirmation in a syngeneic rodent and canine autotransplant model. JOP 2007; 8:628-36.

241. Steinberg MS. Adhesion-guided multicellular assembly: a commentary upon the postulates, real and imagined, of the differential adhesion hypothesis, with special attention to computer simulations of cell sorting. J Theor Biol 1975; 55:431-43.

242. Stendahl JC, Kaufman DB, Stupp SI. Extracellular matrix in pancreatic islets: relevance to scaffold design and transplantation. Cell transplantation 2009; 18:1-12.

243. Suri A, Calderon B, Esparza TJ, Frederick K, Bittner P, Unanue ER. Immunological reversal of autoimmune diabetes without hematopoietic replacement of beta cells. Science 2006; 311:1778-80.

244. Szot GL, Koudria P, Bluestone JA. Transplantation of pancreatic islets into the kidney capsule of diabetic mice. J Vis Exp 2007:404.

245. Takahashi K, Yamanaka S. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell 2006; 126:663-76.

246. Tang Q, Bluestone JA. Regulatory T-cell therapy in transplantation: moving to the clinic. Cold Spring Harb Perspect Med 2013; 3.

247. Toso C, Isse K, Demetris AJ, Dinyari P, Koh A, Imes S, et al. Histologic graft assessment after clinical islet transplantation. Transplantation 2009; 88:1286-93.

248. Turgeon NA, Avila JG, Cano JA, Hutchinson JJ, Badell IR, Page AJ, et al. Experience with a novel efalizumab-based immunosuppressive regimen to facilitate single donor islet cell transplantation. Am J Transplant 2010; 10:2082-91.

249. Tze WJ, Tai J. Preparation of pseudoislets for morphological and functional studies. Transplantation 1982; 34:228-31.

250. Tze WJ, Tai J. Allotransplantation and xenotransplantation of pseudoislets in diabetic rats and mice. Transplantation 1984; 38:438-40.

251. Ungrin MD, Joshi C, Nica A, Bauwens C, Zandstra PW. Reproducible, ultra high-throughput formation of multicellular organization from single cell suspension-derived human embryonic stem cell aggregates. PLoS One 2008; 3:e1565.

252. Vaithilingam V, Bal S, Tuch BE. Encapsulated Islet Transplantation: Where Do We Stand? Rev Diabet Stud 2017; 14:51-78.

253. van der Windt DJ, Bottino R, Casu A, Campanile N, Smetanka C, He J, et al. Long-term controlled normoglycemia in diabetic non-human primates after transplantation with hCD46 transgenic porcine islets. Am J Transplant 2009; 9:2716-26.

254. van der Windt DJ, Bottino R, Kumar G, Wijkstrom M, Hara H, Ezzelarab M, et al. Clinical islet xenotransplantation: how close are we? Diabetes 2012; 61:3046-55.

255. Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, et al. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol 2002; 3:RESEARCH0034.

256. Vanikar AV, Trivedi HL, Thakkar UG. Stem cell therapy emerging as the key player in treating type 1 diabetes mellitus. Cytotherapy 2016; 18:1077-86.

257. Vegas AJ, Veiseh O, Gurtler M, Millman JR, Pagliuca FW, Bader AR, et al. Long-term glycemic control using polymer-encapsulated human stem cell-derived beta cells in immune-competent mice. Nat Med 2016; 22:306-11.

258. Vija L, Farge D, Gautier JF, Vexiau P, Dumitrache C, Bourgarit A, et al. Mesenchymal stem cells: Stem cell therapy perspectives for type 1 diabetes. Diabetes Metab 2009; 35:85-93.

259. Vincenti F, Charpentier B, Vanrenterghem Y, Rostaing L, Bresnahan B, Darji P, et al. A phase III study of belatacept-based immunosuppression regimens versus cyclosporine in renal transplant recipients (BENEFIT study). Am J Transplant 2010; 10:535-46.

260. Voltarelli JC, Couri CE, Stracieri AB, Oliveira MC, Moraes DA, Pieroni F, et al. Autologous nonmyeloablative hematopoietic stem cell transplantation in newly diagnosed type 1 diabetes mellitus. JAMA 2007; 297:1568-76.

261. Wang T, Lacik I, Brissova M, Anilkumar AV, Prokop A, Hunkeler D, et al. An encapsulation system for the immunoisolation of pancreatic islets. Nat Biotechnol 1997; 15:358-62.

262. Wang X, Meloche M, Verchere CB, Ou D, Mui A, Warnock GL. Improving islet engraftment by gene therapy. Journal of transplantation 2011; 2011:594851.

263. Weber CJ, Hardy MA, Pi-Sunyer F, Zimmerman E, Reemtsma K. Tissue culture preservation and intramuscular transplantation of pancreatic islets. Surgery 1978; 84:166-74.

264. Westenfelder C, Gooch A, Hu Z, Ahlstrom J, Zhang P. Durable Control of Autoimmune Diabetes in Mice Achieved by Intraperitoneal Transplantation of "Neo-Islets," Three-Dimensional Aggregates of Allogeneic Islet and "Mesenchymal Stem Cells". Stem Cells Transl Med 2017; 6:1631-43.

265. Wild S, Roglic G, Green A, Sicree R, King H. Global prevalence of diabetes: estimates for the year 2000 and projections for 2030. Diabetes Care 2004; 27:1047-53.

266. Williams JT, Southerland SS, Souza J, Calcutt AF, Cartledge RG. Cells isolated from adult human skeletal muscle capable of differentiating into multiple mesodermal phenotypes. Am Surg 1999; 65:22-6.

267. Williams P.
Notes on diabetes treated with extract and by grafts of sheep's pancreas. Br Med J 1894; 2:1303-4.

268. Wu DC, Hester J, Nadig SN, Zhang W, Trzonkowski P, Gray D, et al. Ex vivo expanded human regulatory T cells can prolong survival of a human islet allograft in a humanized mouse model. Transplantation 2013; 96:707-16.

269. Xin Y, Jiang X, Wang Y, Su X, Sun M, Zhang L, et al. Insulin-Producing Cells Differentiated from Human Bone Marrow Mesenchymal Stem Cells In Vitro Ameliorate Streptozotocin-Induced Diabetic Hyperglycemia. PLoS One 2016; 11:e0145838.

270. Yamamoto T, Horiguchi A, Ito M, Nagata H, Ichii H, Ricordi C, et al. Quality control for clinical islet transplantation: organ procurement and preservation, the islet processing facility, isolation, and potency tests. J Hepatobiliary Pancreat Surg 2009; 16:131-6.

271. Yamamoto T, Ricordi C, Messinger S, Sakuma Y, Miki A, Rodriguez R, et al. Deterioration and variability of highly purified collagenase blends used in clinical islet isolation. Transplantation 2007; 84:997-1002.

272. Yancopoulos GD, Davis S, Gale NW, Rudge JS, Wiegand SJ, Holash J. Vascular-specific growth factors and blood vessel formation. Nature 2000; 407:242-8.

273. Yang L, Guell M, Niu D, George H, Lesha E, Grishin D, et al. Genome-wide inactivation of porcine endogenous retroviruses (PERVs). Science 2015; 350:1101-4.

274. Yeung TY, Seeberger KL, Kin T, Adesida A, Jomha N, Shapiro AM, et al. Human mesenchymal stem cells protect human islets from pro-inflammatory cytokines. PLoS One 2012; 7:e38189.

275. You WP, Henneberg M. Type 1 diabetes prevalence increasing globally and regionally: the role of natural selection and life expectancy at birth. BMJ Open Diabetes Res Care 2016; 4:e000161.

276. Zeng Y, Torre MA, Karrison T, Thistlethwaite JR. The correlation between donor characteristics and the success of human islet isolation. Transplantation 1994; 57:954-8.

277. Zhang N, Richter A, Suriawinata J, Harbaran S, Altomonte J, Cong L, et al. Elevated vascular endothelial growth factor production in islets improves islet graft vascularization. Diabetes 2004; 53:963-70.

278. Zhang N, Schroppel B, Lal G, Jakubzick C, Mao X, Chen D, et al. Regulatory T cells sequentially migrate from inflamed tissues to draining lymph nodes to suppress the alloimmune response. Immunity 2009; 30:458-69.

279. Zhang Y, Li C, Jiang X, Zhang S, Wu Y, Liu B, et al. Human placenta-derived mesenchymal progenitor cells support culture expansion of long-term culture-initiating cells from cord blood CD34+ cells. Exp Hematol 2004; 32:657-64.

280. Zhou Q, Brown J, Kanarek A, Rajagopal J, Melton DA. In vivo reprogramming of adult pancreatic exocrine cells to beta-cells. Nature 2008; 455:627-32.

281. Zhu HT, Wang WL, Yu L, Wang B. Pig-islet xenotransplantation: recent progress and current perspectives. Front Surg 2014; 1:7.

282. Zuellig RA, Cavallari G, Gerber P, Tschopp O, Spinas GA, Moritz W, et al. Improved physiological properties of gravity-enforced reassembled rat and human pancreatic pseudo-islets. J Tissue Eng Regen Med 2017; 11:109-20.