Strategies to Augment Survival and Engraftment in Islet Transplantation

by

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Doctor of Philosophy

in

Experimental Surgery

Department of Surgery

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ABSTRACT

Type 1 diabetes mellitus (T1DM) is characterized by the autoimmune destruction of insulin-producing beta (β)-cells within pancreatic islets of Langerhans. For many patients, frequent blood glucose monitoring and insulin administration are primary therapies aimed to curb hyperglycemia, though long-term complications may still occur. β-cell replacement therapy through islet transplantation has become an accepted treatment modality for select patients suffering from "brittle" T1DM. The establishment of the 'Edmonton Protocol' in 2000 was instrumental in renewing global interest in utilizing islet transplantation as an effective therapy for such patients. Over the course of the last two decades, considerable refinements in islet isolation and culture techniques, as well as the incorporation of anti-inflammatory and novel immunosuppressive therapies have improved long-term islet transplantation outcomes. Despite such refinements, obstacles associated with islet transplantation still exist, as single-donor insulin independence remains elusive, warranting further investigation.

This thesis presents results from multiple studies aimed to augment *in vitro* islet survival to potentiate subsequent engraftment. ***I hypothesize that the administration of therapeutic agents during pancreas procurement, islet culture and/or in the acute post-transplant period can improve in vitro and in vivo islet function***. The thesis is laid out in a paper-based format, based on manuscripts published or under review. Sufficient background is provided to gain an understanding of islet transplantation, beginning with a historical perspective, as well as current limitations and therapeutic interventions employed pre-clinically and clinically to circumvent such obstacles. The reader is also

provided with details of various regulated cell death pathways, those long-existing, as well as newly defined pathways yet to be fully elucidated in islet transplantation.

We present our research to enhance islet viability and potency *in vitro* through the administration of a novel manganese superoxide dismutase (SOD)-mimetic, the metalloporphyrin BMX-001, during organ procurement and 24-hour culture, as well as evaluate whether preserved potency enhances engraftment outcomes in a syngeneic, marginal mass model. We observed the ability of this SOD-mimetic to indeed augment *in vitro* islet function and viability when administering a physiologically relevant dose of 34μM BMX-001. BMX-001-treated islets also exhibited improved engraftment outcomes relative to non-treated control islet recipients.

As a means to expand the availability of donor pancreases, we further developed a murine donation after circulatory death (DCD) model, and further assessed the utility of BMX-001. The findings from this model were striking, notably 15 minutes of warm ischemia (WI) significantly impaired islet isolation yields in mice. The administration of BMX-001 during pancreas procurement did not significantly increase islet yield or viability relative to control DCD islets. BMX-001-cultured DCD islets demonstrated a significant decrease in extra cellular ROS, suggesting some cytoprotection. DCD murine islets, regardless of BMX-001 administration were able to engraft at a similar rate to islets isolated from murine pancreata that did not experience WI.

Shifting focus to cell death in islet transplantation, the novel pan-caspase inhibitor, F573 was investigated. Murine and human islets cultured for 2 or 24 hours, respectively with or without F573 exhibited a marked reduction in terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) and caspase-3 activation *in vitro*. Human

islet engraftment was significantly enhanced in F573-treated recipients relative to non-treated controls. Moreover, F573 was able to augment engraftment in a significant proportion compared with non-treated control recipients in the modified subcutaneous, device-less site, further supporting the potential of this site as an alternative to intra-portal islet infusion.

To expand on the current knowledge of regulated cell death mechanisms that may contribute to islet dysfunction and compromise subsequent engraftment, ferroptosis, a non-apoptotic cell death modality was investigated in human islets. Utilizing ferroptosis inducing agents, erastin and RSL3, islet viability and function was compromised *in vitro*. These effects were abrogated in the presence of the ferroptosis-specific inhibitor, ferrostatin-1, thus confirming that this cell death modality can contribute to islet demise. Further work evaluating alternative regulated cell death mechanisms in islet isolation and transplantation may lead to improved adjuvant therapies to deter islet dysfunction and subsequently improve long-term engraftment outcomes.

Taken together, the results of this body of work reveals that preserving pre-transplant islet potency through the utility of novel agents administered during procurement or in culture can augment islet engraftment outcomes. Such agents hold promise as prospective adjuvant therapies to promote single-donor islet engraftment outcomes.

PREFACE

Dear Reader,

The thesis entitled "Strategies to Augment Survival and Engraftment in Islet Transplantation" is submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Experimental Surgery in the Department of Surgery at the University of Alberta. This body of work evaluates pre-clinical research efforts aimed to improve islet viability and function through the utility of various agents aimed to improve islet potency as a treatment for type 1 diabetes mellitus (T1DM).

The thesis contains pre-clinical studies whereby the author held the leading role within a collaborative team. The chapters are presented from bodies of work prepared for publication. The majority of work has either been published in journal articles or currently under peer-review for publication consideration.

Chapter 1 is presented in two distinct parts. **Part 1** provides a comprehensive introduction to islet transplantation, its therapeutic application for T1DM in select patients, its associated risks and future directions of the field. The chapter is presented from a first-author manuscript published in ***Diabetes, Metabolic Syndrome and Obesity: Targets and Therapy*** (Bruni A, Pepper AR, Gala-Lopez BL, Abualhassan N, and Shapiro AMJ, *Islet Cell Transplantation for the Treatment of Type 1 Diabetes: Recent Advances and Future Challenges* Diabetes Metab Syndr Obes. 2014; 7: 211–223).

. My role for this publication was designing and performing the bibliographic review and writing 90% of the manuscript. ARP and BGL wrote the remaining 10% of the manuscript and provided revisions. NA provided the figures and accompanying legends, as well as revisions to the final manuscript. AMJS performed final edits as the senior

corresponding author. **Part 2** provides new insights into newly defined regulated cell death mechanisms that have been identified in other organ systems and we evaluate their relevance in islet transplantation. The chapter is presented from a first-author manuscript submission currently under peer-review in ***Cell Transplantation*** (Bruni A, Bornstein SR, Linkermann A, and Shapiro AMJ), entitled *Regulated Cell Death Through the Lens of Islet Transplantation*. My roles for this manuscript was designing and performing the bibliographic review, writing 90% of the manuscript, as well as preparing figures and tables. SRB provided critical review of the manuscript and revisions. AL performed final edits as the co-senior corresponding author. I designed the manuscript and figure together with AMJS. AMJS provided final edits and revisions as the senior corresponding author.

Chapter 2 seeks to evaluate the utility of a novel superoxide dismutase (SOD) mimetic, BMX-001 in islet isolation and transplantation. The body of work examines the impact of BMX-001 administration during murine organ procurement and 24-hour culture. Given that islets exhibit reduced antioxidant defenses relative to other tissues, we aimed to examine whether administration of BMX-001 could enhance *in vitro* islet viability, as well as enhance engraftment outcomes in a murine, syngeneic marginal islet transplant model. Based on our murine islet observations, we also cultured human islets with BMX-001 for 24 hours and evaluated engraftment outcomes in a marginal transplant model in immunocompromised murine recipients. The work included in this chapter is currently submitted with revisions to the ***American Journal of Transplantation*** (Bruni A, Pepper AR, Pawlick RL, Gala-Lopez B, Gamble A, Kin T, Malcolm A, Jones C, Piganelli J, Crapo J and Shapiro AMJ). My contribution in this research project was to perform 50% of the murine pancreas distensions and islet isolation procedures, approximately 50% of

the characterization of control and treated islets (recovery, viability and insulin release), performing approximately 70% of the transplant procedures, animal monitoring and assessment, performing all data analysis and writing 90% of the manuscript. ARP and RLP performed the remaining 50% of pancreas distensions, islet isolations and transplants. BGL and AG assisted with the *in vitro* characterization. TK performed all human islet isolations. AM, CJ, JP and JC contributed study design and rationale. CJ, JP and JC are collaborators from BioMimetix and they provided BMX-001 for pre-clinical evaluation. All listed authors reviewed the manuscript and provided corrections. AMJS designed and led the study performed, performed final edits to the manuscript as senior corresponding author.

Chapter 3 further aims to elucidate the utility of BMX-001 specifically within the context of a murine donation after circulatory death (DCD) islet isolation model. The work included in this chapter was accepted for publication in ***Islets*** (Bruni A, Pepper AR, Gala-Lopez BL, Pawlick RL, Abualhassan NS, Piganelli J, Crapo J and Shapiro AMJ; Islets. 2016; 8(4): e1190058). My contribution in this research project was to participate in performing 90% *in vitro* work, animal monitoring, performing all data analysis and writing 90% of the manuscript. ARP, RLP, BG performed all islet isolations and transplants, and review of the manuscript. NA assisted with the remaining *in vitro* work. JP and JC are collaborators from BioMimetix, providing BMX-001 for pre-clinical evaluation. All authors provided final review of the manuscript prior to submission. I jointly designed the study together with AMJS, and AMJS performed final edits to the manuscript as senior corresponding author.

Chapter 4 explores the utility of a novel pan-caspase inhibitor, F573, in improving *in vitro* islet viability as well as subsequent engraftment outcomes. While the utility of anti-apoptotic inhibitors has been previously described, this work further explores this compound in mouse and human islet transplantation in a syngeneic and immune compromised model, respectively, while also exploring engraftment outcomes in various sites of transplantation. Most notably, the administration of F573 augments islet engraftment when transplanted into the modified subcutaneous space utilizing the device-less technique. This work was published in ***Transplantation*** (Pepper AR, Bruni AB, Pawlick RL, Gala-Lopez BL, and Shapiro AMJ; *Transplantation* 2017 Oct; 101(10): 2321-29). Building on my prior experience with subcutaneous islet transplantation and interest in elucidating mechanisms associated with islet cell death, my role in this project was to design 30% of the experiments, perform 30% of murine islet isolations, animal monitoring, perform 50% of IPGTTs and writing 50% of the manuscript. ARP designed 50% of the experiments, conducted *in vitro* TUNEL and caspase-3 assessment, performed 50% of murine islet isolations and IPGTTs, as well as wrote 50% of the manuscript. RLP and BGL assisted with islet isolations and transplants, as well as provided final review of the manuscript. AMJS designed the study, as well as performed final edits to the manuscript as senior corresponding author.

Chapter 5 delves into the identification of alternative regulated necrosis in human islets. Ferroptosis has recently been classified as an iron-dependent form of non-apoptotic cell death, and has been implicated in other organ systems, including renal-ischemia reperfusion injury. Utilizing ferroptosis inducing agents, erastin and RSL3, we were able to compromise islet viability, as well as insulin secretion in response to glucose *in vitro*.

These effects were significantly diminished in the presence of the ferroptosis inhibitor ferrostatin-1. This work is currently under peer-review in ***Cell Death and Disease*** (Bruni AB, Pepper AR, Pawlick RL, Gala-Lopez B, Gamble A, Kin T, Bornstein SR, Linkermann A and Shapiro AMJ). My contribution to this research project was to design the experiments, perform 90% of *in vitro* assays, animal monitoring, data analysis and writing 90% of the manuscript. ARP assisted with islet transplants, data analysis and review of the manuscript. RLP and BGL assisted with islet transplants. AG assisted with animal monitoring. TK performed all human islet isolations. SRB and AL performed final edits to the manuscript. I designed this study together with AMJS. AMJS performed final edits to the manuscript as senior corresponding author.

Chapter 6 provides an overview of the topics highlighted in this thesis. This section incorporates a review of the literature with incorporated personal analyses of interventional strategies in the various stages of islet isolation, culture and transplantation to improve islet potency and function in the acute and peri-transplant period to support long-term, durable engraftment. The chapter also highlights the future of islet transplantation, including the use of alternative β-cell sources, strategies to induce tolerance, as well as approaches to genetically reset the onset of type 1 diabetes mellitus in newly diagnosed patients.

It is my anticipation that you find the work presented of interest and relevance.

DEDICATION

This thesis is dedicated to my wife, Amanda, whose personal sacrifices, unconditional love and support has contributed to my many successes as a graduate student. Thank you for your never-ending encouragement and of course, blessing me with our greatest gift, our beautiful daughter, Abigail.

MAY THERE NEVER
DEVELOP IN ME THE NOTION
THAT MY EDUCATION IS COMPLETE,
BUT GIVE ME THE STRENGTH
AND LEISURE AND ZEAL
CONTINUALLY TO ENLARGE
MY KNOWLEDGE.

~MAIMONIDES~

ACKNOWLEDGEMENTS

I would like to take this opportunity to thank the many individuals who contributed to my growth as a student and researcher here at the University of Alberta and have made this endeavor possible.

My sincerest gratitude to my Supervisor, Dr. James Shapiro, for providing me with the invaluable opportunity to pursue graduate studies in your world-renowned laboratory. Thank you for your guidance and support, which has immensely contributed to my growth as a researcher and scientist. As a beacon in the field of islet transplantation, it has been a privilege and honour to contribute to the endeavours in your laboratory. There is no doubt that my personal and professional growth has been elevated as a result of your unrelenting support in the four years as a trainee in your laboratory. Your tireless dedication to your work and team has been an inspiration to witness and motivation in my own personal pursuits.

Thank you to the members of my supervisory committee Drs. Gregory Korbutt and Peter Senior for your support, encouragement and scientific contributions to the studies presented in this thesis. It has been a pleasure to share with you my progress and expand my knowledge through engaging in scientific discussion. Thank you for your continued support through this journey and your willingness to meet with me outside of our scheduled committee meetings.

I extend my gratitude to Dr. Bruce Verchere for graciously serving as my External Examiner for my final dissertation. It is a privilege and honor to have you review my

thesis, participate in my oral defense examination and engage in constructive scientific discussion.

My sincerest gratitude to Dr. Tom Churchill, former Director of Graduate Education within the Department of Surgery. Thank you for your open-door policy, your unrelenting support for all graduate students within the Department, and your earnest efforts to make the graduate program as strong as it has become. Thank you for also serving as an examiner during my Candidacy and Dissertation examinations.

Thank you to Dr. Jean Buteau for agreeing to be the External Examiner for my Candidacy Exam. You challenged me with thought-provoking questions and certainly contributed to my growth as a budding scientist.

I especially want to thank my colleagues, both past and present, in the pre-clinical experimental laboratory have facilitated my success as a doctoral student. Dr. Andrew Pepper, not only a reliable colleague, but also a true friend who helped instill in me the confidence to propel forward in my studies. For your technical and scientific contributions that have helped me to improve my skillset as a young investigator, as well as numerous opportunities to collaborate on exciting and innovative studies. Thank you for helping me see "the forest for the trees" throughout my research pursuits. An enormous thank you to Mrs. Rena Pawlick whose technical prowess were paramount in assisting me, as well as providing me with the tools to excel in my studies. To Ms. Anissa Gamble, it has been a pleasure working along side you over the last 16 months. I have enjoyed watching you grow as a young scientist and thank you for giving me the opportunity to work alongside you. Thank you to Dr. Mariusz Bral for his encouraging words and anecdotes. Though we did not collaborate much in our studies, I always

appreciated your input and encouragement along the way. To my former colleagues, Drs. Boris Gala-Lopez and Nasser Abualhassan, thank you for your contributions throughout my tenure as a graduate student. It has been a pleasure collaborating with you throughout our time together, and exciting to watch you move forward in your career aspirations. Thanks to James Lyon of the Islet Isolation Research Core for your friendship over the course of the last four years.

It has been an utmost privilege to engage with members of the Clinical Islet Program. Thank you to Dr. Tatsuya Kin and Doug O'Gorman for your tireless efforts in the Clinical Islet Lab, along with your willingness to contribute to my research. A special thank you to Ms. Rayna Bahneva for ensuring appropriate documentation was completed and approved by the appropriate advisory boards to allow conduct of my research. Thank you to Ms. Rosemary Csoma-Kostiuk for attending to our financial and HR needs. Ms. Cherry Robinson for assisting with all scheduling matters to ensure that lab meetings, committee meetings and one-on-one meetings could be organized. A huge thank you to Dr. Andrew Malcolm, Director of Operations for your support throughout the last four years. It was indeed a pleasure to work with you on numerous pre-clinical and clinical grant applications that have contributed to skillsets that transcend the laboratory bench.

I want thank all of our collaborators, which provided indispensable support to my research. Dr. James Crapo, Dr. Jon Piganelli and Ms. Carissa Jones from *BioMimetix Pharmaceutical Inc*., for their continuous collaborative support of our pre-clinical evaluation of BMX-001. Thank you to Drs. Andreas Linkermann and Stefan Bornstein for your collaborative support with our pre-clinical investigation of ferroptosis in human islets, as well as sharing your expertise on regulated cell death pathways, contributing to

our intensive review. It has been a formidable pleasure to engage with titans in the field of regulated cell death.

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Thank you to Dr. Jared Wesley and Mr. Reg Joseph for your mentorship and support outside of the laboratory. You have both provided me with valuable tools to utilize beyond a career in academia.

A special acknowledgement to the numerous funding agencies that provided financial support throughout my tenure as a graduate student in the Program. The Alberta Diabetes Institute Graduate Studentship which was instrumental in initiating my studies, the Canadian Institute of Health Research Banting and Best Canada Graduate Scholarship (CGS-M), the Alberta Diabetes Foundation Matthias Braun Memorial Scholarship for Excellence in Islet Biology, the University of Alberta PhD Recruitment Scholarship, the Blanch and Cantor families for their generous support of the Blanch Award and Ray Rajotte Graduate Award, respectively, as well as the numerous travel awards from the Graduate Student's Association, Faculty of Graduate Studies and Research, Alberta

Diabetes Institute and Department of Surgery for providing me with the opportunity to share my research endeavours abroad.

A resounding thank you to my parents, John and Anna, for leading by example and instilling in me the qualities that have shaped the individual I am today. Thank you for all of your love and support. Thank you to my siblings and in-laws for your unrelenting encouragement throughout my studies.

Lastly, and most importantly, a HUGE THANK YOU to my wife, Amanda, for your unconditional love and support throughout this journey. I love you beyond words.

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α-GAL	galactose-α-1,3-galactose
AHST	Autologous nonmyeloablative hemoatopoeitic stem cell transplantation
ASC	Apoptosis-associated speck-like protein containing a caspase-associated recruitment domain
AUC	Area under curve
β-cell	Beta-cell
BMI	Body mass index
BMX-010	MnTE-2-PyP [Manganese (III) Meso-Tetrakis-(N-Methylpyridinium-2-yl) porphyrin]
BMX-001	MnTnBuOE-2-PyP^{5+} [Mn(III) *meso*-tetrakis(N-b-butoxyethylpyridinium-2-yl)porphyrin]
CARD	Caspase-associated recruitment domain
CIT	Clinical Islet Transplant Consortium
CITR	Collaborative Islet Transplant Registry
CMRL	Connaught Medical Research Laboratories
DAF	Decay accelerating factor
DAMPs	Danger -associated molecular patterns
DAI	DNA-dependent activator of IFN-regulatory factors
DCD	Donation after circulatory death
DFO	Desferroxamine
DISC	death-inducing signaling complex
DL	Device-less
DMSO	Dimethyl sulfoxide
ELISA	Enzyme-linked immunosorbent assay
ESCRT-III	Endosomal sorting complex required for transport-III
FADD	Fas-associated death domain
Fer-1	Ferrostatin-1
FIA	Ferroptosis-inducing agent
GAD65	Glutamate decarboxylase 65

GCSF	Granulocyte colony-stimulating factor
GPX4	Glutathione peroxidase 4
GSH	Glutathione
GSDMD	Gasdermin D
GTKO	Alpha (α)-1,3-galactosyltransferase gene knockout
HbA1c	Glycosylated hemoglobin
HBSS	Hank's Buffered Saline Solution
HMGB1	High-mobility group box 1
hCD46	Humanized CD46
HTK	Histadine-tryptophan-ketoglutarate
IBMIR	Instant-blood mediated inflammatory reaction
IE	Islet equivalents
IFN-γ	Interferon-gamma
IL	Interleukin
IL-1β	Interleukin-1-beta
IL-1Ra	Interleukin-1 receptor agonist
i.p	Intraperitoneal
IPGTT	Intraperitoneal glucose tolerance test
KC	Kidney capsule
LDH	Lactate dehydrogenase
MCP-1	Monocyte chemoattractant protein-1
MLKL	Mixed lineage kinase domain-like protein
MMF	Mycophenolate mofetil
MnP	metalloporphyrin anti-inflammatory and catalytic antioxidant functions
MnSOD	Manganese superoxide dismutase
NDD	Neurological determination of death
Nec-1	Necrostatin-1
Nec-1s	Necrostatin-1 stable
NLR	Nod-like receptors
NLRP	Nod-like receptors containing a pyrin domain
PBS	Phosphate buffered saline

PE	Polyethylene
PERV	Porcine endogenous retrovirus
PV	Portal vein
PYD	Pyrin
RIPK1	Receptor interaction protein kinase 1
RIPK3	Receptor interaction protein kinase 3
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
T2DM	Type 2 diabetes mellitus
TBS	Tris-buffered saline
TLRs	Toll-like receptors
TNF-α	Tumor necrosis factor-alpha
TNF-α-i	TNF-α-inhibition
TNFR1	TNF receptor-1
Treg	Regulatory T-cell
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling
WI	Warm Ischemia
zVAD-FMK	N-benzyloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone
zVD-FMK	N-benzyloxycarbonyl-Val-Asp-fluoromethyl ketone

CHAPTER 1

INTRODUCTION

PART 1

ISLET CELL TRANSPLANTATION FOR THE TREATMENT OF TYPE 1 DIABETES: RECENT ADVANCES AND FUTURE CHALLENGES

CHAPTER 1 PART 1 – ISLET CELL TRANSPLANTATION FOR THE TREATMENT OF TYPE 1 DIABETES: RECENT ADVANCES AND FUTURE CHALLENGES

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REVIEW

Islet cell transplantation for the treatment of type 1 diabetes: recent advances and future challenges

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Abstract: Islet transplantation is a well-established therapeutic treatment for a subset of patients with complicated type I diabetes mellitus. Prior to the Edmonton Protocol, only 9% of the 267 islet transplant recipients since 1999 were insulin independent for >1 year. In 2000, the Edmonton group reported the achievement of insulin independence in seven consecutive patients, which in a collaborative team effort propagated expansion of clinical islet transplantation centers worldwide in an effort to ameliorate the consequences of this disease. To date, clinical islet transplantation has established improved success with insulin independence rates up to 5 years post-transplant with minimal complications. In spite of marked clinical success, donor availability and selection, engraftment, and side effects of immunosuppression remain as existing obstacles to be addressed to further improve this therapy. Clinical trials to improve engraftment, the availability of insulin-producing cell sources, as well as alternative transplant sites are currently under investigation to expand treatment. With ongoing experimental and clinical studies, islet transplantation continues to be an exciting and attractive therapy to treat type I diabetes mellitus with the prospect of shifting from a treatment for some to a cure for all.

Keywords: islet transplantation, type I diabetes mellitus, Edmonton Protocol, engraftment, immunosuppression

Video abstract

Point your SmartPhone at the code above. If you have a QR code reader the video abstract will appear. Or see: http://dx.doi.org/10.2147/DMSO.S65598

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Introduction

Type 1 diabetes mellitus (T1DM) is a chronic, progressive autoimmune disorder characterized by destruction of insulin-producing β-cells within the pancreatic islets of Langerhans. Diabetes is a major source of morbidity and mortality due to progressive chronic micro- and macrovascular complications. The discovery of insulin by Banting et al has allowed diabetes to become a chronically manageable condition.[1,2] Today intensive blood glucose monitoring and frequent daily administration of exogenous insulin delays progression of microvascular diseases, including retinopathy and neuropathy, but does not entirely prevent these complications.[3] Concerted efforts to ameliorate the symptoms and complications of diabetes have spanned beyond administration of exogenous insulin to restoration of β-cell mass through islet transplantation.

The pioneering experiments by Lacy and Kostianovsky provided the fundamental means to introduce islet transplantation as an effective therapy to correct hyperglycemia through the ability to isolate a sufficient number of metabolically active and intact islets from rodent pancreata.[4] While several authors reported correction of hyperglycemia in diabetic mice using varied islet doses and success via the intraperitoneal route, Reckard et al in 1973 were the first to effectively cure diabetes in a chemically induced model.[5] Yet despite these successes, the same principles of isolation and purification could not

A version of this section was published as a Review in *Diabetes, Metabolic Syndrome and Obesity: Targets and Therapy* 2016 Jun; (7): 211-23.

ORIGINAL ARTICLE

Title: Islet Cell Transplantation for the treatment of type-1 diabetes: recent advances and future challenges

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1.1.1 – ABSTRACT

Islet transplantation is a well-established therapeutic treatment for a subset of patients with complicated Type 1 diabetes mellitus (T1DM). Prior to the Edmonton Protocol, only 9% of the 267 islet transplant recipients before 1999 were insulin independent for >1 year. In 2000, the Edmonton group reported the achievement of insulin independence in 7 consecutive patients, which in a collaborative team effort propagated expansion of clinical islet transplantation centers worldwide in an effort to ameliorate the consequences of this disease. To date, clinical islet transplantation has established improved success with insulin independence at 5 years post-transplant with minimal complications. In spite of marked clinical success, donor availability and selection, engraftment, and side effects of immunosuppression remain existing obstacles to be addressed to further improve this therapy. Clinical trials to improve engraftment, the availability of insulin-producing cell sources, as well as alternative transplant sites are currently under investigation to expand treatment. With on-going experimental and clinical studies, islet transplantation continues to be an exciting and attractive therapy to treat T1DM, with the prospect of shifting from a treatment for some to a cure for all.

1.1.2 – INTRODUCTION

Type 1 diabetes mellitus (T1DM) is a chronic, progressive autoimmune disorder characterized by destruction of insulin-producing β-cells within the pancreatic islets of Langerhans. Diabetes is a major source of morbidity and mortality due to progressive chronic micro- and macrovascular complications. The discovery of insulin by Banting, Best, Collip and McLeod has allowed diabetes to become a chronically manageable condition.[1,2] Today intensive blood glucose monitoring and frequent daily administration of exogenous insulin delays progression of microvascular diseases, including retinopathy and neuropathy, but does not entirely prevent these complications.[3] Concerted effort to ameliorate the symptoms and complications of diabetes have spanned beyond administration of exogenous insulin to restoration of β-cell mass through islet transplantation.

The pioneering experiments by Lacy et al. provided the fundamental means to introduce islet transplantation as an effective therapy to correct hyperglycemia through the ability to isolate a sufficient number of metabolically active and intact islets from rodent pancreata.[4] While several authors reported correction of hyperglycemia in diabetic mice using varied islet doses and success via the intraperitoneal route, Reckard and Barker in 1973 were the first to effectively cure diabetes in a chemically induced model.[5] Yet despite these successes, the same principles of isolation and purification could not be applied to larger animals or humans, whose glands are more dense and fibrous.[6]

Refinements in the methods of islet isolation and purification for islet transplantation continued for decades with improved success in isolating significant

quantities of highly pure islet preparations. The intraductal injection of collagenase proved an effective method for successful islet isolation from large animals and humans[6,7] and modifications to this procedure progressed. However, the development of the Ricordi chamber in 1988 introduced a semi-automated process that was instrumental in consistently isolating and purifying large islet quantities.[6,8] This method of islet isolation, in conjunction with improvements in islet purification and transplantation techniques, were paramount in the translation of islet transplantation from an experimental concept to an efficient clinical treatment modality for a selected group of patients suffering from T1DM.[6]

With a rising prevalence of T1DM and a limited supply of donor pancreata from scarce organ donors, ongoing efforts are being made to improve islet isolation practices and prevent islet loss, especially in the immediate post-transplant period through a series of strategies.[9] Herein, we outline the current status of clinical islet transplantation, the obstacles associated with this practice and strategies to improve islet transplantation outcomes. Lastly, we introduce the prospect of modulating the immune system in an attempt to abolish the onset of T1DM, to circumvent the necessity of the implementation of therapeutic strategies including the administration of exogenous insulin.

1.1.3 – The evolution of clinical islet transplantation

Outcomes in clinical islet transplantation have progressed significantly since its inception, in part due to improved islet manufacturing processes, coupled with more effective induction and maintenance immunosuppression to protect against both auto- and alloreactivity.[10] Islet-alone transplantation has recently become an accepted practice to stabilize frequent hypoglycemia or severe glycemic lability in highly selected subjects

with poor glycemic control.[11] While Lacy's work established the liver as an ideal site for islet transplantation[12], further work by Najarian et al. in 1977 reported the first successful clinical islet transplant, paired with the administration of azathioprine and corticosteroids.[13] In spite of these advancements, 9% of the 267 islet transplant recipients before 1999 were insulin independent for >1 year.[14] It was not until 2000 that the 'Edmonton Protocol' reported insulin independence in seven consecutive T1D patients over a median follow-up of 11.9 months with sustained C-peptide.[10] Of particular importance, patients had received at least two different islet transplants and a mean islet mass of 13,000 IEQ/kg, as well as received a steroid-free immunosuppressive regimen with anti-interleukin (IL)-2 receptor antagonist antibody therapy, daclizumab. These results were pivotal in driving forward both interest and activity in clinical islet transplantation over the subsequent decade, which through remarkable inter-center collaboration, resulted in the expansion of islet transplantation programs in North America and abroad.[15]

1.1.4 – Current status of clinical islet transplantation

Over the last decade, over 750 islet transplants have been performed in over 30 International transplant centers. Without doubt, islet transplantation has evolved from an experimental strategy to ameliorate the consequences of T1DM, to a recognized, standard clinical therapy. The therapy is only suitable in its current form for patients with unstable glycemic control that cannot be corrected by standard conventional and intensive insulin therapies. Patients with good glycemic control, and children, are not considered currently on account of a need for lifelong chronic immunosuppression. On this account, a recent trial by Ly and colleagues reported that sensor-augmented pump

therapy with automated insulin suspension reduced the rate of moderate and severe hypoglycemia, as well as impaired hypoglycemia awareness over a 6-month period in trial participants. However, when compared to the standard insulin pump control group, no change in glycosylated hemoglobin (HbA1c) was observed.[16] To the contrary, islet transplantation has the ability to correct HbA1c to levels that can predictably reverse the secondary consequences of diabetes.[17] In a one-way crossover study conducted by Thompson and colleagues, it was demonstrated that clinical islet transplantation was more effective in reducing progression of diabetic retinopathy and nephropathy than intensive medical therapy.[18] To this extent, the lifelong need for immunosuppressive therapy may be readily justified in this therapeutic setting.

In the most recent report released from the Collaborative Islet Transplant Registry (CITR), 677 allogeneic islet transplants have been reported. Results from the CITR indicate that 44% of recipients were insulin independent at three years post-transplant in 'new era' of islet transplantation, year 2007 – 2010, as compared to 27% of clinical islet transplant recipients in 1999 – 2002.[15,19] Moreover, marked improvements in clinical islet transplantation have been observed from 2007 – 2010, as evidenced by retained C-peptide levels, reduction of HbA1c and reduced islet reinfusion rates.[19] This success, in part, can be attributed to shifts in immunosuppression strategies. However, improvements to islet engraftment and subsequent survival are critical in achievement of durable insulin-independence (**Figure 1.1.1**).

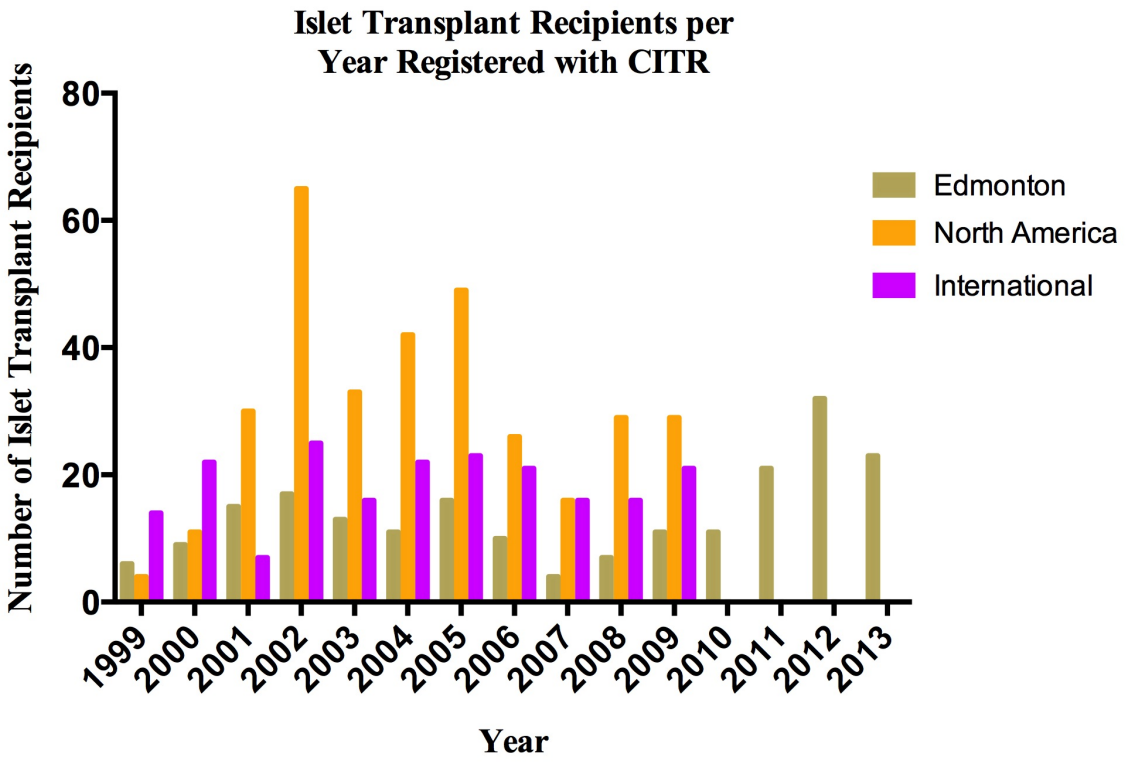

Figure 1.1.1 Number of islet transplant recipients from 1999 – 2013 in Edmonton, North America and International Islet Transplant Centers. 2010 CITR Seventh Annual Report.[15]

This data is kindly reproduced with express permission from the 2010 CITR. At the time of publication, reported data from 2010 – 2013 for North America and International Islet Transplant Centers was not available.

Within North America, few islet transplant centers are currently active, despite the substantial transplant activity of international islet transplant centers. This is reflected in the classification of islet transplantation as an experimental therapy in the United States, resulting in a lack of available funds to conduct and support large-scale clinical trials. Two pivotal Phase III clinical trials conducted in specialized islet transplantation centers through the Clinical Islet Transplant (CIT) Consortium (CIT-06 & CIT-07, Clinical Trials.gov NCT00468117 and NCT00434811, respectively), have been established in an effort to support the FDA biological license application mandate. Encouraging results from CIT-07 will indeed contribute to successful licensure, which will inevitably recognize islet transplantation as a clinical therapy, expanding its therapeutic benefit for patients with T1DM in the United States.

The University of Alberta's Clinical Islet Transplant Program continues to be the most active center participating within the CITR. In 2013 alone, 66 islet transplants were conducted at the Edmonton site (**Figure 1.1.1**). The Edmonton group also reports that of over 200 patients transplanted with more than 400 intraportal islet preparations, 79% of recipients continue to show full or partial islet graft function.[20] The median duration of insulin independence is 34.6 and 11 months for subjects with full or partial graft function, respectively, whereas the duration of C-peptide is 53.3 and 70.4 months, respectively, for those same patients.[21-23]

Prior to the 'Edmonton Protocol', insulin independence was an uncommon achievement. Though clinical success have improved markedly over the past 14 years, further obstacles must be overcome if islet transplantation is to be more broadly applied in the T1DM population. Such remaining challenges include expansion of the islet

donor supply, improving islet isolation techniques, strategies to improve engraftment, mediating the anti-inflammatory response post-transplant, and improving recipient immunosuppression regimens. Several clinical trials are currently under active investigation to address these obstacles in an attempt to improve this important therapy (**Table 1.1.1**).

1.1.5 – Clinical islet transplantation: obstacles and refinements

1.1.5.1 – Donor selection and availability

The number of pancreas donors required to treat one recipient limits the number of transplants that can occur. A component of islet transplantation that may improve clinical outcomes is donor selection. Retrospective studies at single-centers have identified several donor-related variables that may contribute to islet isolation outcomes. These variables include donor age, cause of death, body mass index (BMI), cold ischemia time, length of hospitalization, use of vasopressors, and blood glucose levels[24-31]. In most cases, a larger pancreas contains a larger β-cell mass, however, pancreas weight is not a donor-selection criterion since a value cannot be obtained prior to procurement[30,32]. In a study analyzing data from 345 deceased donors, it was determined that BMI correlates with pancreas weight, but body surface area is a better predictor of pancreas weight than BMI[32]. Several groups have indicated that BMI positively affects islet yield[33], which leads many to consider BMI as an important donor factor influencing islet isolation outcome.[28-30] However, this view has led to the misconception that an obese donor is a good candidate for successful islet isolation and transplantation. To date, "optimal" pancreata are allocated for whole organ transplantation in most centers, as this procedure has historically established success in single-donor transplant scenarios,

though this procedure is not without inherent perioperative risks. Supporting this notion is a recent report by Berney and Johnson who conclude that islet mass transplanted does not unequivocally correlate with islet graft function; therefore argue that donor selection criteria for islet transplantation, and hence allocation rules (pancreas for whole organ or islet transplant), may need to be redefined.[34]

A scoring system based on donor characteristics that can predict islet isolation outcomes was previously developed by O'Gorman and colleagues and has been an instrumental tool in assessing whether a pancreas should be processed for islet isolation[35 36]. Though this tool has been sufficient in determining organs for islet isolation, it does not predict islet transplant outcome. Similarly, other published studies dealing with donor factors do not take transplant outcome into consideration.[24,25,27-31] A prospective scoring system that takes both islet isolation and transplantation outcomes into consideration would be more advantageous.

Expansion of organs available for islet transplantation can be made possible through the use of donation after cardiac death (DCD) donors. The use of DCD donors, compared to their brain dead counterparts, has varied results and may not be entirely promising. Japan has extensive experiences with DCD donors for organ transplantation and have optimized retrieval practices in these donors, as well as the Kyoto preservation solution and the two-layer preservation method.[37] In a most recent report for islet transplantation from DCD donors, overall graft survival was 76.5%, 47.1%, and 33.6% at 1, 2, and 3 years, respectively. Moreover, corresponding graft survival after multiple transplantations was 100%, 80.0%, and 57.1%, respectively. Islet transplant recipients remained free from severe hypoglycemic episodes, while three achieved insulin

independence for 14, 79, and 215 days.[38] These encouraging results suggest the benefit of DCD as an expanded organ source for islet transplantation, particularly in countries where heart-beating donors may not be readily available, though strict release criteria may be imperative to achieve desirable and consistent transplant outcomes.

1.1.5.2 – Pancreas digestion and islet isolation

Due to the multi-faceted composition of the pancreas, islet isolation from the pancreas involves dissociation of islets from the exocrine pancreas by enzymatic digestion combined with mechanical agitation. Successful islet transplantation is initially contingent on the isolation of high islet yields, ensuring that this process inflicts significantly minimal damage. Subsequent to isolation, islets are then purified by density gradient centrifugation. To ensure optimal isolation will provide a sufficient islet yield without compromising high purity, integrity, and viability, a critical balance of composition, process, and duration of collagenase digestion is required important.[39] The enzymatic digestion process disrupts islet-to-exocrine tissue adhesive contact (**Figure 1.1.2**). Suboptimal collagenase composition leads to incomplete digestion of islets from exocrine tissue along with reduced yield, decreased purity, increased duration of collagenase exposure adversely affects within-islet cell to-cell adhesion, leading to loss of islet integrity and viability. Thus, the use of highly pure and intact collagenase preparations is desirable to isolate pure islets with the least possible damage to the islets themselves.[40] The culturing of islets post-isolation is critical for their recovery from isolation-induced damage, however this may be at the cost of impaired revascularization subsequent to transplant, due to the loss of intra-islet endothelial cells during this culture

period. Sufficient oxygen and nutrient supply is a primary aim of culture conditions for human islet preparations. Moreover, the maintenance of the tri-dimensional islet cluster, as well as preventing islet mass loss should also be accomplished during the culturing phase. Though sufficient investigation of optimal culture conditions has occurred to date, protocols have yet to be standardized, and culture conditions may vary between islet isolation centers.[41] Other considerations like media composition, seeding density and incubation temperature play a significant role in maintaining viability and recovery.[41]

Further to extracellular culture requirements, the use of slightly impure islet preparations and co-culture with extracellular matrix components like collagen were shown to enhance the viability and function of isolated islets.[42] In addition, islet co-culture with pancreatic ductal epithelial cells were also shown to maintain islet viability and function post-isolation.[43] An essential component of the extracellular matrix, pancreatic ductal epithelial cells have been considered as putative stem cells for islets. The cells have been shown to play a critical role in secreting appropriate growth factors that support islet viability. In a pivotal study by Gatto et al., culturing techniques, like long-term culture and cryopreservation, had a negative impact on the viability of human islet preparations. These events were shown to be ameliorated when co-cultured with ductal epithelial cells at 33°C.[44] It has also been established that co-culture of islets with ductal epithelial cells assisted with the maintenance of structural integrity and prolonged viability.[40] Due to the conditions of the islet isolation procedure, islets become disconnected from their blood supply. As a result, hypoxic events during culture impact islet viability.[45,46] Although it may be difficult to prevent a hypoxic condition of the inner islet cell mass during *in vitro* culture, genetic modulation of islets to express genes

that promote rapid revascularization upon transplantation and reduced culture time could play an important role in preventing hypoxic damage to the islets.[47] Furthermore, the use of chemical agents that attenuate the downstream effects of hypoxia during culture may also be a feasible strategy to improve islet viability post-isolation and prior to transplantation.

1.1.5.3 – Islet engraftment

After transplantation, to adequately survive and function, islets heavily depend on the diffusion of oxygen and nutrients from the surrounding microenvironment. In order to regain proper islet function, new capillaries and blood vessels develop from their old capillary network. The new network derives from both, the recipient blood vessels but also from the remnant donor islet endothelium. This revascularization process may initiate as soon as 1 – 3 days post-transplant and may conclude round day 14.[48] Multiple studies report the enormous stress to which the islets are exposed during the first days after transplant.[40,48,49] Multiple factors combine and contribute to apoptosis and cell death, resulting in islet tissue loss of around 60%.[48]

Research efforts to improve intrahepatic islet delivery have identified multiple mechanisms that limit islet engraftment and long-term function. Intrahepatic transplantation is a minimally invasive portal infusion that results in islet entrapment within hepatic sinusoids. This vascular space provides nutritional and physical support for islets; an essential role given that isolation strips the islets of their dense vasculature and specialized extracellular matrix.[50,51] However, the hepatic portal vasculature can be considered as a hostile environment that limits islet engraftment and function[52]. Since

many more islets must be transplanted to reverse diabetes, a significant portion of the transplanted islets fails to engraft and become functional. It has been estimated that up to 70% of the transplanted β-cell mass may be destroyed in the early post-transplant period.

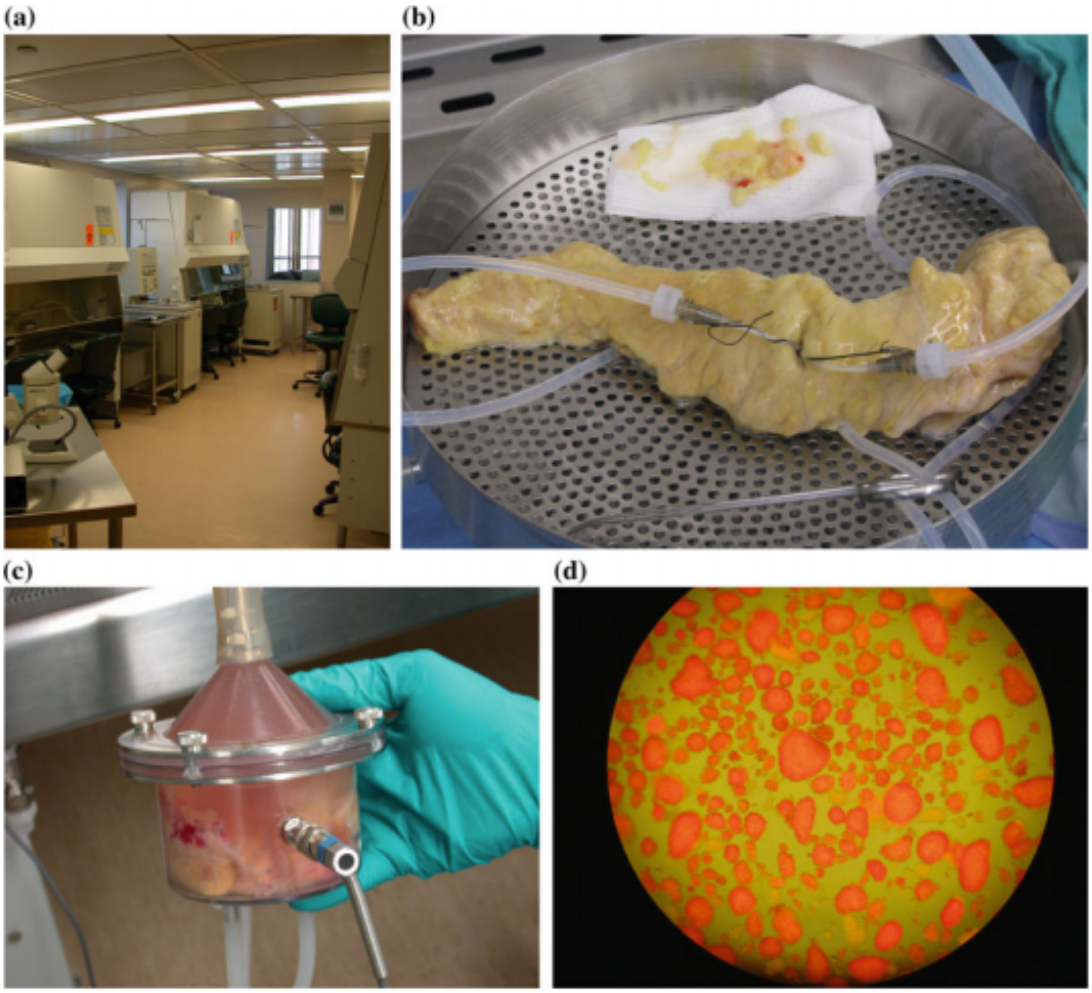

Figure 1.1.2 The islet isolation process.

(A) Highly specialized islet isolation facility. (B) Donor pancreas is cannulated and distended with collagenase via the primary duct. (C) The pancreas is sectioned and placed within the Ricordi chamber where it undergoes mechanical and enzymatic digestion. (D) Brightfield microscope image of a highly purified islet preparation stained with the zinc-chelator, dithizone.

Table 1.1.1 Summary of current clinical trials with refinements in islet transplantation. (Adapted from ClinicalTrials.gov at the time of publication)

Category	Trial ID	Description	Institution	Estimated Completion Date
Islet Alone	NCT00434811	Islet Transplantation in Type 1 Diabetes – Phase 3 (CIT-07)	National Institute of Allergy and Infectious Diseases	May 2014
Islet - Kidney	NCT01123187	Islet Cell Transplantation in Patients With Type 1 Diabetes With Previous Kidney Transplantation	University Hospital, Lille	March 2015
	NCT01241864	Islet Transplantation in Type 1 Diabetic Kidney Allograft	University of Chicago	December 2016
	NCT01705899	Islet Allotransplantation in Type 1 Diabetes: phase I trial comparing Islet alone vs Islet After kidney transplantation	Ohio State University	May 2017
	NCT00784966	Islet After Kidney Transplant for Type 1 Diabetes	Virginia Commonwealth University	September 2017
	NCT00468117	Efficacy of Islet After Kidney Transplantation – Phase 3 (CIT-06)	National Institute of Allergy and Infectious Diseases	December 2018
Alternative Transplant Sites	NCT00790257	Safety and Efficacy Study of Encapsulated Human Islets Allotransplantation to Treat Type 1 Diabetes (Subcutaneous Space)	Cliniques universitaires Saint Luc Université Catholique de Louvain	December 2013
	NCT01722682	Bone Marrow vs Liver as Site for Islet Transplantation	Ospedale San Raffaele	November 2014
	NCT01652911	A Phase I/II Study of the Safety and Efficacy of Sernova's Cell Pouch™ For Therapeutic Islet Transplantation (Subcutaneous Space)	University of Alberta	December 2014
	NCT01379729	Functional Survival of Beta Cell Allografts After Transplantation in the Peritoneal Cavity of Non-uremic Type 1 Diabetic Patients	Ziekenhuis Brussel / Ziekenhuizen Leuven Belgium	May 2018
Complications	NCT01148680	Trial Comparing Metabolic Efficiency of Islet Graft to Intensive Insulin Therapy for Type 1 Diabetes's Treatment	University Hospital, Grenoble	December 2014
	NCT00853424	A Comparison of Islet Cell Transplantation With Medical Therapy for the Treatment of Diabetic Eye Disease	University of British Columbia	June 2015
Refined Portal Vein Protocols	NCT00679042	Islet Transplantation in Type 1 Diabetic Patients Using the University of Illinois at Chicago Protocol	University of Illinois	December 2013
	NCT00789308	Safety and Effectiveness of Low Molecular Weight Sulfated Dextran in Islet Transplantation	National Institute of Allergy and Infectious Diseases	August 2014
	NCT01817959	Study to Assess Efficacy and Safety of Reparixin in Pancreatic Islet Transplantation	Multi-Centers International	November 2014
	NCT00530686	Pancreatic Islet Cell Transplantation - A Novel Approach to Improve Islet Quality and Engraftment	Baylor Research Institute	December 2014
	NCT01653899	Caspase Inhibition in Islet Transplantation	University of Alberta	June 2015
	NCT01897688	A Phase 2 Single Center Study of Islet Transplantation in Non-uremic Diabetic Patients	Northwestern University	June 2015
	NCT01186562	Sitagliptin Therapy to Improve Outcomes After Islet Autotransplant	University of Minnesota	September 2015
	NCT01630850	Islet Transplantation in Patients With (Brittle) Type 1 Diabetes	University of Chicago	June 2019
	NCT01974674	Allogeneic Islet Transplantation for the Treatment of Type 1 Diabetes (GRIIF)	Assistance Publique - Hôpitaux de Paris	January 2021
	NCT01909245	Islet Cell Transplant for Type 1 Diabetes (TCD)	City of Hope Medical Center	July 2021
Alternative Cellular Therapies	NCT00646724	Co-transplantation of Islet and Mesenchymal Stem Cell in Type 1 Diabetic Patients	Fuzhou General Hospital	January 2014
	NCT01736228	Open-label Investigation of the Safety and Efficacy of DIABECELL in Patients With T1DM: Xenotransplantation of Encapsulated Porcine Islets into the Peritoneal Cavity	Living Cell Technologies	December 2014
	NCT01350219	Stem Cell Educator Therapy in Type 1 Diabetes	Tianhe Stem Cell Biotechnologies Inc.	September 2014
	NCT01341899	Efficacy and Safety Study of Autologous Hematopoietic Stem Cell Transplantation to Treat New Onset Type 1 Diabetes	Nanjing University Medical School	December 2015
	NCT01285934	A Trial of High Dose Immunosuppression and Autologous Hematopoietic Stem Cell Support Versus Intensive Insulin Therapy in Adults With Early Onset T1DM	University of Sao Paulo General Hospital	December 2016

The culprit of this acute graft loss is the instant blood-mediated inflammatory reaction (IBMIR), which negatively influences islet engraftment through expression of tissue factor, resulting in platelet adherence, activation, clot formation and lymphocyte recruitment.[9,53] The direct impact of IBMIR on early loss of islet function and mass has yet to be fully characterized. However, given that platelet activation is one of the primary contributing factors in the generation of an inflammatory response, IBMIR is most likely one of the key processes that elicits an early immune response.[54-58] In a study conducted by Korsgren and colleagues, it was demonstrated that IBMIR is initiated upon intraportal infusion.[59] Specifically, in animal models and in recent clinical reports, marked activation of coagulation has been noted after islet infusion, despite the presence of heparin in the infusate.

During engraftment, transplanted islets are continuously exposed to immunosuppressive drugs, including tacrolimus and sirolimus, which are known to adversely impact β-cell survival and function.[60] Taken together, these negative effects are likely compounded by the proximity of the transplanted islets and high concentrations of these drugs in the hepatoportal circulation, further contributing to loss in β-cell mass over time.[61]

1.1.5.4 – Alternative islet transplantation sites

Today, intrahepatic islet infusion via the portal vein, accounts for virtually all clinical islet transplants conducted worldwide. While percutaneous portal vein infusion offers a minimally invasive procedure, with the ability to regulate glycemic levels through portal insulin delivery [62], it is not without potential procedural risks such as

portal thrombosis and bleeding.[63] A significant amount of intraportal islet mass is lost immediately post-transplant due to innate immune pathways involving platelet and complement activation described above. As such, extrahepatic islet transplantation has drawn focused attention recently to identify an optimal site to achieve sustained post-transplant insulin independence.

An ideal engraftment site should provide an adequate space to accommodate a large volume of transplanted islets, within close proximity to vascular networks supplying sufficient oxygen and nutrients during the revascularization period. Moreover, the site should prevent early islet loss due to host inflammatory reactions, while also providing accessibility for transplantation procedures and retrievability, if necessary.[64] The latter of which is paramount should insulin-producing stem cells therapies be translated into clinical practice.

Numerous sites have been proposed and tested, both experimentally and in some cases, clinically, including the liver, kidney subcapsule, spleen, pancreas, omentum, gastrointestinal wall, immune privileged sites and subcutaneous spaces. While some alternative sites may be advantageous in experimental models, their feasibility and translation into clinical settings is limited to date. For example, when compared to intraportal infusion in mice, a smaller islet mass is required to reverse hyperglycemia in the renal subcapsular space.[65,66] Clinically, however, the subcapsular site is less favorable, as a greater islet mass is required than intraportal infusion, as well as being much more invasive surgically.[67] Pepper et al. and Vériter et al. have summarized the utility of alternative transplant sites in experimental models and their prospective applicability to the clinical setting.[68,69]

Of the alternative transplant sites studied to date, the subcutaneous space may be considered attractive for multiple reasons. The subcutaneous site offers accessibility, and potential for biopsy access.[62] Subcutaneous transplantation of islets have been developed using alternative approaches including pre-implantation and vascularization of subcutaneous devices, encapsulation of islets, or a combination of both approaches.[69] Subcutaneous devices can be easily implanted, accessed for subsequent transplantation, as well as retrieved.[64,70] The subcutaneous space is limited however by its poor blood supply, which may considerably compromise islet function and engraftment. Experimental studies support this notion, as prevascularized devices prior to transplantation increased islet survival, as evidenced by improvements in hyperglycemia.[71,72] When immune-isolating devices are placed under the skin, they may require an enhanced oxygen-supply from an external source, as they are impermeable to vascular ingrowth. Currently, this technology is being tested in preclinical and clinical studies through the use of an implantable bioartificial pancreas.[73,74] Barkai and colleagues have reported that enhanced subcutaneous bioartificial pancreas containing a refillable oxygen reservoir was capable of maintaining islet function, as well as demonstrating immunoprotective characteristics in allogeneic and xenogeneic models.[73] Moreover, in an allogeneic human islet transplant setting using the same subcutaneous device, the authors demonstrated prolonged graft function and regulated insulin secretion without the need for immunosuppressive therapy[74]. The prospect of such a device is an attractive option in that it can be easily retrieved, reduces need for chronic immune suppression and may expand the utility of insulin-producing cells from an alternative supply, including stem cells and xenogeneic sources.

While intraportal islet infusion has clinically demonstrated the ability to abrogate T1DM, there continues to be an ongoing need to identify an alternative transplant site to optimize long-term clinical outcomes. Experimental investigation has provided potential alternatives to restore normoglycemia, although some approaches have suggested technical and/or physiological limitations. Conversely, other extrahepatic sites may hold promise in promoting β-cell viability, restoration of indefinite normoglycemia, as well as the prospect of eliminating immunosuppression in the allograft recipient.

1.1.5.5 – Improvements in immunosuppression

The 'Edmonton Protocol' established the immunosuppression scheme that utilized the combination of sirolimus, low-dose tacrolimus and daclizumab, in an effort to prevent the deleterious effects of calcineurin inhibitors and steroids.[75] However, insulin independence was not durable long-term, as most patients returned to modest amounts of insulin despite the elimination of recurrent hypoglycemia, by 5 years post-transplant, clearly indicating room for improvement.[76] Undoubtedly, a primary challenge to islet transplantation is the prevention of alloreactivity in addition to the recurrence of autoimmunity against insulin producing β-cells.[76]

It is unlikely that a monotherapy will optimize clinical islet transplantation outcomes and lead to single-donor recipients, due to the multiple pathways known to contribute to β-cell attrition, as well as the alloresponse to foreign antigens.[76] The implementation of highly potent and selective biological agents for the initiation and maintenance of immunosuppression has made significant progress in reducing the frequency of acute rejection, prolonging graft survival and minimizing the

complications of these therapeutic schemes.[77,78] Improvements to single-donor success rates were reported at the University of Minnesota through combining anti-inflammatory biologics to maintenance immunosuppression. [79,80] In addition, peritransplant insulin and heparin administration greatly increased the success rate of single-donor islet transplants from 10 to 40%.[81] Furthermore, the blockade of tumor necrosis factor-alpha with etanercept also has also enhanced single-donor islet transplant outcomes. [80-84]

The clonal depletion of alloreactive T cells appears to promote a hyporesponsive environment and peripheral mechanisms of anergy, thus driving the shift towards tolerance [85,86]. Substantial improvements in long-term insulin independence (>5 years) have been made possible through induction agents such as alemtuzumab in conjunction with tacrolimus/mycophenolate mofetil (MMF) that drive the process of T-cell depletion [17]. Combined immunosuppressive strategies have shown significant therapeutic benefit as reported by Posselt and colleagues. In the absence of calcineurin inhibitors, co-stimulation blockage using belatacept (inhibiting CD80-CD86 interactions) in conjunction with T-cell depletion induction led to insulin independence with islets from a single donor and prolonged allograft survival.[87]

The long-term success of islet transplantation, in part, is contingent on the successful establishment of an immunosuppressive regimen that promotes self-tolerance. A tolerizing regimen that utilizes biologics and techniques that selectively target donor-reactive T-cells while expanding populations of regulatory T cells, in an 'islet friendly' manner will undoubtedly lead to the definitive cure of T1DM.

1.1.6 – Immunomodulation : A method to prevent T1DM?

While obstacles and risks associated with islet transplantation still exist, alternative strategies to eliminate T1DM through immunomodulation have been proposed in experimental and clinical settings. Investigation of such strategies has been implemented in an effort to prevent the progression of β-cell destruction and clinical disease onset, without the need to transplant islets to restore euglycemia. To effectively establish such a feat would require identification of genetic, immunologic and metabolic parameters linked to T1DM.[88] Although extensive efforts have been made to identify such markers, success in these studies has been limited.[89] This can be attributed to the multi-component and heterogeneous immunologic response between patients that renders single-component therapies useless in preventing disease onset.[88] The aim of immunomodulation in T1DM, albeit through pharmacological or cellular replacement therapies, is to enhance regulatory immune cells to restore self-tolerance or eliminate pathogenic cells responsible for the destruction of pancreatic β-cells.

Pharmacological approaches of immune modulation to date include antigen-specific agents, as well as non-antigen specific agents. Examples of antigen-specific immune therapies undergoing clinical investigation include glutamate decarboxylase (GAD)65, as well as DiaPep227. GAD65 was identified over 20 years ago as an autoantigen expressed in β-cells[90]. Experimental and clinical studies have provided some insight into this prospective pharmacological tool, though conflicting results have been reported between animal and human trials. For example, GAD65 has been shown to prevent the spontaneous onset of T1DM in non-obese diabetic mice[91,92], while recent human trials have established that immunization with GAD65 were ineffective in

ameliorating β-cell destruction in patients with recent onset diabetes[93]. A critical component to these contradictory findings is the temporal relationship between therapeutic administration and disease onset. In NOD mice, treatment occurred prior to the onset of disease, while administration in humans occurred after the clinical manifestation of the disease. Taken together, these observations emphasize the importance of identifying markers for disease onset so that such therapies can be useful in rescuing from disease.

An example of non-antigen specific pharmacological agents used as a prospective immunomedulatory therapy is the use of teplizumab, an FcR non-binding anti-CD3 monoclonal antiobody. FcR non-binding of anti-CD3 induces adaptive regulatory T cells as evidenced from preclinical and clinical studies.[94,95] Compelling preclinical results in diabetic NOD mice elucidated prolonged remission of disease and achievement of immunologic tolerance.[95,96]. Clinical trials further established a protective effect on β-cell function for 1 to 2 years, however, protective effects diminished and disease progression ensued.[97] While this treatment may hold promise as a potential combination therapy, side effects like reactivation of Epstein-Barr virus and flu-like symptoms have been reported in some instances.[97]

The evaluation of pharmacological agents as an immune intervention to prevent residual β-cell loss and re-establish the autoimmune response has been evaluated in many clinical trials.[98-101] Though these therapies showed an improvement in C-peptide levels when compared to placebo groups, these effects were not maintained when immunosuppressive therapies were discontinued. As a means to circumvent this event, a pivotal study by Voltarelli and colleagues established a therapy of high-dose

cyclophosphamide administration followed by autologous nonmyeloablative hemoatopoeitic stem cell transplantation (AHST) in newly diagnosed T1DM patients. The compelling results of this clinical trial established self-tolerance, as evidenced by increased C-peptide levels and insulin independence in 93% of the study participants. It should be noted, however, that the mechanism of tolerance in this instance is not fully understood and cannot be definitively attributed to either T-regulatory suppression or clonal deletion.[102] While this study does show promise, the use of a potentially toxic immunosuppressive agent like cyclophosphamide may not warrant this as a prospective therapy to prevent T1DM due to long-term complications related to high-dose cyclophosphamide. The goal to establish "immunological reset" to prevent β-cell destruction and the onset of T1DM is an attractive therapy that may be attainable with the use of more safe and effective immunosuppressive therapies and anti-inflammatory agents, paired with autologous stem cell transplantation.

1.1.7 – CONCLUSION

Undoubtedly, islet isolation and transplantation, introduction of the 'Edmonton Protocol' and subsequent important developments internationally have played a major role in improving the results and activity in clinical islet transplantation. While islet transplantation cannot currently be defined as a cure for T1DM, the therapy can offer remarkable stability of glycemic control, correction of HbA1c, and an increasing number of patients can enjoy sustained periods of complete independence from insulin. Prevention of life-threatening hypoglycemia is a major advance that can often not be sustained by optimized exogenous insulin therapy. In parallel to the strategies implemented to overcome limitations associated with islet transplantation, alternative

methods to drive self-tolerance and prevent the onset of T1DM are also under investigation. Nevertheless, concerted efforts to improve the lives of those afflicted with T1DM rapidly drive the transition from experimental research to clinical care.

1.1.8 - REFERENCES

1. Polonsky KS. The past 200 years in diabetes. *The New England journal of medicine.* 2012;367(14):1332-1340.
2. Banting FG, Best CH, Collip JB, Campbell WR, Fletcher AA. Pancreatic Extracts in the Treatment of Diabetes Mellitus. *Canadian Medical Association journal.* 1922;12(3):141-146.
3. Agarwal A, Brayman KL. Update on islet cell transplantation for type 1 diabetes. *Seminars in interventional radiology.* 2012;29(2):90-98.
4. Lacy PE, Kostianovsky M. Method for the isolation of intact islets of Langerhans from the rat pancreas. *Diabetes.* 1967;16(1):35-39.
5. Reckard CR, Ziegler MM, Barker CF. Physiological and immunological consequences of transplanting isolated pancreatic islets. *Surgery.* 1973;74(1):91-99.
6. Shapiro AM. A historical perspective on experimental and clinical islet transplantation. *Informa Health Care.* 2007:1.
7. Lakey JR, Warnock GL, Shapiro AM, et al. Intraductal collagenase delivery into the human pancreas using syringe loading or controlled perfusion. *Cell transplantation.* 1999;8(3):285-292.
8. Ricordi C, Lacy PE, Scharp DW. Automated islet isolation from human pancreas. *Diabetes.* 1989;38 Suppl 1:140-142.
9. Emamaullee JA, Shapiro AM. Interventional strategies to prevent beta-cell apoptosis in islet transplantation. *Diabetes.* 2006;55(7):1907-1914.

10. Shapiro AM, Lakey JR, Ryan EA, 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(4):230-238.

11. Ryan EA, Bigam D, Shapiro AM. Current indications for pancreas or islet transplant. *Diabetes, obesity & metabolism.* 2006;8(1):1-7.

12. Scharp DW, Kemp CB, Knight MJ, Ballinger WF, Lacy PE. The use of ficoll in the preparation of viable islets of langerhans from the rat pancreas. *Transplantation.* 1973;16(6):686-689.

13. Najarian JS, Sutherland DE, Matas AJ, Steffes MW, Simmons RL, Goetz FC. Human islet transplantation: a preliminary report. *Transplantation proceedings.* 1977;9(1):233-236.

14. Brendel M HB, Shulz A, Bretzel R. International Islet Transplant Registry Report. 1999.

15. The CITR Coordinating Center and Investigators. The Collaborative Islet Transplant Registry (CITR) 2011 Seventh Annual Report

16. Ly TT, Nicholas JA, Retterath A, Lim EM, Davis EA, Jones TW. Effect of sensor-augmented insulin pump therapy and automated insulin suspension vs standard insulin pump therapy on hypoglycemia in patients with type 1 diabetes: a randomized clinical trial. *JAMA : the journal of the American Medical Association.* 2013;310(12):1240-1247.

17. Shapiro AM. Strategies toward single-donor islets of Langerhans transplantation. *Current opinion in organ transplantation.* 2011;16(6):627-631.

18. Thompson DM, Meloche M, Ao Z, et al. Reduced progression of diabetic microvascular complications with islet cell transplantation compared with intensive medical therapy. *Transplantation.* 2011;91(3):373-378.

19. Barton FB, Rickels MR, Alejandro R, et al. Improvement in outcomes of clinical islet transplantation: 1999-2010. *Diabetes care.* 2012;35(7):1436-1445.

20. Senior PA, Kin T, Shapiro AMJ, Koh A. Islet Transplantation at the University of Alberta: Status Update and Review of Progress over the Last Decade. *Canadian Journal of Diabetes.* 2012;36:32-37.

21. Merani S, Shapiro AM. Current status of pancreatic islet transplantation. *Clin Sci (Lond).* 2006;110(6):611-625.

22. Pepper AR, Gala-Lopez B, Ziff O, Shapiro AJ. Current status of clinical islet transplantation. *World J Transplant.* 2013;3(4):48-53.

23. Senior PA KT, Shapiro AMJ, Koh A. Islet transplantation at the University of Alberta: Status update and review of progress over the last decade. *Can J Diabetes* 2012(36):32 - 37.

24. Benhamou PY, Watt PC, Mullen Y, et al. Human islet isolation in 104 consecutive cases. Factors affecting isolation success. *Transplantation.* 1994;57(12):1804-1810.

25. Goto M, Eich TM, Felldin M, 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(9):1367-1375.

26. Ihm SH, Matsumoto I, Sawada T, et al. Effect of donor age on function of isolated human islets. *Diabetes.* 2006;55(5):1361-1368.

27. Lakey JR, Rajotte RV, Warnock GL, Kneteman NM. Human pancreas preservation prior to islet isolation. Cold ischemic tolerance. *Transplantation.* 1995;59(5):689-694.

28. Lakey JR, Warnock GL, Rajotte RV, et al. Variables in organ donors that affect the recovery of human islets of Langerhans. *Transplantation.* 1996;61(7):1047-1053.

29. Matsumoto I, Sawada T, Nakano M, et al. Improvement in islet yield from obese donors for human islet transplants. *Transplantation.* 2004;78(6):880-885.

30. Nano R, Clissi B, Melzi R, et al. Islet isolation for allotransplantation: variables associated with successful islet yield and graft function. *Diabetologia.* 2005;48(5):906-912.

31. Zeng Y, Torre MA, Karrison T, Thistlethwaite JR. The correlation between donor characteristics and the success of human islet isolation. *Transplantation.* 1994;57(6):954-958.

32. Kin T, Murdoch TB, Shapiro AM, Lakey JR. Estimation of pancreas weight from donor variables. *Cell transplantation.* 2006;15(2):181-185.

33. Brandhorst H, Brandhorst D, Hering BJ, Federlin K, Bretzel RG. Body mass index of pancreatic donors: a decisive factor for human islet isolation. *Exp Clin Endocrinol Diabetes.* 1995;103 Suppl 2:23-26.

34. Berney T, Johnson PR. Donor pancreata: evolving approaches to organ allocation for whole pancreas versus islet transplantation. *Transplantation.* 2010;90(3):238-243.

35. O'Gorman D, Kin T, Murdoch T, et al. The standardization of pancreatic donors for islet isolation. *Transplantation proceedings.* 2005;37(2):1309-1310.

36. Witkowski P, Liu Z, Cernea S, et al. Validation of the scoring system for standardization of the pancreatic donor for islet isolation as used in a new islet isolation center. *Transplantation proceedings.* 2006;38(9):3039-3040.

37. Noguchi H. Pancreas procurement and preservation for islet transplantation: personal considerations. *Journal of transplantation.* 2011;2011:783168.

38. Saito T, Gotoh M, Satomi S, et al. Islet transplantation using donors after cardiac death: report of the Japan Islet Transplantation Registry. *Transplantation.* 2010;90(7):740-747.

39. Wolters GH, Vos-Scheperkeuter GH, van Deijnen JH, van Schilfgaarde R. An analysis of the role of collagenase and protease in the enzymatic dissociation of the rat pancreas for islet isolation. *Diabetologia.* 1992;35(8):735-742.

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

41. 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.

42. Nagata NA, Inoue K, Tabata Y. Co-culture of extracellular matrix suppresses the cell death of rat pancreatic islets. *Journal of biomaterials science. Polymer edition.* 2002;13(5):579-590.

43. Lakey JR, Woods EJ, Zieger MA, et al. Improved islet survival and in vitro function using solubilized small intestinal submucosa. *Cell and tissue banking.* 2001;2(4):217-224.

44. Gatto C, Callegari M, Folin M, et al. Effects of cryopreservation and coculture with pancreatic ductal epithelial cells on insulin secretion from human pancreatic islets. *International journal of molecular medicine.* 2003;12(6):851-854.

45. Velmurugan K, Balamurugan AN, Loganathan G, Ahmad A, Hering BJ, Pugazhenthi S. Antiapoptotic actions of exendin-4 against hypoxia and cytokines are augmented by CREB. *Endocrinology.* 2012;153(3):1116-1128.

46. Bloch K, Vennang J, Lazard D, Vardi P. Different susceptibility of rat pancreatic alpha and beta cells to hypoxia. *Histochemistry and cell biology.* 2012;137(6):801-810.

47. Narang AS, Cheng K, Henry J, et al. Vascular endothelial growth factor gene delivery for revascularization in transplanted human islets. *Pharmaceutical research.* 2004;21(1):15-25.

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

49. Merani S, Toso C, Emamaullee J, Shapiro AM. Optimal implantation site for pancreatic islet transplantation. *The British journal of surgery.* 2008;95(12):1449-1461.

50. Gibly RF, Graham JG, Luo X, Lowe WL, Jr., Hering BJ, Shea LD. Advancing islet transplantation: from engraftment to the immune response. *Diabetologia.* 2011;54(10):2494-2505.

51. Wang RN, Rosenberg L. Maintenance of beta-cell function and survival following islet isolation requires re-establishment of the islet-matrix relationship. *The Journal of endocrinology.* 1999;163(2):181-190.

52. Barshes NR, Wyllie S, Goss JA. Inflammation-mediated dysfunction and apoptosis in pancreatic islet transplantation: implications for intrahepatic grafts. *Journal of leukocyte biology.* 2005;77(5):587-597.

53. Plesner A, Verchere CB. Advances and challenges in islet transplantation: islet procurement rates and lessons learned from suboptimal islet transplantation. *Journal of transplantation.* 2011;2011:979527.

54. Goto M, Tjernberg J, Dufrane D, et al. Dissecting the instant blood-mediated inflammatory reaction in islet xenotransplantation. *Xenotransplantation.* 2008;15(4):225-234.

55. Vivot K, Jeandidier N, Dollinger C, et al. Role of islet culture on angiogenic and inflammatory mechanisms. *Transplantation proceedings.* 2011;43(9):3201-3204.

56. Nilsson B, Ekdahl KN, Korsgren O. Control of instant blood-mediated inflammatory reaction to improve islets of Langerhans engraftment. *Current opinion in organ transplantation.* 2011;16(6):620-626.

57. Ji M, Yi S, Smith-Hurst H, et al. The importance of tissue factor expression by porcine NICC in triggering IBMIR in the xenograft setting. *Transplantation.* 2011;91(8):841-846.

58. Ma X, Ye B, Gao F, et al. Tissue factor knockdown in porcine islets: an effective approach to suppressing the instant blood-mediated inflammatory reaction. *Cell transplantation.* 2012;21(1):61-71.

59. Ozmen L, Ekdahl KN, Elgue G, Larsson R, Korsgren O, Nilsson B. Inhibition of thrombin abrogates the instant blood-mediated inflammatory reaction triggered by isolated human islets: possible application of the thrombin inhibitor melagatran in clinical islet transplantation. *Diabetes.* 2002;51(6):1779-1784.

60. Bloom SR, Polak JM. Somatostatin. *British medical journal.* 1987;295(6593):288-290.

61. Korsgren O, Lundgren T, Felldin M, et al. Optimising islet engraftment is critical for successful clinical islet transplantation. *Diabetologia.* 2008;51(2):227-232.

62. Rajab A. Islet transplantation: alternative sites. *Curr Diab Rep.* 2010;10(5):332-337.

63. Barshes NR, Lee TC, Goodpastor SE, et al. Transaminitis after pancreatic islet transplantation. *J Am Coll Surg.* 2005;200(3):353-361.

64. Veriter S, Gianello P, Dufrane D. Bioengineered Sites for Islet Cell Transplantation. *Curr Diab Rep.* 2013.

65. Lacy PE, Ricordi C, Finke EH. Effect of transplantation site and alpha L3T4 treatment on survival of rat, hamster, and rabbit islet xenografts in mice. *Transplantation.* 1989;47(5):761-766.

66. Mellgren A, Schnell Landstrom AH, Petersson B, Andersson A. The renal subcapsular site offers better growth conditions for transplanted mouse pancreatic islet cells than the liver or spleen. *Diabetologia.* 1986;29(9):670-672.

67. Jindal RM, Sidner RA, McDaniel HB, Johnson MS, Fineberg SE. Intraportal vs kidney subcapsular site for human pancreatic islet transplantation. *Transplantation proceedings.* 1998;30(2):398-399.

68. Pepper AR, Gala-Lopez B, Ziff O, Shapiro AM. Revascularization of transplanted pancreatic islets and role of the transplantation site. *Clinical & developmental immunology.* 2013;2013:352315.

69. Veriter S, Gianello P, Dufrane D. Bioengineered sites for islet cell transplantation. *Curr Diab Rep.* 2013;13(5):745-755.

70. Dufrane D, Goebbels RM, Gianello P. Alginate macroencapsulation of pig islets allows correction of streptozotocin-induced diabetes in primates up to 6 months without immunosuppression. *Transplantation.* 2010;90(10):1054-1062.

71. Rafael E, Wu GS, Hultenby K, Tibell A, Wernerson A. Improved survival of macroencapsulated islets of Langerhans by preimplantation of the immunoisolating device: a morphometric study. *Cell transplantation.* 2003;12(4):407-412.

72. Sorenby AK, Kumagai-Braesch M, Sharma A, Hultenby KR, Wernerson AM, Tibell AB. Preimplantation of an immunoprotective device can lower the curative dose of islets to that of free islet transplantation: studies in a rodent model. *Transplantation.* 2008;86(2):364-366.

73. Barkai U, Weir GC, Colton CK, et al. Enhanced oxygen supply improves islet viability in a new bioartificial pancreas. *Cell transplantation.* 2013;22(8):1463-1476.

74. Ludwig B, Reichel A, Steffen A, et al. Transplantation of human islets without immunosuppression. *Proceedings of the National Academy of Sciences of the United States of America.* 2013;110(47):19054-19058.

75. Ryan EA, Paty BW, Senior PA, et al. Five-year follow-up after clinical islet transplantation. *Diabetes.* 2005;54(7):2060-2069.

76. Gala-Lopez B, Pepper AR, Shapiro AM. Biologic agents in islet transplantation. *Curr Diab Rep.* 2013;13(5):713-722.

77. Shapiro AM. A historical perspective on experimental and clinical islet transplantation. In: Shapiro AM, Shaw J.A., ed. *Islet transplantation and beta cell replacement therapy*. New York, London: Informa Healthcare; 2007.

78. Gabardi S, Martin ST, Roberts KL, Grafals M. Induction immunosuppressive therapies in renal transplantation. *American journal of health-system pharmacy : AJHP : official journal of the American Society of Health-System Pharmacists.* 2011;68(3):211-218.

79. Hering BJ. Repurification: rescue rather than routine remedy. *Am J Transplant.* 2005;5(1):1-2.

80. Hering BJ, Kandaswamy R, Ansite JD, et al. Single-donor, marginal-dose islet transplantation in patients with type 1 diabetes. *JAMA : the journal of the American Medical Association.* 2005;293(7):830-835.

81. Koh A, Senior P, Salam A, et al. Insulin-heparin infusions peritransplant substantially improve single-donor clinical islet transplant success. *Transplantation.* 2010;89(4):465-471.

82. Matsumoto S, Takita M, Chaussabel D, et al. Improving Efficacy of Clinical Islet Transplantation with Iodixanol Based Islet Purification, Thymoglobulin Induction and Blockage of IL-1-beta and TNF-alpha. *Cell transplantation.* 2011.

83. Shapiro AM, Ricordi C. Unraveling the secrets of single donor success in islet transplantation. *Am J Transplant.* 2004;4(3):295-298.

84. Xenos ES, Farney AC, Widmer MB, et al. Effect of tumor necrosis factor alpha and of the soluble tumor necrosis factor receptor on insulin secretion of isolated islets of Langerhans. *Transplantation proceedings.* 1992;24(6):2863-2864.

85. Bhatt S, Fung JJ, Lu L, Qian S. Tolerance-inducing strategies in islet transplantation. *International journal of endocrinology.* 2012;2012:396524.

86. Shapiro AM, Nanji SA, Lakey JR. Clinical islet transplant: current and future directions towards tolerance. *Immunological reviews.* 2003;196:219-236.

87. Posselt AM, Szot GL, Frassetto LA, 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(12):1595-1601.

88. Skyler JS, Ricordi C. Stopping type 1 diabetes: attempts to prevent or cure type 1 diabetes in man. *Diabetes.* 2011;60(1):1-8.

89. Skyler JS, Type 1 Diabetes TrialNet Study G. Update on worldwide efforts to prevent type 1 diabetes. *Annals of the New York Academy of Sciences.* 2008;1150:190-196.

90. Baekkeskov S, Aanstoot HJ, Christgau S, et al. Identification of the 64K autoantigen in insulin-dependent diabetes as the GABA-synthesizing enzyme glutamic acid decarboxylase. *Nature.* 1990;347(6289):151-156.

91. Tian J, Clare-Salzler M, Herschenfeld A, et al. Modulating autoimmune responses to GAD inhibits disease progression and prolongs islet graft survival in diabetes-prone mice. *Nature medicine.* 1996;2(12):1348-1353.

92. Pleau JM, Fernandez-Saravia F, Esling A, Homo-Delarche F, Dardenne M. Prevention of autoimmune diabetes in nonobese diabetic female mice by treatment with recombinant glutamic acid decarboxylase (GAD 65). *Clinical immunology and immunopathology.* 1995;76(1 Pt 1):90-95.

93. Wherrett DK, Bundy B, Becker DJ, et al. Antigen-based therapy with glutamic acid decarboxylase (GAD) vaccine in patients with recent-onset type 1 diabetes: a randomised double-blind trial. *Lancet.* 2011;378(9788):319-327.

94. Belghith M, Bluestone JA, Barriot S, Megret J, Bach JF, Chatenoud L. TGF-beta-dependent mechanisms mediate restoration of self-tolerance induced by antibodies to CD3 in overt autoimmune diabetes. *Nature medicine.* 2003;9(9):1202-1208.

95. Chatenoud L, Primo J, Bach JF. CD3 antibody-induced dominant self tolerance in overtly diabetic NOD mice. *Journal of immunology.* 1997;158(6):2947-2954.

96. Chatenoud L, Thervet E, Primo J, Bach JF. Anti-CD3 antibody induces long-term remission of overt autoimmunity in nonobese diabetic mice. *Proceedings of the National Academy of Sciences of the United States of America.* 1994;91(1):123-127.

97. Keymeulen B, Walter M, Mathieu C, et al. Four-year metabolic outcome of a randomised controlled CD3-antibody trial in recent-onset type 1 diabetic patients

depends on their age and baseline residual beta cell mass. *Diabetologia.* 2010;53(4):614-623.

98. Cook JJ, Hudson I, Harrison LC, et al. Double-blind controlled trial of azathioprine in children with newly diagnosed type I diabetes. *Diabetes.* 1989;38(6):779-783.

99. Elliott RB, Crossley JR, Berryman CC, James AG. Partial preservation of pancreatic beta-cell function in children with diabetes. *Lancet.* 1981;2(8247):631-632.

100. Harrison LC, Colman PG, Dean B, Baxter R, Martin FI. Increase in remission rate in newly diagnosed type I diabetic subjects treated with azathioprine. *Diabetes.* 1985;34(12):1306-1308.

101. Silverstein J, Maclaren N, Riley W, Spillar R, Radjenovic D, Johnson S. Immunosuppression with azathioprine and prednisone in recent-onset insulin-dependent diabetes mellitus. *The New England journal of medicine.* 1988;319(10):599-604.

102. Voltarelli JC, Couri CE, Stracieri AB, et al. Autologous nonmyeloablative hematopoietic stem cell transplantation in newly diagnosed type 1 diabetes mellitus. *JAMA : the journal of the American Medical Association.* 2007;297(14):1568-1576.

CHAPTER 1

INTRODUCTION

PART 2

REGULATED CELL DEATH SEEN THROUGH THE LENS OF ISLET TRANSPLANTATION

ORIGINAL ARTICLE

REGULATED CELL DEATH SEEN THROUGH THE LENS OF ISLET TRANSPLANTATION

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1.2.1 - ABSTRACT

Clinical islet transplantation effectively restores euglycemia and corrects glycosylated hemoglobin in labile type 1 diabetes. Despite marked improvements in islet transplantation outcomes, acute islet cell death remains a substantial obstacle that compromises long-term engraftment outcomes. Multiple organ donors are routinely required to achieve insulin independence. Therapeutic agents that ameliorate cell death and/or control injury-related inflammatory cascades offer potential to improve islet transplant success. Apoptotic cell death has been identified as a major contributor to cellular demise and therapeutic strategies that subvert initiation and consequences of apoptotic cell death have shown promise in pre-clinical models. Indeed, apoptosis has been the most extensively described form of regulated cell death in numerous pathologies and diseases. However, recent identification of novel, alternative regulated cell death pathways in other disease states and solid organ transplantation suggest that these additional pathways may also have substantial relevance in islet transplantation. These regulated, non-apoptotic cell death pathways exhibit distinct biochemical characteristics but have yet to be fully characterized within islet transplantation. We review herein the various regulated cell death pathways and highlight their relative potential contributions to islet viability, engraftment failure and islet dysfunction.

1.2.2 – INTRODUCTION

Inroads in clinical islet transplantation have demonstrated repeatedly that this therapy protects against hypoglycemia, corrects hemoglobin A1C, improves overall glycemic control, and to a more variable degree can secure and maintain insulin independence for periods of time. Studies by the Clinical Islet Transplant Consortium (CIT) and a comprehensive Collaborative Islet Transplant Registry (CITR) strongly endorse these findings.[1,2] Several studies suggest that both pancreas and islet transplantation may impede evolution of several long-term secondary complications associated with type1 diabetes mellitus (T1DM). However, despite marked improvements in outcome over the past two decades, a consistent finding is that except in highly selected series, multiple organ donors and a cumulative islet implant mass ≥10,000 islet equivalents per kilogram recipient weight (IE/kg) are consistently required.[3] While both auto- and allo-immune-mediated mechanisms clearly contribute to long-term graft failure, mounting evidence strongly suggests that acute islet cell death in the immediate and peri-transplant period severely compromises engraftment outcomes. Islet transplantation is unique across organ transplantation as the complex enzymatic process required to mechanically separate islets away from their extracellular matrix, the purification and culture steps cumulatively result in injury. The subsequent transplantation to the hypoxic, intrahepatic portal site and many days to establishment of neovascularization render islets far more susceptible to injury than solid organ grafts.

Apoptosis is generally considered the primary form of regulated cell death, mediating biological functions such as homeostasis, development and pathogenesis.[4,5] Necrotic cell death was once considered as an all-encompassing, uncontrolled modality

that occurred in response to unabated environmental triggers, hypoxia and physiological stress, resulting in the release of intracellular contents of dying cells.[6] Indeed, in instances of extreme stimuli, such as high temperatures, resultant necrotic cell death can occur in an accidental manner.[6] However, recent evidence has identified the existence of several alternative forms of regulated cell death, collectively termed 'regulated necrosis' (RN), elicited by pathophysiological conditions that occur in a genetically controlled fashion.[6] The recent identification of these cell death pathways has revealed non-apoptotic mechanisms, some of which are caspase-independent, characterized by morphologically and biochemically distinct events.[7] (**Figure 1.2**) In contrast to apoptosis, which is immunologically silent at least in the initial phase, RN-pathways inevitably release damage associated molecular patterns (DAMPs) when the plasma membrane ruptures, and thereby trigger an inflammatory cascade. Whereas all RN-pathways are to some extent immunogenic, active production of cytokines during the death process modulates the immunogenic response and may provide an evolutionary advantage to conserve different RN-subroutines.[6] These pathways include, but are not limited to, pyroptosis, ferroptosis, necroptosis and parthanatos. (**Table 1.2.1**)

Within the context of islet isolation and transplantation, our group has examined several therapeutic interventions that truncate apoptotic cell death, either with pan-caspase inhibitors or agents that ameliorate onset of apoptosis.[3,8-12] The field of islet transplantation has identified apoptosis as the primary culprit of programmed cell death in experimental and clinical investigation.[13] Therapeutic strategies that dampen the inflammatory response in the acute transplant period have also been employed with considerable focus to subvert apoptotic cell death. With the emergence of recently

defined, genetically and biochemically distinct pathways, re-examination of cell death modalities that contribute to islet loss is warranted. Indeed, some of the mechanisms attributed to the various regulated cell death pathways have been identified in β-cell death and islet transplantation, yet these modalities have not been exclusively defined. Herein, we discuss regulated cell death and its potential contributions to islet cell death in islet transplantation, and highlight prospective interventional strategies to ameliorate the consequences associated with islet loss.

1.2.3 - REGULATED CELL DEATH – IMPLICATIONS IN ISLET TRANSPLANTATION

1.2.3.1 Caspase-Dependent Cell Death

1.2.3.1.1 Apoptosis

Once considered the only form of 'programmed cell death', apoptosis can be triggered by multiple stimuli. A distinct set of cysteine proteases termed caspases, are key mediators of apoptosis that become activated by pro-apoptotic stimuli.[14] Activation of initiator caspases (caspase-8 and -9) results in the downstream cleavage and further activation of executioner caspases (caspase-3, -6 and -7) with subsequent cellular morphological changes including DNA fragmentation and membrane blebbing.[15] Apoptosis is considered the least immunogenic form of programmed cell death, as it does not lead to plasma membrane rupture, however, it is not entirely immunologically silent.[6,16]

Apoptosis can be triggered by extracellular (extrinsic) or intracellular (intrinsic) cues. Binding of ligands to death receptors initiates the extrinsic pathway. Death receptors are members of the tumor necrosis factor (TNF) superfamily, which includes

TNF receptor-1 (TNFR1) and CD95 (also called Fas and APO-1).[15] Ligand-receptor binding (TNF-α to TNFR1) results in receptor clustering, adaptor molecule recruitment (i.e. Fas-associated death domain; FADD), and the formation of the death-inducing signaling complex (DISC). The initiator caspase, caspase-8, associates with the DISC complex, where it is activated. Caspase-8 initiates apoptosis by cleaving and activating executioner caspases.[15] It is now clear, however, that the most important function of caspase-8 is the prevention of necroptotic cell death.[17-19]

Apoptosis has largely been identified as the primary form of programmed cell death contributing to islet loss. Intrinsic cues, such as reactive oxygen species (ROS) and hypoxia, as well as extrinsic cues, instant-blood mediated inflammatory reaction (IBMIR) and inflammatory cytokine stimulation such as TNF-α and interleukin (IL)-1β, have demonstrated ability to initiate the apoptotic cascade. Methods of mitigating apoptosis activation in islets through inhibition of circulating inflammatory cytokines, IL-1β and TNF-α, have demonstrated improved engraftment outcomes in the experimental setting. In an immunocompromised murine model, human islet transplant recipients synergistically administered IL-1β receptor agonist (Anakinra) and TNF-α receptor fusion protein (Etanercept) exhibited improved islet engraftment outcomes.[20] Islet grafts harvested 24 hours post-transplant exhibited reduced apoptosis levels, as measured by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining, suggesting these agents mitigated activation of the extrinsic apoptotic pathway. Interventional strategies aimed to inhibit caspase activation directly, thus preventing the downstream apoptotic cascade have been utilized in the preclinical setting to improve islet engraftment outcomes.

Table 1.2.1 Regulated cell death pathways influenced by key mediators, morphological characteristics associated with the cell death modality and their immunogenic potential.

Regulated Cell Death Pathway	Key Mediator(s)	Morphological Features	Immunogenicity
Apoptosis	Initiator Caspases (Caspase-8 and -10 Executor Caspases (Caspase-3, -6 and -7)	Nuclear chromatin condensation Cellular shrinkage Membrane Blebbing	Absent
Pyroptosis	Caspase-1 and Caspase-11	Cellular necrosis Membrane rupture Release of IL-1b and IL-18	+++
Ferroptosis	GPX4	Cellular necrosis membrane rupture	Unknown
Necroptosis	RIPK3	Cellular necrosis Membrane rupture Release of IL-33 and CXCL1	+
Parthanatos	PARP1	Cellular necrosis Membrane rupture	Unknown

GPX4 - Glutathione peroxidase-4; PARP1 – poly(ADP-ribose) polymerase 1; RIPK3 – Receptor-interacting protein kinase 3; + moderately immunogenic; +++ highly immunogenic

Early generation pan-caspase-inhibitors, such as N-benzyloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone (zVAD-FMK) or N-benzyloxycarbonyl-Val-Asp-fluoromethyl ketone (zVD-FMK), have demonstrated the ability to augment islet graft survival and long-term outcomes when administered systemically in the acute transplant period.[3,9] When administered concomitantly with the co-stimulatory blockade agent, high affinity CTLA-4Ig (Belatacept), zVD-FMK enhanced islet engraftment outcomes in a murine allotransplantation model.[8] The development of novel, potent inhibitors of caspases, such as IDN-6556, have also demonstrated the ability to improve engraftment outcomes using marginal islet doses in a murine syngeneic model and porcine autograft model.[10,11] Most recently, our group demonstrated that the potent pan-caspase inhibitor, F573, could effectively reduce the incidence of apoptosis in murine and human islets *in vitro.* Furthermore, when administered to the recipient in the acute post-transplant period, F573 was able to deter apoptosis and improve long-term marginal islet engraftment outcomes in a murine transplant model. Most notably, the administration of F573 was able to augment engraftment in the clinically relevant portal site, as well as under the modified subcutaneous space.[12]

The utility of pan-caspase and other inhibitors in islet transplantation has identified apoptosis as a key player that influences islet viability and contributes to islet engraftment outcomes. Therapeutic strategies aimed at reducing apoptosis have clearly demonstrated efficacy pre-clinically and merits the utility of pan-caspase inhibitors as an adjuvant clinical therapy in the acute transplant period. While these studies highlighted the impact of apoptosis, most often overlooked other regulated cell death pathways and impact upon islet engraftment. With identification of newly defined, biochemically

distinct, regulated cell death pathways, alternative interventions should now also be examined in islet transplantation to explore their potential as therapeutic targets for islet protection in the translational clinical setting.

1.2.3.1.2 Pyroptosis

Pyroptosis is a caspase-dependent form of regulated cell death that is biochemically and phenotypically distinct from apoptosis and other regulated necrosis pathways. Dependent on caspase-1 and caspase-11, pyroptosis is unique from apoptosis in that it does not require the activation of caspase-3, -7, -8 or -9.[21] Activation of caspase-1 or caspase-11 is reliant on the formation of multi-protein signaling complexes termed inflammasomes that assemble in response to various stimuli, including intracellular microbial ligands or cellular perturbations.[22] While inflammasomes are encompassed within three gene families, for the purpose of this review we will focus on the Nod-like receptors (NLRs), more specifically NLRs that contain a pyrin domain (PYD) that mediates the signaling event. The NLRs containing a PYD (NLRP) signal through the apoptosis-associated speck-like protein containing a CARD (caspase-associated recruitment domain) which is responsible for the recruitment of caspase-1. Activation of caspase-1 or caspase-11 results in the maturation of the pyroptosis-specific cytokines, IL-1β and IL-18, and in the cleavage of the pyroptosis-mediating molecule gasdermin D (GSDMD) the translocation of which to the plasma membrane is required for subsequent cell death and IL-1b/IL-18 release.[17,23-25] Pyroptosis has largely been implicated in the host's innate defense against intracellular pathogens,[26] however, it has also been identified in the promotion of chronic liver injury.[27,28] Within the context of

islet transplantation, pyroptosis has not been elucidated, although emerging evidence suggests that mediators of pyroptosis may contribute to compromised β-cell function and viability, particularly within the context of type 2 diabetes mellitus (T2DM), which may have translational implications in islet transplantation.

Inflammation plays a critical role leading to β-cell dysfunction and death.[29,30] IL-1β, a key inflammatory cytokine has also been implicated in type-2 diabetes mellitus. Clinical studies inhibiting IL-1β either by IL-1 receptor antagonist (IL-1Ra) or IL-1β antibody, suggest improved glycemic control and β-cell function in type 2 diabetes (T2DM).[31,32] Given that NLRP3 activation drives IL-1β secretion, it is at least plausible that this inflammasome contributes to islet dysfunction and death. Islet amyloid polypeptide, a protein that forms amyloid deposits in islets of patients with T2DM, has demonstrated the capacity to trigger NLRP3 inflammasome activation and subsequent maturation and secretion of IL-1β.[33] Within the context of islet transplantation, amyloid deposition in human islets has been observed as early as 2 weeks in an immune-compromised murine transplant model.[34] Moreover, amyloid deposition was discovered in liver sections at the time of autopsy in clinical patients that exhibited marginal graft function at the time of death.[17,35,36] These findings suggest that amyloid deposition is not restricted to patients with T2DM and may trigger NLRP3 activation in islet transplant recipients, thus contributing to graft failure. Potter and colleagues revealed that human *in vitro* islet viability was preserved when treated with a potent inhibitor of amyloid formation.[37] Ongoing studies will determine if caspase-1-specific inhibitors or NLRP3-specific inhibitors can mitigate the deleterious effects associated with amyloid deposition in islets. In previous studies employing pan-caspase inhibitors, pyroptosis

may have also been impaired in tandem with apoptosis, since these inhibitors indiscriminately inhibit multiple caspases, including caspase-1. Further studies evaluating the role of inflammasome activation and pyroptosis in islet dysfunction and death may play a critical role in identifying prospective therapeutic targets, thus preserving islet function and viability.

1.2.3.2 - Caspase-Independent Regulated Cell Death – A Role in Islet Transplantation?

1.2.3.2.1 – Ferroptosis

With the recent identification and expansion of cell death modalities in other disease states, it is likely that islets are indeed susceptible to non-caspase-dependent, regulated cell death mechanisms. Ferroptosis has recently emerged as a distinct form of regulated cell death that is morphologically, biochemically, and genetically distinct from apoptosis and alternative forms of regulated necrosis.[38] Recently discovered using a pharmacological approach, ferroptosis is defined by the iron-dependent accumulation of lipid ROS.[38,39] The accumulation of toxic lipid ROS can be initiated by the inhibition of intracellular glutathione (GSH) synthesis or the GSH-dependent antioxidant enzyme, glutathione peroxidase 4 (GPX4).[38-40] In mammals, ferroptosis has recently been implicated in numerous pathological conditions including stroke, traumatic brain injury, ischemia-reperfusion injury and kidney degeneration, as well as degenerative diseases, including Alzheimer's and Huntington's diseases.[41] Despite its observations in such pathologies, the discovery of ferroptosis occurred through a pharmacological approach.[39] The first ferroptosis inducing compounds identified were erastin[42] and

RSL3[43]. Erastin, a small potent molecule, demonstrated the ability to induce ferroptosis by selectively inhibiting the X_c^-cystine/glutamate antiporter required for GSH biosynthesis.[39,40] The depletion of intracellular GSH results in the accumulation of lipid-based ROS molecules due to the impaired ability of the GSH-dependent, lipid repair enzyme glutathione peroxidase 4 (GPX4).[38-40,44,45] RSL3 has the ability to induce ferroptosis by directly inhibiting the enzymatic activity of GPX4, with the biochemical hallmarks of ferroptosis ensuing, including elevated lipid peroxides. GPX4 has been implicated in models of ischemia-reperfusion-related diseases. Moreover, cancer cells in a high-mesenchymal therapy-resistant cell state are dependent GPX4.[40] Loss of GPX4 function in these resistant cell types are susceptible to ferroptosis-induced cell death *in vitro*.[46]

The role of ferroptosis has yet to be clearly defined in context to islet loss, however, prior pre-clinical studies suggest that this regulated cell death pathway may have implications in islet transplantation. As a tri-peptide, GSH is synthesized from glutamate, cysteine and glycine, and has been implicated as a crucial antioxidant alleviating oxidative stress in islets.[47] In the presence of lipid peroxidation byproducts, islets exhibit impaired glucose-induced insulin secretion.[48] When elevated in β-cells, fatty acids impair insulin gene expression, glucose-stimulated insulin secretion, and increase cell death.[49] The administration of GSH precursors have demonstrated improved insulin secretion in response to glucose, as well as reduce lipid peroxidation levels.[21,50,51] Koulajian *et al.* demonstrated improved *in vitro* and *in vivo* β-cell function in islets over-expressing GPX4 in the presence of lipid peroxidation products,[52] further substantiating the necessity to deter lipid peroxidation for improved islet function. Most

recently, our group has revealed that human islets exposed to the ferroptosis-inducing agents, erastin and RSL3, exhibit compromised islet function and viability. When islets were pre-treated with the ferroptosis-specific inhibitor, ferrostatin-1, the affects of erastin and RSL3 were abolished (unpublished data). These results suggest that islets are indeed susceptible to ferroptosis, at least in part, through pharmacological induction. The utility of inhibitors of ferroptosis capable of reducing intracellular lipid peroxidation may be attractive therapies to employ in islet isolation and transplantation.[53]

Given that ferroptosis requires abundant and accessible cellular iron, iron chelators, such desferrioxamine (DFO) have demonstrated the ability to protect from ferroptosis in other disease models.[44] Since DFO has led to improved islet function and engraftment outcomes, ferroptosis likely plays at least some role in in islet injury after isolation and transplantation. In murine islet transplant models, DFO-treated islets and recipients exhibit improved engraftment outcomes due to preserved islet mass[54,55] Furthermore, Vaithilingam *et al* demonstrated that encapsulated human islets cultured in the presence of DFO exhibited enhanced insulin secretion relative to non-treated control islets. DFO-treated islets restored euglycemia in immunocompromised NOD/SCID recipients at marginal doses relative to control islet recipients.[56] While ferroptosis was not identified as the cell death modality contributing to reduced islet function or engraftment, this regulated cell death pathway had yet to be defined.

Within the context of islet isolation and transplantation, the relative contribution of ferroptosis has yet to be fully elucidated, however, prior preclinical observations has revealed that key contributors to ferroptosis, such as increased lipid peroxidation, iron

accumulation and compromised GPX4 function, contribute to cellular demise. Interventional strategies, such as ferrostatins or newer potent longer-acting inhibitors of ferroptosis may potentially mitigate islet lipid peroxidation, improve islet function and thereby preserve islet mass. This newly identified regulated cell death pathway has garnered much interest in other disease pathologies and organ systems, and its role in islet transplantation is being actively investigated.

1.2.3.2.2 – Necroptosis

Necroptosis is a newly identified form of regulated necrosis induced by ligand binding to death receptors, TNF-receptor 1 (TNFR1) and Fas, Toll-like receptors (TLRs) or intracellular receptors such as DNA-dependent activator of IFN-regulatory factors (DAI). Emerging evidence suggests that TNF binding to TNFR1 in concomitant inhibition of caspase-8 through pan-caspase inhibition (such as, zVAD-FMK) induces necroptosis.[57] The upstream signaling elements of apoptosis and necroptosis are shared, and hence are tightly regulated. While TNFR1-induced apoptosis requires the activation of caspase-8, necroptosis requires caspase-8 function to be inhibited or disrupted.[53] The necroptosis signaling cascade requires the involvement of receptor interaction protein kinase 1 and 3 (RIPK1 and RIPK3, respectively). Subsequent RIPK3-mediated phosphorylation of mixed lineage kinase domain-like protein (MLKL) results in plasma membrane rupture, though the mechanism of MLKL-plasma membrane rupture remains unknown.[6] However, it is now clear that the ESCRT-3 complex is downstream of pMLKL and facilitates for plasma membrane rupture.[17] Early work identifying the embryonic lethality of caspase-8-null genes in mice led researchers to believe that

apoptosis was a process required for vertebrate viability.[58] Further investigation reversing the lethal phenotype of caspase-8-deficient mice on a RIPK3-deficient background revealed other functions of caspase-8, not just as a mediator of the apoptotic pathway, but also as a key controller of RIPK3.[19,58-60] These observations revealed that the primary function of caspase-8 was not solely for the execution of extrinsic apoptosis, but also in mediating the prevention of RIPK3-dependent necroptosis.[58]

In various whole organ murine transplant models, the role of RIPK3 has been identified as an important mediator of necroptosis. For example, Pavlosky *et al* demonstrated delayed graft rejection when hearts from RIPK3-deficient mice were transplanted in rapamycin-immunosuppressed recipients when compared to mice receiving wild-type hearts.[61] In a kidney transplant model void of immunosuppression, kidneys from RIPK3-deficient C57Bl/6 mice transplanted into Balb/c recipients exhibited improved organ function and overall survival, in comparison to wild-type kidney recipients.[62,63] Given the importance of RIPK3 as a therapeutic target in whole organ transplantation, further investigation certainly permits the determination of necroptosis in islet transplantation.

Given that TNF-α has demonstrated to be toxic to β-cells, it is plausible that necroptosis contributes to islet cell death in islet transplantation. An initial study by Farney *et al* demonstrated benefit of TNF-α blockade in a murine syngeneic islet transplant model.[64] Subsequently, in a single-donor clinical transplant protocol utilizing Etanercept, a TNF-α fusion protein, Hering *et al.* achieved insulin independence in all 8 patients transplanted.[65] Bellin *et al.* also revealed that islet transplant recipients receiving an induction therapy T-cell depleting antibodies with TNF-α-inhibition (TNF-

α-i) exhibited significantly improved insulin-independence rates up to 5 years post-transplant than recipients who did not receive TNF-α-i, regardless of maintenance immunosuppression.[66] Though these findings do not specifically address whether necroptosis or apoptosis account for TNF-α-induced β-cell death, this clearly merits comprehensive further delineation in experimental islet transplantation.

As previously noted, our laboratory examined the concerted administration of anti-inflammatory agents Etanercept and the IL-1 receptor agonist (IL-1Ra) Anakinra in a murine syngeneic islet transplant model, as well as in a human islet immunocompromised murine model.[20] The results from this study revealed that when administered alone, these agents could not augment engraftment outcomes in either model. However, when administered together, a significant proportion of islet transplant recipients became euglycemic as compared to non-treated control recipients. In the clinical setting, Matsumoto and colleagues achieved single-donor success in 3 islet transplant patients receiving Etanercept and Anakinra in a sirolimus-free immunosuppression regimen.[67]

While pre-clinical and clinical studies utilizing these anti-inflammatory agents suggest the role of reducing apoptosis to confer engraftment efficacy, it seems likely that necroptosis was also ameliorated in this setting. During necroptosis, IL-1α is actively produced.[68] Given that IL-1α and IL-1β bind to the same cell-surface receptor, and that Anakinra demonstrates the ability to prevent IL-1α and IL-1β activity,[69] it is plausible that the administration of Anakinra may have ameliorated the consequences of necroptosis.

The release of intracellular danger-associated molecular patterns (DAMPs) from dying cells into the extracellular milieu has been identified as a downstream event associated with necroptosis, and other regulated necrosis pathways.[70,71] In normal conditions, high-mobility group box 1 (HMGB1) has been associated with DNA winding and promotes protein assembly.[72] However, HMGB1 has also been implicated as a DAMP.[73] A novel classification of DAMPs has recently been introduced.[74,75] In a study by Itoh and colleagues, greater HMGB1 release from human and mouse islets correlated with poorer islet engraftment outcomes.[76] Matsumoto *et al* further corroborated these experimental findings in a clinical autotransplantation model.[77] Peredes-Juarez *et al.* further established that when cultured in low oxygen conditions, human islets exhibit robust HMGB1 release into the extracellular milieu *in vitro.* In parallel, treatment with necrostatin-1 (Nec-1), a once perceived inhibitor of necroptosis, revealed the ability to significantly reduce HMGB1 release in islets.[73] When islets were challenged with nitric oxide, Tamura and colleagues revealed the release of HMGB1, as well as compromised islet viability, which could be completely abrogated in the presence of Nec-1.[78] (**Table 2**) A caveat to these studies in pinpointing necroptosis as a defined cell death modality in islets is that Nec-1 has demonstrated the ability to potently inhibit necroptosis and ferroptosis.[58] Therefore, these results, and others employing Nec-1 to confer cytoprotection in islets, permits further evaluation to effectively delineate the contribution of necroptosis and/or ferroptosis in islet cell death. This can be accomplished through utilizing necroptosis-specific inhibitors, like Nec-1 stable (Nec-1s), which may truly elucidate the role of necroptosis in solid organ and prospectively, islet transplantation.

1.2.3.2.3 – Parthanatos

The over-activation of poly(ADP-ribose) polymerase 1 (PARP1) triggers parthanatos, a regulated necrosis pathway that has been implicated in neurodegenerative disorders, such as Parkinson's disease.[79] PARP1 has been shown to be involved in DNA repair, chromosome stability and the inflammatory response.[80] Moreover, while other isoforms of PARP have been identified, namely PARP2 and PARP3, specific inhibition of PARP1 solely prevents parthanatos. PARP1 activity has been demonstrated in response to stimuli, such as DNA damage and ROS production.[81] Under oxidative stress, activated PARP1 consumes NAD+, depleting cellular ATP, leading to eventual cellular energy collapse. PARP1 hyperactivation results in the translocation of apoptosis-inducing factor (AIF) from mitochondria to the nucleus, fragmenting DNA.[82] Given that islet viability is susceptible to both stimuli, it is conceivable that parthanatos may play a role in β-cell loss.

Murine studies have revealed that mice deficient in PARP1 exhibit resistance to single-bolus treatment of streptozotocin (STZ),[83,84] a known β-cell toxin that induces DNA damage through alkylation.[85,86] Further work has also revealed that inhibition of PARP1 protects islets against free radical- and cytokine-mediated islet damage.[87-89] Islets deficient in PARP1 have also been associated with reduced cytokine and endotoxin signaling, as evidenced by reduced NF-κB activation and its inflammatory gene targets, such as inducible nitric oxide (NO) synthase (iNOS).[90] Andreone *et al* revealed that islets isolated from PARP1-deficient mice prevented islet cell death when exposed to inflammatory cytokines, IL-1β and interferon (IFN)-γ, suggesting a role of parthanatos in inflammatory injury to islets.[90] In a study by Heller et al, islets pre-treated

with the PARP1 inhibitor, 3-aminobenzamide, were partially protected when subsequently challenged with NO or ROS, further supporting a role of PARP1 in islet cell death[91] (**Table 2**). As a contributor to islet cell death, PARP1 and other molecular targets in this pathway may serve as important opportunities for intervention.

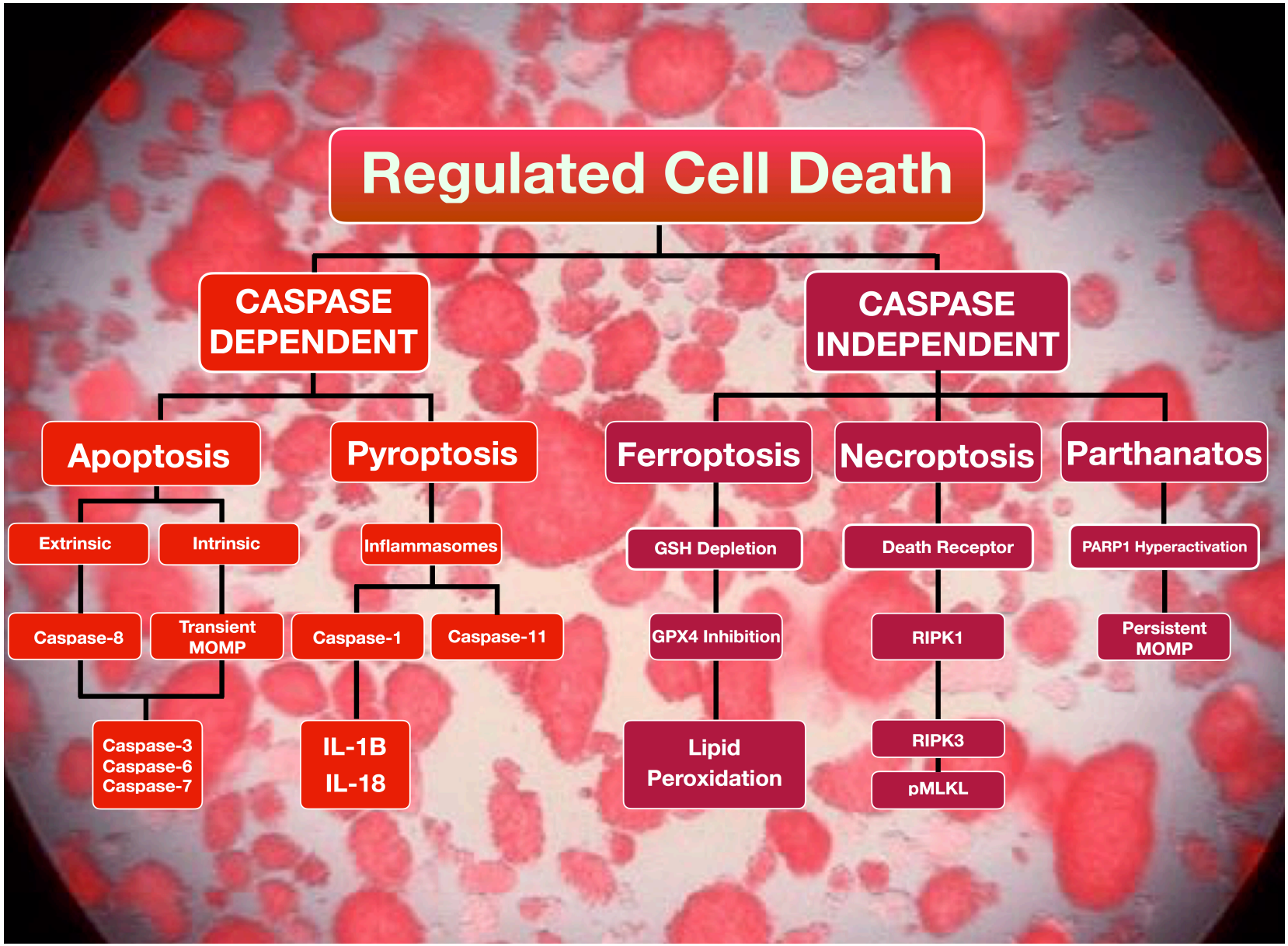
Regulated Cell Death
CASPASE DEPENDENT
CASPASE INDEPENDENT
Apoptosis
Pyroptosis
Ferroptosis
Necroptosis
Parthanatos
Extrinsic
Intrinsic
Inflammasomes
GSH Depletion
Death Receptor
PARP1 Hyperactivation
Caspase-8
Transient MOMP
Caspase-1
Caspase-11
GPX4 Inhibition
RIPK1
Persistent MOMP
Caspase-3
Caspase-6
Caspase-7
IL-1B
IL-18
Lipid Peroxidation
RIPK3
pMLKL

Figure 1.2.1 Regulated cell death signaling pathways.

Regulated cell death pathways may be differentiated by their dependence on caspase activity. Apoptosis, a caspase-dependent regulated cell death pathway, can be initiated by extrinsic or intrinsic cellular cues. Death receptor (DR) binding from appropriate signals, including TNF-α, initiates the extrinsic pathway. Alternatively, internal signals, including hypoxia and ROS, activate the intrinsic pathway. Both pathways converge on caspase-3 activation and result in morphological changes, such as plasma membrane blebbing. Since membrane integrity is conserved during apoptosis, this cell death modality is nonimmunogenic. Pyroptosis, a caspase-dependent form of regulated cell death requires the formation of multi-protein complexes, termed inflammasomes which results in the activation of caspase-1 or caspase-11. Caspase-1 activation results in the activation and secretion of the pro-inflammatory cytokines IL-1β and IL-18. As such, pyroptosis is an immunogenic form of regulated cell death. Regulated necrosis pathways are caspase-independent forms of immunogenic cell death with distinct biochemical features. Ferroptosis results in the accumulation of lipid peroxides as a result of glutathione depletion and inhibition of GPx4. Necroptosis can be initiated by DR-ligand binding resulting in the activation of RIPK1 and RIPK3 with subsequent phosphorylation of MLKL. Parthanatos is triggered by diverse stimuli, including ROS production, resulting in the hyperactivation of PARP1 resulting in the prospective release of AIF. AIF, apoptosis-inducing factor; GPx4, Glutathione peroxidase 4; IL – interleukin; MOMP, mitochondrial outer membrane Permeabilization; PARP1, Poly(ADP-ribose) polymerase 1; pMLKL, phosphorylated mixed lineage kinase domain-like protein; RIPK, receptor-interacting serine/threonine-protein kinase.

Table 1.2.2. Relevance of regulated cell death pathway in islet isolation and transplantation.

Regulated Cell Death Pathway	Evidence in Islet Transplantation	Known Inhibitor(s) of Islet Cell Death	Reference
Apoptosis	+++	EP1013 zVAD-FMK zVD-FMK IDN-6556 F573	3, 8, 9, 10, 11, 12
Pyroptosis	+	TBD	
Ferroptosis	?	Ferrostatin-1	*Unpublished*
Necroptosis	+	Necrostatin-1	73, 78
Parthanatos	+	Nicotinamide	87, 88

1.2.4 – CROSS-TALK BETWEEN REGULATED CELL DEATH PATHWAYS

As described above, there are numerous regulated necrosis pathways that can be triggered by several molecular pathways. As such, there is considerable cross-talk between components in different forms of these pathways. For example, RIPK3 has been implicated in the processing of pro-IL-1β as a result of promoting the NLRP3 inflammasome, independent of necroptotic cell death.[92] Regulated cell death mechanisms have also been implicated in chronic kidney injury, as inflammasome activation and pyroptosis has been demonstrated to occur.[27,92] Moreover, Nec-1 has also demonstrated the capacity to inhibit ferroptosis, prospectively suggesting implications in off-target, to be determined mechanisms.[7] Within the context of islet transplantation, cross talk of the various regulated cell death pathways has yet to be fully elucidated. It is conceivable that multiple regulated pathways can contribute to islet dysfunction and cell death, given that islets are susceptible to numerous stimuli that act as key contributors to the various regulated cell death mechanisms. Elucidating key molecules contributing to islet demise will prove crucial for the development of therapeutic treatments.

1.2.5 – CONCLUSION

Despite substantial advances in clinical islet transplantation over the past two decades, islet loss in the acute and peri-transplant period remains a substantial obstacle to long-term success. As such, single-donor transplant success rates still remain elusive for the majority of islet recipients. Therapeutic strategies to ameliorate islet cell death in the acute and peri-transplant period provide an attractive approach to preserve early islet mass, potentially improving long-term engraftment outcomes. Apoptosis has been

identified in numerous pathological conditions and transplant settings, including islet transplantation. Numerous pre-clinical and clinical strategies have been employed to ameliorate the deleterious events associated with apoptosis. However, recent research endeavors have identified other notable, regulated cell death modalities that are genetically and biochemically distinct from apoptosis. The identification of such regulated necrosis pathways, including but not limited to, ferroptosis and necroptosis, exhibit distinct biochemical hallmarks with defined molecular machinery contributing to cellular demise.

Our detailed review of published literature reveals that hallmarks of various regulated cell death pathways contribute to islet β-cell death, but these pathways have not been fully characterized to date. Studies aimed to identify the key contributors of ferroptosis, necroptosis, and other regulated necrotic pathways may be new and exciting arenas to explore in islet transplantation. The key molecules identified in these regulated cell death modalities may be ideal targets for therapeutic intervention in the early isolation and acute transplant period. With the potential crosstalk of these cell death modalities, employing a single therapy to abate early islet death either post-isolation or in the acute transplant period may be of limited benefit. A multi-therapeutic approach is likely required, as targeted inhibition of some molecules may drive the incidence of other cell death pathways. Insights in pre-clinical and clinical investigations have revealed that a multi-therapeutic strategy to combat various biochemical pathways may be imperative to improve single-donor engraftment outcomes. The administration of these drugs to multiorgan donors and subsequently to transplant recipients may also have considerable implications in supporting islet viability and deterring the onset of

islet cell death. The window at which these drugs are administered will likely be attributed to the time at which these regulated cell death processes occur, as well as the homeostatic importance of these pathways in the host. The efficacy of any interventions aimed at controlling regulated cell death will depend heavily on their half-life and durability of action. It will also be important to establish that any of these new target therapeutics do not have direct toxicity to islet beta cells, or to the engraftment and neovascularization process. Emerging evidence of alternative, regulated cell death pathways in other pathological conditions will continue to garner relevance in islet transplantation. However, elucidating the mechanisms that contribute to islet death in islet transplantation will be of much benefit to ameliorate graft attrition thus improving long-term engraftment outcomes.

The results from this thesis reveal that the administration of therapeutic agents during organ procurement and/or islet culture preserve pre-transplant islet function and improve engraftment in murine transplant models. Further work elucidating the application of these agents may warrant their utility in the clinical setting as adjuvant therapies to enhance single-donor islet engraftment outcomes.

1.2.6 – REFERENCES

1. Hering BJ, Clarke WR, Bridges ND, et al. Phase 3 Trial of Transplantation of Human Islets in Type 1 Diabetes Complicated by Severe Hypoglycemia. *Diabetes Care.* 2016;39(7):1230-1240.
2. The CITR Coordinating Center and Investigators. *The Collaborative Islet Transplant Registry (CITR)* 2016 Ninth Annual Report. US Department of Health and Human Services. Bethesda, MD, USA; 2016. Available from https://citregistry.org/system/files/9AR_Report.pdf. Accessed June 3, 2017. 2016.
3. Emamaullee JA, Davis J, Pawlick R, et al. The caspase selective inhibitor EP1013 augments human islet graft function and longevity in marginal mass islet transplantation in mice. *Diabetes.* 2008;57(6):1556-1566.
4. Suzanne M, Steller H. Shaping organisms with apoptosis. *Cell Death Differ.* 2013;20(5):669-675.
5. Taylor RC, Cullen SP, Martin SJ. Apoptosis: controlled demolition at the cellular level. *Nat Rev Mol Cell Biol.* 2008;9(3):231-241.
6. Linkermann A, Green DR. Necroptosis. *N Engl J Med.* 2014;370(5):455-465.
7. Friedmann Angeli JP, Schneider M, Proneth B, et al. Inactivation of the ferroptosis regulator Gpx4 triggers acute renal failure in mice. *Nat Cell Biol.* 2014;16(12):1180-1191.
8. Emamaullee JA, Davis J, Pawlick R, et al. Caspase inhibitor therapy synergizes with costimulation blockade to promote indefinite islet allograft survival. *Diabetes.* 2010;59(6):1469-1477.

9. Emamaullee JA, Stanton L, Schur C, Shapiro AM. Caspase inhibitor therapy enhances marginal mass islet graft survival and preserves long-term function in islet transplantation. *Diabetes.* 2007;56(5):1289-1298.

10. McCall M, Toso C, Emamaullee J, et al. The caspase inhibitor IDN-6556 (PF3491390) improves marginal mass engraftment after islet transplantation in mice. *Surgery.* 2011;150(1):48-55.

11. McCall MD, Maciver AM, Kin T, et al. Caspase inhibitor IDN6556 facilitates marginal mass islet engraftment in a porcine islet autotransplant model. *Transplantation.* 2012;94(1):30-35.

12. Pepper AR, Bruni A, Pawlick R, et al. Engraftment Site and Effectiveness of the Pan-Caspase Inhibitor F573 to Improve Engraftment in Mouse and Human Islet Transplantation in Mice. *Transplantation.* 2017;101(10):2321-2329.

13. Paraskevas S, Maysinger D, Wang R, Duguid TP, Rosenberg L. Cell loss in isolated human islets occurs by apoptosis. *Pancreas.* 2000;20(3):270-276.

14. Hengartner MO. The biochemistry of apoptosis. *Nature.* 2000;407(6805):770-776.

15. McIlwain DR, Berger T, Mak TW. Caspase functions in cell death and disease. *Cold Spring Harb Perspect Biol.* 2013;5(4):a008656.

16. Vanden Berghe T, Linkermann A, Jouan-Lanhouet S, Walczak H, Vandenabeele P. Regulated necrosis: the expanding network of non-apoptotic cell death pathways. *Nat Rev Mol Cell Biol.* 2014;15(2):135-147.

17. 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(14):977-986.

18. Kaiser WJ, Upton JW, Long AB, et al. RIP3 mediates the embryonic lethality of caspase-8-deficient mice. *Nature.* 2011;471(7338):368-372.

19. Oberst A, Dillon CP, Weinlich R, et al. Catalytic activity of the caspase-8-FLIP(L) complex inhibits RIPK3-dependent necrosis. *Nature.* 2011;471(7338):363-367.

20. McCall M, Pawlick R, Kin T, Shapiro AM. Anakinra potentiates the protective effects of etanercept in transplantation of marginal mass human islets in immunodeficient mice. *Am J Transplant.* 2012;12(2):322-329.

21. Avila J, Barbaro B, Gangemi A, et al. Intra-ductal glutamine administration reduces oxidative injury during human pancreatic islet isolation. *Am J Transplant.* 2005;5(12):2830-2837.

22. Jorgensen I, Zhang Y, Krantz BA, Miao EA. Pyroptosis triggers pore-induced intracellular traps (PITs) that capture bacteria and lead to their clearance by efferocytosis. *J Exp Med.* 2016;213(10):2113-2128.

23. Jorgensen I, Miao EA. Pyroptotic cell death defends against intracellular pathogens. *Immunol Rev.* 2015;265(1):130-142.

24. Shi J, Zhao Y, Wang K, et al. Cleavage of GSDMD by inflammatory caspases determines pyroptotic cell death. *Nature.* 2015;526(7575):660-665.

25. Aglietti RA, Estevez A, Gupta A, et al. GsdmD p30 elicited by caspase-11 during pyroptosis forms pores in membranes. *Proc Natl Acad Sci U S A.* 2016;113(28):7858-7863.

26. Sangiuliano B, Perez NM, Moreira DF, Belizario JE. Cell death-associated molecular-pattern molecules: inflammatory signaling and control. *Mediators Inflamm.* 2014;2014:821043.

27. Vilaysane A, Chun J, Seamone ME, et al. The NLRP3 inflammasome promotes renal inflammation and contributes to CKD. *J Am Soc Nephrol.* 2010;21(10):1732-1744.

28. Lawlor KE, Khan N, Mildenhall A, et al. RIPK3 promotes cell death and NLRP3 inflammasome activation in the absence of MLKL. *Nat Commun.* 2015;6:6282.

29. Bluestone JA, Herold K, Eisenbarth G. Genetics, pathogenesis and clinical interventions in type 1 diabetes. *Nature.* 2010;464(7293):1293-1300.

30. Butler AE, Janson J, Bonner-Weir S, Ritzel R, Rizza RA, Butler PC. Beta-cell deficit and increased beta-cell apoptosis in humans with type 2 diabetes. *Diabetes.* 2003;52(1):102-110.

31. Sloan-Lancaster J, Abu-Raddad E, Polzer J, et al. Double-blind, randomized study evaluating the glycemic and anti-inflammatory effects of subcutaneous LY2189102, a neutralizing IL-1beta antibody, in patients with type 2 diabetes. *Diabetes Care.* 2013;36(8):2239-2246.

32. Larsen CM, Faulenbach M, Vaag A, et al. [Interleukin-1 receptor antagonist-treatment of patients with type 2 diabetes]. *Ugeskr Laeger.* 2007;169(45):3868-3871.

33. Masters SL, Dunne A, Subramanian SL, et al. Activation of the NLRP3 inflammasome by islet amyloid polypeptide provides a mechanism for enhanced IL-1beta in type 2 diabetes. *Nat Immunol.* 2010;11(10):897-904.

34. Westermark GT, Westermark P, Nordin A, Tornelius E, Andersson A. Formation of amyloid in human pancreatic islets transplanted to the liver and spleen of nude mice. *Ups J Med Sci.* 2003;108(3):193-203.

35. Westermark GT, Westermark P, Berne C, Korsgren O, Nordic Network for Clinical Islet T. Widespread amyloid deposition in transplanted human pancreatic islets. *N Engl J Med.* 2008;359(9):977-979.

36. Westermark GT, Davalli AM, Secchi A, et al. Further evidence for amyloid deposition in clinical pancreatic islet grafts. *Transplantation.* 2012;93(2):219-223.

37. Potter KJ, Scrocchi LA, Warnock GL, et al. Amyloid inhibitors enhance survival of cultured human islets. *Biochim Biophys Acta.* 2009;1790(6):566-574.

38. Dixon SJ, Stockwell BR. The role of iron and reactive oxygen species in cell death. *Nat Chem Biol.* 2014;10(1):9-17.

39. Dixon SJ, Lemberg KM, Lamprecht MR, et al. Ferroptosis: an iron-dependent form of nonapoptotic cell death. *Cell.* 2012;149(5):1060-1072.

40. Linkermann A, Skouta R, Himmerkus N, et al. Synchronized renal tubular cell death involves ferroptosis. *Proc Natl Acad Sci U S A.* 2014;111(47):16836-16841.

41. Stockwell BR, Friedmann Angeli JP, Bayir H, et al. Ferroptosis: A Regulated Cell Death Nexus Linking Metabolism, Redox Biology, and Disease. *Cell.* 2017;171(2):273-285.

42. Dolma S, Lessnick SL, Hahn WC, Stockwell BR. Identification of genotype-selective antitumor agents using synthetic lethal chemical screening in engineered human tumor cells. *Cancer Cell.* 2003;3(3):285-296.

43. Yang WS, Stockwell BR. Synthetic lethal screening identifies compounds activating iron-dependent, nonapoptotic cell death in oncogenic-RAS-harboring cancer cells. *Chem Biol.* 2008;15(3):234-245.

44. Yang WS, Stockwell BR. Ferroptosis: Death by Lipid Peroxidation. *Trends Cell Biol.* 2016;26(3):165-176.

45. Reed JC, Pellecchia M. Ironing out cell death mechanisms. *Cell.* 2012;149(5):963-965.

46. Hangauer MJ, Viswanathan VS, Ryan MJ, et al. Drug-tolerant persister cancer cells are vulnerable to GPX4 inhibition. *Nature.* 2017;551(7679):247-250.

47. do Amaral AS, Pawlick RL, Rodrigues E, 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(2):e55288.

48. Miwa I, Ichimura N, Sugiura M, Hamada Y, Taniguchi S. Inhibition of glucose-induced insulin secretion by 4-hydroxy-2-nonenal and other lipid peroxidation products. *Endocrinology.* 2000;141(8):2767-2772.

49. Robertson RP, Harmon J, Tran PO, Poitout V. Beta-cell glucose toxicity, lipotoxicity, and chronic oxidative stress in type 2 diabetes. *Diabetes.* 2004;53 Suppl 1:S119-124.

50. Carobbio S, Ishihara H, Fernandez-Pascual S, Bartley C, Martin-Del-Rio R, Maechler P. Insulin secretion profiles are modified by overexpression of glutamate dehydrogenase in pancreatic islets. *Diabetologia.* 2004;47(2):266-276.

51. Li C, Buettger C, Kwagh J, et al. A signaling role of glutamine in insulin secretion. *J Biol Chem.* 2004;279(14):13393-13401.

52. Koulajian K, Ivovic A, Ye K, et al. Overexpression of glutathione peroxidase 4 prevents beta-cell dysfunction induced by prolonged elevation of lipids in vivo. *Am J Physiol Endocrinol Metab.* 2013;305(2):E254-262.

53. Skouta R, Dixon SJ, Wang J, et al. Ferrostatins inhibit oxidative lipid damage and cell death in diverse disease models. *J Am Chem Soc.* 2014;136(12):4551-4556.

54. Bradley B, Prowse SJ, Bauling P, Lafferty KJ. Desferrioxamine treatment prevents chronic islet allograft damage. *Diabetes.* 1986;35(5):550-555.

55. Nomikos IN, Prowse SJ, Carotenuto P, Lafferty KJ. Combined treatment with nicotinamide and desferrioxamine prevents islet allograft destruction in NOD mice. *Diabetes.* 1986;35(11):1302-1304.

56. Vaithilingam V, Oberholzer J, Guillemin GJ, Tuch BE. Beneficial effects of desferrioxamine on encapsulated human islets--in vitro and in vivo study. *Am J Transplant.* 2010;10(9):1961-1969.

57. Vercammen D, Beyaert R, Denecker G, et al. Inhibition of caspases increases the sensitivity of L929 cells to necrosis mediated by tumor necrosis factor. *J Exp Med.* 1998;187(9):1477-1485.

58. Linkermann A. Nonapoptotic cell death in acute kidney injury and transplantation. *Kidney Int.* 2016;89(1):46-57.

59. Oberst A, Green DR. It cuts both ways: reconciling the dual roles of caspase 8 in cell death and survival. *Nat Rev Mol Cell Biol.* 2011;12(11):757-763.

60. Kang TB, Yang SH, Toth B, Kovalenko A, Wallach D. Caspase-8 blocks kinase RIPK3-mediated activation of the NLRP3 inflammasome. *Immunity.* 2013;38(1):27-40.

61. Pavlosky A, Lau A, Su Y, et al. RIPK3-mediated necroptosis regulates cardiac allograft rejection. *Am J Transplant.* 2014;14(8):1778-1790.

62. Linkermann A, Hackl MJ, Kunzendorf U, Walczak H, Krautwald S, Jevnikar AM. Necroptosis in immunity and ischemia-reperfusion injury. *Am J Transplant.* 2013;13(11):2797-2804.

63. Lau A, Wang S, Jiang J, et al. RIPK3-mediated necroptosis promotes donor kidney inflammatory injury and reduces allograft survival. *Am J Transplant.* 2013;13(11):2805-2818.

64. Farney AC, Xenos E, Sutherland DE, et al. Inhibition of pancreatic islet beta cell function by tumor necrosis factor is blocked by a soluble tumor necrosis factor receptor. *Transplant Proc.* 1993;25(1 Pt 2):865-866.

65. Hering BJ. Achieving and maintaining insulin independence in human islet transplant recipients. *Transplantation.* 2005;79(10):1296-1297.

66. Bellin MD, Barton FB, Heitman A, et al. Potent induction immunotherapy promotes long-term insulin independence after islet transplantation in type 1 diabetes. *Am J Transplant.* 2012;12(6):1576-1583.

67. Matsumoto S, Takita M, Chaussabel D, et al. Improving efficacy of clinical islet transplantation with iodixanol-based islet purification, thymoglobulin induction, and blockage of IL-1beta and TNF-alpha. *Cell Transplant.* 2011;20(10):1641-1647.

68. England H, Summersgill HR, Edye ME, Rothwell NJ, Brough D. Release of interleukin-1alpha or interleukin-1beta depends on mechanism of cell death. *J Biol Chem.* 2014;289(23):15942-15950.

69. Dinarello CA, Simon A, van der Meer JW. Treating inflammation by blocking interleukin-1 in a broad spectrum of diseases. *Nat Rev Drug Discov.* 2012;11(8):633-652.

70. Kono H, Rock KL. How dying cells alert the immune system to danger. *Nat Rev Immunol.* 2008;8(4):279-289.

71. Kaczmarek A, Vandenabeele P, Krysko DV. Necroptosis: the release of damage-associated molecular patterns and its physiological relevance. *Immunity.* 2013;38(2):209-223.

72. Daly KA, Liu S, Agrawal V, et al. Damage associated molecular patterns within xenogeneic biologic scaffolds and their effects on host remodeling. *Biomaterials.* 2012;33(1):91-101.

73. Paredes-Juarez GA, Sahasrabudhe NM, Tjoelker RS, et al. DAMP production by human islets under low oxygen and nutrients in the presence or absence of an immunoisolating-capsule and necrostatin-1. *Sci Rep.* 2015;5:14623.

74. Land WG, Agostinis P, Gasser S, Garg AD, Linkermann A. DAMP-Induced Allograft and Tumor Rejection: The Circle Is Closing. *Am J Transplant.* 2016;16(12):3322-3337.

75. Land WG, Agostinis P, Gasser S, Garg AD, Linkermann A. Transplantation and Damage-Associated Molecular Patterns (DAMPs). *Am J Transplant.* 2016;16(12):3338-3361.

76. Itoh T, Takita M, SoRelle JA, et al. Correlation of released HMGB1 levels with the degree of islet damage in mice and humans and with the outcomes of islet transplantation in mice. *Cell Transplant.* 2012;21(7):1371-1381.

77. Itoh T, Iwahashi S, Kanak MA, et al. Elevation of high-mobility group box 1 after clinical autologous islet transplantation and its inverse correlation with outcomes. *Cell Transplant.* 2014;23(2):153-165.

78. Tamura Y, Chiba Y, Tanioka T, et al. NO donor induces Nec-1-inhibitable, but RIP1-independent, necrotic cell death in pancreatic beta-cells. *FEBS Lett.* 2011;585(19):3058-3064.

79. Conrad M, Angeli JP, Vandenabeele P, Stockwell BR. Regulated necrosis: disease relevance and therapeutic opportunities. *Nat Rev Drug Discov.* 2016;15(5):348-366.

80. Curtin NJ, Szabo C. Therapeutic applications of PARP inhibitors: anticancer therapy and beyond. *Mol Aspects Med.* 2013;34(6):1217-1256.

81. Virag L, Robaszkiewicz A, Rodriguez-Vargas JM, Oliver FJ. Poly(ADP-ribose) signaling in cell death. *Mol Aspects Med.* 2013;34(6):1153-1167.

82. Jang KH, Do YJ, Son D, Son E, Choi JS, Kim E. AIF-independent parthanatos in the pathogenesis of dry age-related macular degeneration. *Cell Death Dis.* 2017;8(1):e2526.

83. Yamamoto H, Uchigata Y, Okamoto H. DNA strand breaks in pancreatic islets by in vivo administration of alloxan or streptozotocin. *Biochem Biophys Res Commun.* 1981;103(3):1014-1020.

84. Yamamoto H, Uchigata Y, Okamoto H. Streptozotocin and alloxan induce DNA strand breaks and poly(ADP-ribose) synthetase in pancreatic islets. *Nature.* 1981;294(5838):284-286.

85. Lenzen S. The mechanisms of alloxan- and streptozotocin-induced diabetes. *Diabetologia.* 2008;51(2):216-226.

86. Wilson GL, Hartig PC, Patton NJ, LeDoux SP. Mechanisms of nitrosourea-induced beta-cell damage. Activation of poly (ADP-ribose) synthetase and cellular distribution. *Diabetes.* 1988;37(2):213-216.

87. Andersen HU, Jorgensen KH, Egeberg J, Mandrup-Poulsen T, Nerup J. Nicotinamide prevents interleukin-1 effects on accumulated insulin release and nitric oxide production in rat islets of Langerhans. *Diabetes.* 1994;43(6):770-777.

88. Rabinovitch A, Suarez-Pinzon WL, Strynadka K, et al. Human pancreatic islet beta-cell destruction by cytokines is independent of nitric oxide production. *J Clin Endocrinol Metab.* 1994;79(4):1058-1062.

89. Sandler S, Bendtzen K, Borg LA, Eizirik DL, Strandell E, Welsh N. Studies on the mechanisms causing inhibition of insulin secretion in rat pancreatic islets exposed to human interleukin-1 beta indicate a perturbation in the mitochondrial function. Endocrinology. 1989;124(3):1492-1501.

90. Andreone T, Meares GP, Hughes KJ, Hansen PA, Corbett JA. Cytokine-mediated beta-cell damage in PARP-1-deficient islets. Am J Physiol Endocrinol Metab. 2012;303(2):E172-179.

91. Heller B, Wang ZQ, Wagner EF, et al. Inactivation of the poly(ADP-ribose) polymerase gene affects oxygen radical and nitric oxide toxicity in islet cells. J Biol Chem. 1995;270(19):11176-11180.

92. Anders HJ, Muruve DA. The inflammasomes in kidney disease. J Am Soc Nephrol. 2011;22(6):1007-1018.

CHAPTER 2.

BMX-001, A NOVEL REDOX-ACTIVE METALLOPORPHYRIN, IMPROVES ISLET FUNCTION AND ENGRAFTMENT IN A MURINE TRANSPLANT MODEL

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ORIGINAL ARTICLE

BMX-001, a novel redox-active metalloporphyrin, improves islet function and engraftment in a murine transplant model

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2.1 – ABSTRACT

Islet transplantation has become a well-established therapy for select patients suffering from type 1 diabetes. Viability and engraftment can be compromised by the generation of oxidative stress encountered during isolation and culture.

We evaluate whether administration of BMX-001, and its earlier derivative, BMX-010 could improve islet function and engraftment outcomes. Long-term culture of human islets with BMX-001, but not BMX-010, exhibited preserved *in vitro* viability. Murine islets isolated and cultured for 24 hours with 34μM BMX-001 exhibited improved insulin secretion (n=3 isolations, $p<0.05$) in response to glucose relative to control islets. 34μM BMX-001-supplemented murine islets exhibited significantly reduced apoptosis as indicated by TUNEL, compared to non-treated control islets ($p<0.05$). Murine syngeneic islets transplanted under the kidney capsule (KC) at a marginal dose of 150 islets, revealed 57.9% of 34μM BMX-001-treated islet recipients became euglycemic (n=11 of 19) as compared to 19% of non-treated control islet recipients (n=3 of 19, $p<0.05$). 92.3% of murine recipients receiving a marginal dose of human islets cultured with 34μM BMX-001 (n=12 of 13) achieved euglycemia compared to 57.1% of control recipients (n=8 of 14, $p=0.11$). These results demonstrate that the administration of BMX-001 enhances *in vitro* viability and augments murine marginal islet mass engraftment.

2.2 – INTRODUCTION

In select patients, islet cell transplantation has become an attractive clinical therapy to restore glycemic control and ameliorate the secondary complications associated with type 1 diabetes mellitus (T1DM).[1] The 'Edmonton Protocol' by Shapiro and colleagues in 2000 was the first to achieve sustainable insulin independence up to one year post-islet transplant in seven consecutive patients, owing in part, to more potent immunosuppressive drugs, the avoidance of corticosteroids, as well as higher quality islet preparations.[2] Despite initial successes, five year follow-up revealed that insulin independence was not durable, as most patients returned to moderate exogenous insulin administration, albeit with 80% of patients maintaining C-peptide.[3] Within recent years, inroads in clinical islet transplantation have improved long-term transplantation outcomes, including but not limited to the introduction of selective and potent therapeutic agents in the initiation and maintenance of immunosuppression.[4]

Improvements in clinical islet transplantation have translated to 5-year insulin independence rates matching that of whole pancreas transplantation.[5] However, the sustainability of insulin independence in most patients requires multiple islet infusions, with single-donor insulin-independence being accomplished at few centers world-wide.[6] Results from a recent multicenter, single-arm phase III study completed by the Clinical Islet Transplant Consortium revealed 87.5% and 71% of participants at 1 year and 2 year follow-ups, respectively, exhibited restoration of glycemic control and hypoglycemic awareness when receiving one to two islet infusions.[7] Considerable evidence suggests that islet function and viability can be compromised during organ procurement, islet isolation and culture, thus contributing to islet cell death,

consequently compromising long-term engraftment outcomes.[8-10] The events contributing to cellular demise during procurement and isolation have been attributed, at least in part, to oxidative stress, the imbalance between free radical production and antioxidant availability.[11] Compared to other tissues within the body, islets exhibit reduced inherent antioxidant expression, including manganese superoxide dismutase (MnSOD).[12,13] As such, islets are considerably susceptible to the deleterious events associated with oxidative stress, including impaired β-cell metabolic function and islet loss.[11] Accordingly, strategies to augment endogenous antioxidant expression by utilizing therapeutic agents have been undertaken to mitigate events associated with oxidative stress thus promoting islet survivability and engraftment. Recent strategies have included natural antioxidants, such as red ginseng and anthocyanin, to improve islet viability *in vitro,* as well as improve engraftment in rodent transplant models.[14,15] Alternatively, the efficacy of low-molecular-weight, metalloporphyrin MnSOD mimics have gained considerable interest due to their ability to dismutate superoxide with a high rate constant.[16,17] Previous studies evaluating early-generation metalloporphyrin MnSOD mimics demonstrated an inhibition of NF-κB activation and preservation of islet mass *in vivo* in diabetic mice.[11,18,19] However, the translation of early generation MnSODs in islet isolation and transplantation have yet to confer significant benefit in the clinical setting.[20] Subsequent generation of a newly synthesized, more potent, MnSOD, MnTnBuOE-2-PyP^{5+}[Mn(III) *meso*-tetrakis(N-b-butoxyethylpyridinium-2-yl)porphyrin (BMX-001), has demonstrated the ability to reduce the generation of reactive oxygen species (ROS) in a murine donation after circulatory death islet

transplantation model, thus supporting further investigation of MnSODs in islet isolation and transplantation preclinical models.[21]

Herein, we examined whether the administration of a novel, redox-active metalloporphyrin, BMX-001 could confer greater cytoprotection than the earlier generation MnSOD, BMX-010, in human islets. We also evaluate whether administration of BMX-001 during organ procurement and islet culture could preserve *in vitro* islet function and subsequent engraftment outcomes in a syngeneic, marginal murine transplant model. We further evaluated whether human islets supplemented with BMX-001 could improve engraftment outcomes in an immunecompromised murine transplant model.

2.3 – MATERIALS AND METHODS

2.3.1 – BMX-010 and BMX-001

The metalloporphyrin SOD-mimetics, BMX-010 (Manganese (III) Meso-Tetrakis-(N-Methylpyridinium-2-yl)porphyrin and BMX-001 (MnTnBuOE-2-PyP^{5+}Mn(III) *meso*-tetrakis(N-b-butoxyethylpyridinium-2-yl)porphyrin) were obtained from BioMimetix Inc. (Denver, Colorado, USA). A stock preparation of 1 mM BMX-010 and BMX-001 was prepared by dissolving each drug in phosphate buffered saline (PBS).

2.3.2 – Human islet culture and *in vitro* assessment

Human islets were prepared by the Clinical Islet Laboratory at Alberta Health Services. Deceased donor pancreata were processed for islet isolation with appropriate

ethical approval and consent obtained from next-of-kin of the donor. Islets were isolated from 5 donor pancreata, implementing a modified Ricordi technique [22,23]. Studies were conducted with permission granted by the Health Research Ethics Board of the University of Alberta, Edmonton, Alberta, Canada. For long-term *in vitro* viability assessment, human islets were counted and subsequently distributed into standard culture media supplemented with 0 (Control), 34μM BMX-010 or 34μM BMX-001 and cultured for 7 days at 37°C and 5% CO_2. For *in vivo* transplant studies, human islets were cultured in standard culture media ± 34μM BMX-001 for 24 hours at 37°C and 5% CO_2. Standard culture media consists of Connaught Medical Research Laboratories (CMRL-1066, Mediatech, Manassas, VA, USA) supplemented with fetal bovine serum (10%), L-glutamine (100 mg/l), penicillin (112 kU/l), streptomycin (112 mg/l) and HEPES (25 mmol/l) at pH 7.4.

2.3.3 – Mouse islet isolation

Pancreatic islets were isolated from 8 to 12 week old male BALB/c mice (Jackson Laboratories, Canada). Animals were housed under conventional conditions having access to food and water *ad libitum*. Mouse care was in accordance with the guidelines approved by the Canadian Council on Animal Care. Prior to pancreatectomy, the common bile duct was cannulated with a 30G needle and the pancreas was distended with 0.125 mg/mL cold Liberase TL Research Grade enzyme (Roche Diagnostics, Laval, QC, CA) in Hank's Balanced Salt Solution (Sigma-Aldrich Canada Co., Oakville, ON, CA) supplemented with 0 (Control), 10 or 34μM BMX-001. Islets were isolated by digesting the pancreases in a 50ml Falcon tube placed in a 37°C water bath for 14 minutes with light agitation. Following the pancreatic digestion phase, islets were

purified using histopaque-density gradient centrifugation (1.108, 1.083 and 1.069 g/mL, Sigma-Aldrich Canada Co., Oakville, ON, Canada).

2.3.4 – Mouse islet yield and culture

Immediately post-isolation, islets from each group were harvested to determine islet yield. Islet isolation yield is expressed as total number of islets isolated per pancreas (islets/pancreas). Immediately post-isolation, islets were either assessed for *in vitro* viability and function or cultured for 24 hours at 37°C/5% CO_2 in standard Connaught Medical Research Laboratories (CMRL)-1066 medium supplemented with 10% fetal bovine serum, L-glutamine (100mg/l), penicillin (112kU/l units), streptomycin (112mg/l), HEPES (25mmol/l), nicotinamide (10mM), sodium pyruvate (5mM), and additionally supplemented with 0 (Control), 10 or 34μM BMX-001.

2.3.5 – Murine islet insulin secretion assessment

Subsequent to 24-hour culture (D_1), islets from each culture condition were handpicked (50 islets per group in triplicate) and subjected to static glucose-stimulated insulin secretion (sGSIS) or dynamic insulin perifusion. For sGSIS, each islet replicate was incubated in a 15 ml Falcon tube in 6ml of RPMI-1640 containing low glucose (2.8 mmol/L) for one hour at 37°C. Islets were washed three times in glucose-free RPMI-1640, and incubated in 6 ml of RPMI containing high glucose (16.7 mmol/L) for an additional hour at 37°C. Subsequent to each glucose challenge, cell-free supernatants were harvested and stored at -20°C. Insulin was quantified by enzyme-linked immunosorbent assay (ELISA) (Mercodia, Uppsala, Sweden). Data is represented as

insulin secretion (μg/L) and as stimulation index (SI) (ratio of insulin secreted in response to high glucose/insulin secreted in response to low glucose).

2.3.6 – Apoptosis analysis

For murine islets, apoptosis was assessed between treatment groups in islets harvested 24 hours post-culture. For human islets, apoptosis was assessed between groups in islets harvested for 7 days. Apoptosis was assayed in all islets groups using a terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining (DeadEnd Apoptosis Detection System, Promega, Madison, WI), following formalin fixation (10%), agar embedding, processing, paraffinizing and sectioning. In addition to TUNEL staining, islets were co-stained with insulin and DAPI. Subsequent to deparaffinization, islet sections were washed with phosphate buffered saline (PBS) supplemented with 1% goat serum, followed by blocking with 20% goat serum in PBS for 30 minutes. The sections were treated with a primary antibody of guinea pig anti-pig insulin (Dako A0564) diluted 1:100 (PBS with 1% goat serum) for 2 hours. Samples were rinsed with PBS with 1% goat serum followed by secondary antibody treatment consisting of goat anti-guinea pig (Alexa 568) diluted 1:500 (PBS with 1% goat serum) for 1 hour at room temperature. Samples were rinsed with PBS and counter stained with DAPI in anti-fade mounting medium (ProLong®, LifeTechnologies). Samples were protected from light and stored overnight at 4°C until microscopy. Using a fluorescent microscope, the resulting microphotographs were taken using the appropriate filter with AxioVision imaging software.

Islet apoptosis was quantified as a percentage of positive-TUNEL staining nuclei per islet (+TUNEL/Total Nuclei) using ImageJ software (freeware ImageJ v1.33 and Cell Counter plug-in, both downloaded from the NIH website [http://rsb.info.nih.gov/ij]).

2.3.7 – Percent islet recovery

For murine studies, subsequent to 24-hour culture, islets were harvested and counted to determine islet recovery post-culture. For human islet studies, islets were harvested 7 days post-culture. Percent islet recovery was determined as the ratio of total islets harvested 24 hours post-culture relative to the number of islets harvested immediately post-isolation.

2.3.8 – Membrane integrity

Mouse Islet viability was assessed immediately after isolation and 24 hours post-culture for control, 10, and 34μM BMX-001 supplemented islets. Human islets were harvested 7 days post-culture and membrane integrity was assessed 7 days post-culture. Simultaneous staining of live and dead cells using a membrane integrity fluorescence assay (SytoGreen 13 and ethidium bromide, Invitrogen, Oregon, USA) was used to determine islet viability. The percentage of viable cells is expressed for each sample on the day of isolation (D_0) and 24 hours post-culture (D_1).

2.3.9 – Diabetes induction and islet transplantation

One week prior to transplantation, recipient mice, either BALB/c (syngeneic studies) or B6-RAG$^{-/-}$ (B6.129S7-*Rag1*^{tm1Mom}/J) (human islet studies), were rendered

diabetic by chemical induction with intraperitoneal streptozotocin (Sigma-Aldrich Canada Co., Oakville, ON, Canada), at 200 mg/kg or 180 mg/kg, respectively, in acetate phosphate buffer, pH 4.5. Diabetes was confirmed when two consecutive daily blood glucose levels exceeded 15 mmol/L. For mouse islet transplants, islets were harvested 24 hours post-culture from CMRL media supplemented with 0, 10 or 34μM BMX-001 and were transplanted under the kidney capsule (KC) at a marginal dose of 150 islets ± 10%, with a purity of approximately 95%, per diabetic recipient. For human islet transplants, 24 hours post-culture ± 34μM BMX-001 media supplementation, islets were counted and transplanted under the KC at a marginal islet dose of 750 islet equivalents (± 10%) per diabetic recipient. In both transplant models, islets were aspirated into polyethylene (PE-50) tubing using a micro-syringe, and centrifuged into a pellet suitable for transplantation. Immediately prior to transplantation, all recipients received a 0.1 mg/kg subcutaneous bolus of buprenorphine. A left lateral paralumbar incision was made and the left kidney delivered. The KC was incised and the islets were infused.

2.3.10 – Evaluation of islet graft function

Non-fasting blood glucose measurements (mmol/L) were assessed three times weekly using a portable glucometer (FreeStyle InsuLinx, Abbott Diabetes Care Ltd., Oxon, UK) in the three transplant groups tested. Graft function and reversal of diabetes was defined as two consecutive readings ≤11.1 mmol/L and maintained until study completion. Intraperitoneal glucose tolerance tests (IPGTTs) were conducted 60 days post-transplant in all islet transplant recipients. Mice were fasted overnight prior to receiving an intraperitoneal 25% glucose bolus (3g/kg). Blood glucose levels were

evaluated at baseline (time 0), 15, 30, 60, 90 and 120 minutes post-injection. Blood glucose area under the curve (Blood glucose AUC) was calculated and analyzed between transplant groups. At the time of study completion, islet-bearing kidney grafts were retrieved by recovery nephrectomy. For euglycemic recipients, non-fasting blood glucose measurements were monitored up to 7 days subsequent to graft removal to confirm a return to hyperglycemia.

2.3.11 – Pro-inflammatory assessment and caspase-3 activation

Mouse islet-bearing kidney grafts (3 recipients per group receiving 150 islets each) were harvested 24 hours post-transplant to determine pro-inflammatory cytokine concentrations and cleaved caspase-3 activation. Islet grafts were excised and stored at -80°C until assessment. Tissue samples were subsequently lysed in acid buffer as reported previously [25]. Pro-inflammatory cytokines and chemokines interferon (IFN)-γ, interleukin (IL)-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12p70, keratinocyte chemoattractant, and tumor necrosis factor (TNF)-α were measured using a ProInflammatory Panel 1 V-Plex kit and analyzed on a SECTOR Imager (Meso Scale Discovery®, Gaithersburg, MD, USA). Results are normalized per gram of tissue (pg/g tissue). Cytosolic cleaved caspase-3 activation was determined according to the manufacturer's specifications for protease activity by the addition of a caspase-specific peptide conjugated to the color reporter substrate p-nitroaniline (BF3100; R&D Systems, Minneapolis, MN). Caspase activity was quantified spectrophotometrically at 405nm. Results are expressed as absorbance normalized per gram of tissue (Abs/g tissue).

2.3.11 – Statistical analysis

Mean insulin secretion, glucose stimulation index, non-fasting daily blood glucose, IPGTT blood glucose responses, blood glucose area under the curve (AUC) and percent apoptosis data are represented as the mean ± standard error of mean (s.e.m.). Comparison between mean absolute insulin secretion in low and high glucose within groups was conducted by paired student's t-test. The difference between mean stimulation index values was conducted by one-way ANOVA. Tukey's post-hoc tests were used following the analysis of variances for multiple comparisons between study groups. Kaplan-Meier survival function curves were compared using the log-rank statistical method (Mantel-Cox). $p<0.05$ was considered significant.

2.4 – RESULTS

2.4.1 – BMX-001, but not BMX-010, reduces apoptosis in human islets cultured for 7 days

To determine the cytoprotective effects of BMX-010 and/or BMX-001, human islets harvested 7 days post-culture were assessed for apoptosis via terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining. Control islets exhibited a significantly greater number of apoptotic cells (65.5 ± 4.3%) relative to islets cultured in the presence of 34μM BMX-001 (52.7 ± 2.3%) ($p<0.05$, One-way ANOVA) (**Figure 2.1A,B**). Islets cultured in the presence of 34μM BMX-010 exhibited no significant difference relative to non-treated control islets (61.3 ± 3.1%) ($p>0.05$, One-way ANOVA). BMX-010 and BMX-001 did not affect islet yield or islet recovery 7

days post-culture. Percent islet recovery for control islets cultured in standard media (41.0 ± 8.9%) was comparable to islets cultured in media supplemented with 34μM BMX-010 (53.6 ± 6.5%). Islets cultured in the presence of 34μM BMX-001 exhibited a trend towards increased recovery (71.2 ± 12.5%)($p>0.05$, one-way ANOVA) (**Figure 2.1C**). Islet viability, as assessed by dual-fluorescence staining revealed no discernable difference between groups 7 days post-culture. (Control: 67.5 ± 9.1 vs. 34μM BMX-010: 45.4 ± 5.7 vs. 34μM BMX-001: 55.8 ± 9.2, $p>0.05$, one-way ANOVA) (**Figure 2.1D**).

2.4.2 – Mouse islets supplemented with BMX-001 exhibit improved function 24 hours post-culture

Islets harvested 24-hours post-culture were assessed for function by static glucose stimulated insulin secretion (sGSIS). The insulin secretory capacity in response to glucose challenge in islets was assessed 24 hours post-culture. All islet groups exhibited an *in vitro* physiological response to extracellular glucose challenge as exhibited by insulin secretion. However, islets isolated and cultured in the presence of 34μM BMX-001 exhibited a significant elevation in mean insulin secretion when challenged with high glucose relative to low glucose (34μM BMX-001 low glucose: 9.6 ± 1.9 μg/L vs. high glucose: 33.2 ± 7.4 μg/L; $p<0.05$, paired t-test) (**Figure 2.2A**). Stimulation index (SI) assessment revealed a dose-dependent enhanced insulin response with significance achieved in islets supplemented with 34μM BMX-001 (Control SI: 2.1 ± 0.4 vs 10μM BMX-001 SI: 3.3 ± 0.8; $p>0.05$, One-way ANOVA) (Control SI: 2.1 ± 0.4 vs 34μM BMX-001 SI: 4.1 ± 0.5; $p<0.05$, One-way ANOVA, n=3 islet preparations

tested in triplicate) (**Figure 2.2B**). When assessed for insulin secretion via dynamic insulin perifusion assay, no statistical difference was observed between culture groups (one-way ANOVA; **Figure 2.2 C & D**).

2.4.3 – 24-hour culture with BMX-001 reduces murine islet apoptosis

Murine islets harvested 24 hours post-culture in standard culture conditions or supplemented with 10 or 34μM BMX-001 were assessed histologically for apoptosis by TUNEL. Control islets exhibited a significantly greater number of apoptotic cells (9.64 ± 1.40%) relative to islets cultured in the presence of 34μM BMX-001 (2.72 ± 0.4%) ($p<0.05$, One-way ANOVA) (**Figure 2.3A,B**). Islets cultured in the presence of 10μM BMX-001 exhibited no significant difference relative to non-treated control islets (9.90 ± 1.4%) ($p>0.05$, One-way ANOVA), but were significantly higher than 34μM BMX-001-treated islets ($p<0.05$, One-way ANOVA).

2.4.4 – Isolation and culture with BMX-001 is non-toxic to mouse islets

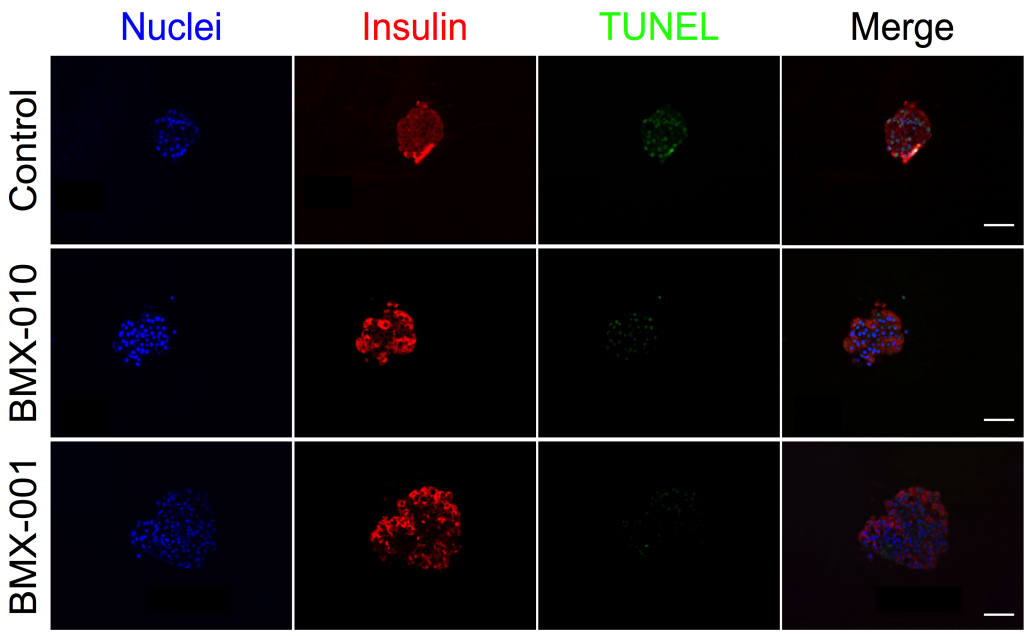
The administration of 10 or 34μM BMX-001 during islet isolation did not enhance islet yield immediately post-isolation. For standard control isolation conditions, 198.8 ± 33.2 islets/pancreas was achieved. Similarly, for islets isolated in the presence of 10 or 34μM BMX-001, 208.5 ± 28.8 and 183.8 ± 25.6, islets/pancreas, respectively ($p>0.05$) (**Figure 2.4A**). When harvested 24 hours post-culture, percent islet recovery for control islets cultured in standard media (71.1 ± 11.4%) was comparable to islets cultured in media supplemented with 10μM BMX-001 (68.9 ± 17.0%). Islet recovery for 34μM BMX-001-cultured islets exhibited a trend towards increased percent recovery

relative to control (88.8 ± 3.4%) ($p>0.05$) (**Figure 2.4B**). Islet viability, as assessed by dual-fluorescence staining revealed no discernable difference between groups on the day of isolation (D_0) (Control D_0: 94.4 ± 0.4 vs. 10μM BMX-001 D_0: 96.8 ± 1.1 vs. 34μM BMX-001 D_0: 95.2 ± 0.6, $p>0.05$, one-way ANOVA) (**Figure 2.4C**). Similarly, when harvested 24 hours post-culture (D_1), no difference in membrane integrity staining was observed between groups (Control D_1: 95.8 ± 0.2% vs. 10μM BMX-001 D_1: 93.5 ± 4.6% vs. 34μM BMX-001 D_1: 99.0 ± 0.5%, $p>0.05$, one-way ANOVA) (**Figure 2.4C**).

2.4.5 – BMX-001 augments long-term murine syngeneic islet engraftment

To determine whether the administration of BMX-001 during organ procurement and culture could improve islet engraftment, marginal, syngeneic islet transplants were conducted in STZ-induced diabetic BALB/c recipients. Subsequent to 24-hour culture, 150 islets from media supplemented with 0 (control), 10 or 34μM BMX-001 were transplanted under the renal capsule of diabetic recipients. Of the control islet recipients, 3 of 19 (15.8%) mice became euglycemic subsequent to transplant. Conversely, a significantly higher proportion of recipients transplanted with islets isolated and cultured in the presence of 10μM BMX-001, 10 of 19 (52.6%) and 34μM BMX-001, 11 of 19 (57.9%), respectively, became euglycemic post-transplant (log-rank, $p<0.05$ and $p<0.01$, respectively) (**Figure 2.5A**). Both BMX-001 treatment groups exhibited an overall reduced daily non-fasting blood glucose profile compared to control-islet recipients (**Figure 2.5B**). Recipients in all groups reverted back to hyperglycemia upon graft recovery nephrectomy, thus confirming graft-dependent euglycemia.

A


Nuclei
Insulin
TUNEL
Merge
Control
BMX-010
BMX-001

B

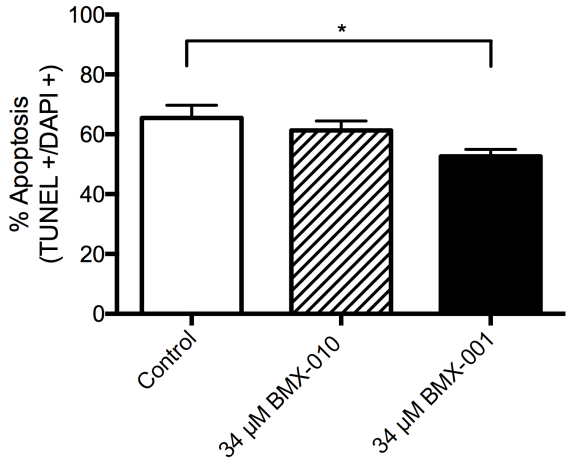
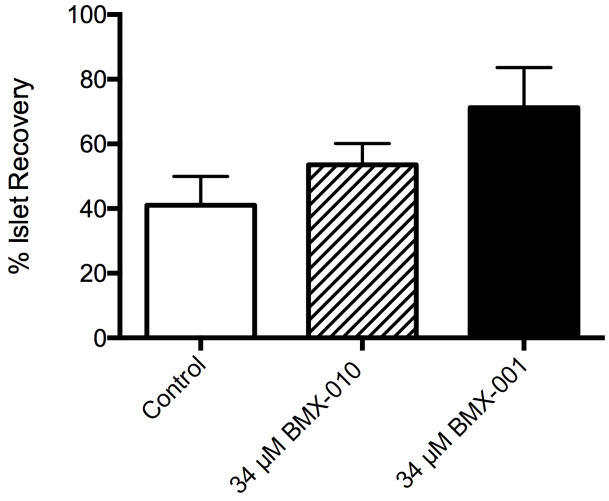

% Apoptosis
(TUNEL +/DAPI +)
100
80
60
40
20
0
*
Control
34 μM BMX-010
34 μM BMX-001

Figure 2.1 (A – B). Comparison of human islet viability 7 days post-culture with BMX-010 or BMX-001.

(A) Representative fluorescent microphotographs of islets stained for insulin (red), TUNEL (apoptosis) (green) and nuclei (blue). Data from 5 human islet preparations, triplicate samples per isolation. Data represented as mean ± SEM.

(B) 7 days post-culture, human islets cultured in BMX-001 exhibit significantly reduced TUNEL-positive cells relative to control islets (*$p<0.05$, one-way ANOVA).

C


% Islet Recovery
100
80
60
40
20
0
Control
34 µM BMX-010
34 µM BMX-001

D

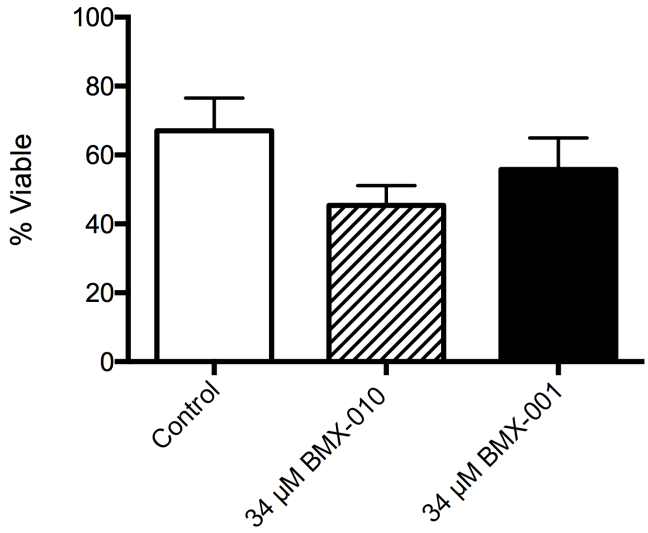
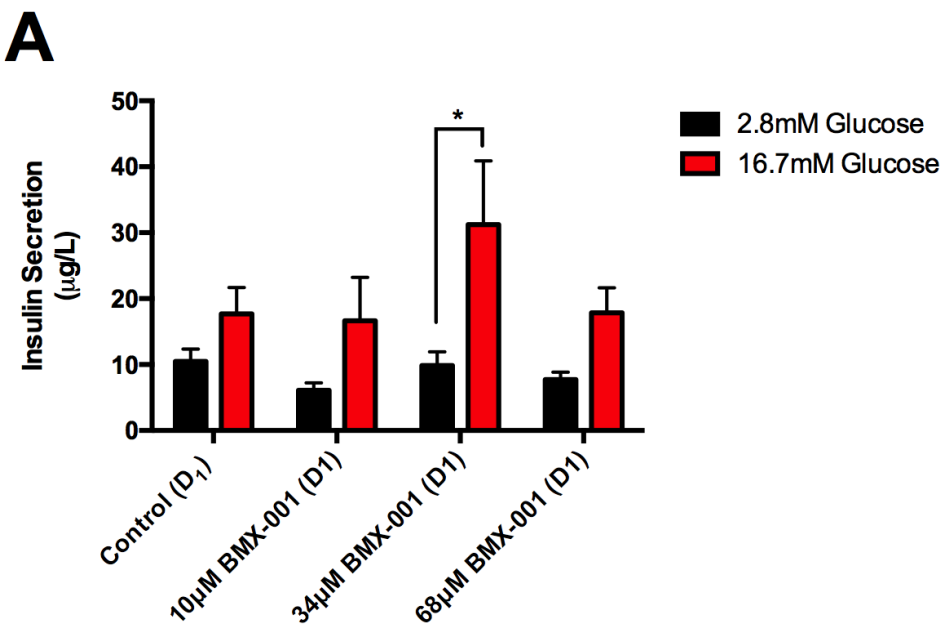

% Viable
100
80
60
40
20
0
Control
34 µM BMX-010
34 µM BMX-001

Figure 2.1 (C – D). Comparison of human islet viability 7 days post-culture with BMX-010 or BMX-001.

(C) Percent islet recovery was similar between groups ($p>0.05$, one-way ANOVA). (D) Percent viability, as assessed by dual-fluorescence membrane integrity staining, reveals no discernable difference between groups on 7 days post-culture ($p>0.05$, one-way ANOVA). Data from 5 human islet preparations, triplicate samples per isolation. Data represented as mean ± SEM.


A
Insulin Secretion (μg/L)
50
40
30
20
10
0
*
2.8mM Glucose
16.7mM Glucose
Control (D1)
10μM BMX-001 (D1)
34μM BMX-001 (D1)
68μM BMX-001 (D1)

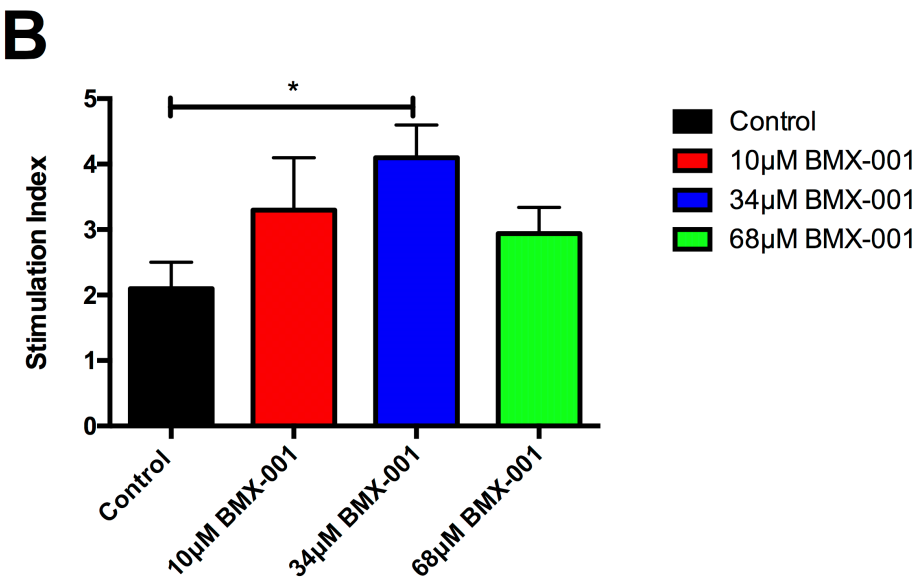
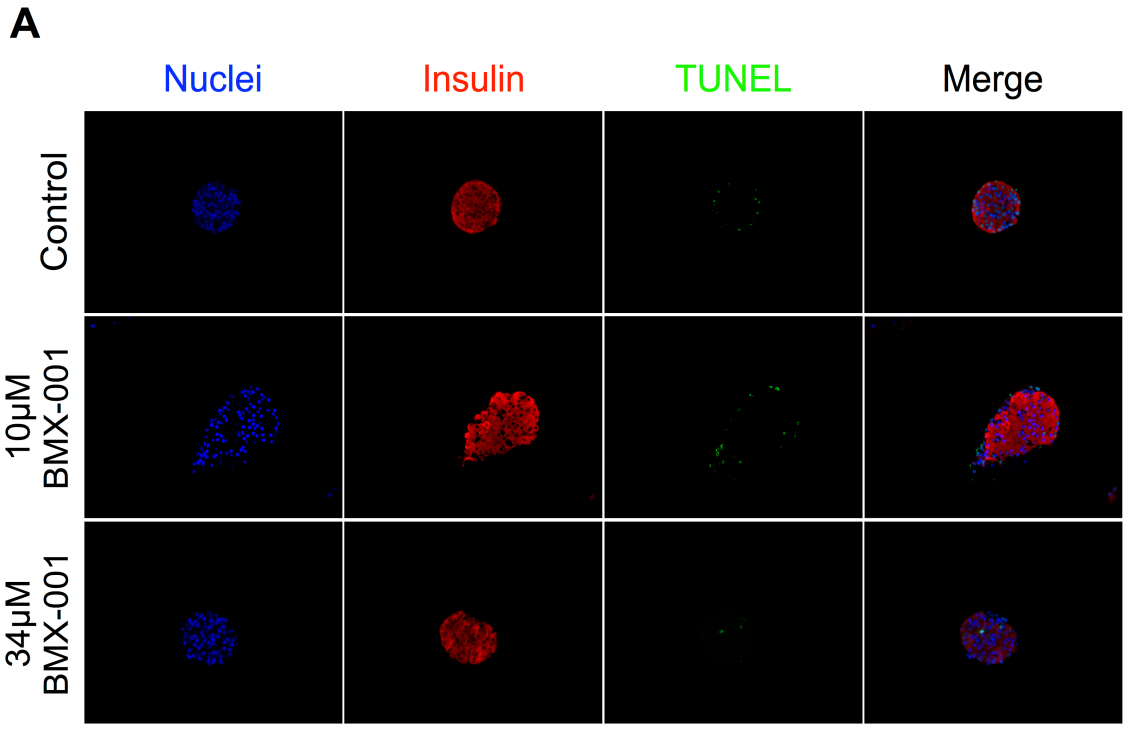

B
Stimulation Index
5
4
3
2
1
0
*
Control
10μM BMX-001
34μM BMX-001
68μM BMX-001
Control
10μM BMX-001
34μM BMX-001
68μM BMX-001

Figure 2.2. Evaluation of *in vitro* insulin secretion in murine islets harvested 24 hours post-culture.

(A) 24 hours post-culture, 34μM BMX-001-treated islets exhibit a significant increase in insulin secretion in response to high glucose relative to low glucose when assessed by sGSIS. (B) 34μM BMX-001-treated islets exhibit improved insulin-secretory capacity in response to glucose challenge relative to control islets as represented by stimulation index (*$p<0.05$, one-way ANOVA, n=3 isolations, triplicate samples per isolation). Data represented as mean ± SEM.


A
Nuclei
Insulin
TUNEL
Merge
Control
10μM BMX-001
34μM BMX-001

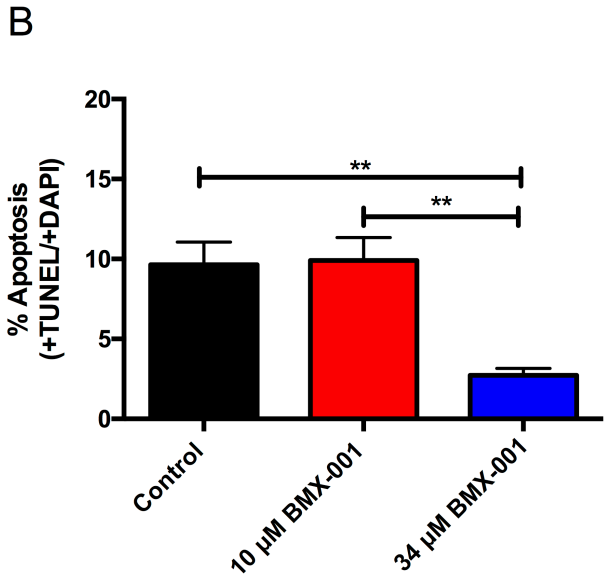
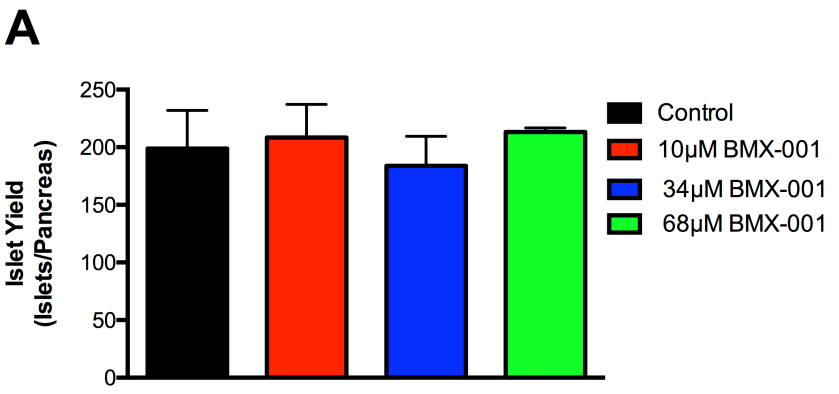
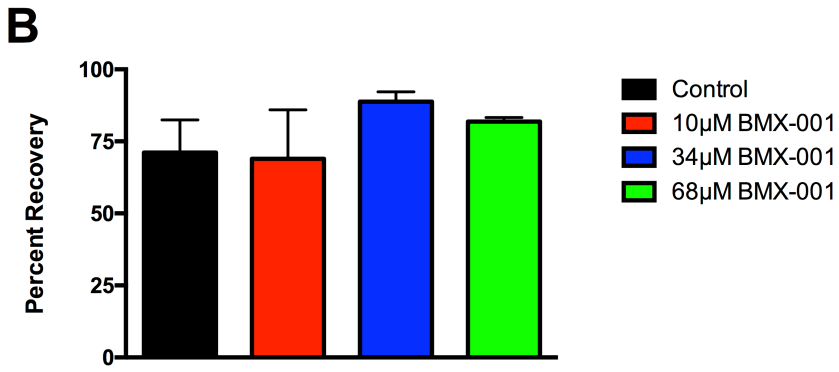

B
% Apoptosis (+TUNEL/+DAPI)
20
15
10
5
0
**
**
Control
10 μM BMX-001
34 μM BMX-001

Figure 2.3. Evaluation of apoptosis subsequent to 24-hour culture in control and BMX-001 supplemented islets.

(A) Representative fluorescent microphotographs of islets stained for insulin (red), TUNEL (apoptosis) (green) and nuclei (blue). Data is from three isolations with triplicate samples per isolation. Data represented as mean ± SEM. Percentage of (B) TUNEL-positive cells in islets harvested 24-hours post-culture. (Data points represent mean ± SEM, n=9/group, **$p<0.01$, one-way ANOVA). Data represented as mean ± SEM.


A
Islet Yield
(Islets/Pancreas)
250
200
150
100
50
0
Control
10μM BMX-001
34μM BMX-001
68μM BMX-001


B
Percent Recovery
100
75
50
25
0
Control
10μM BMX-001
34μM BMX-001
68μM BMX-001

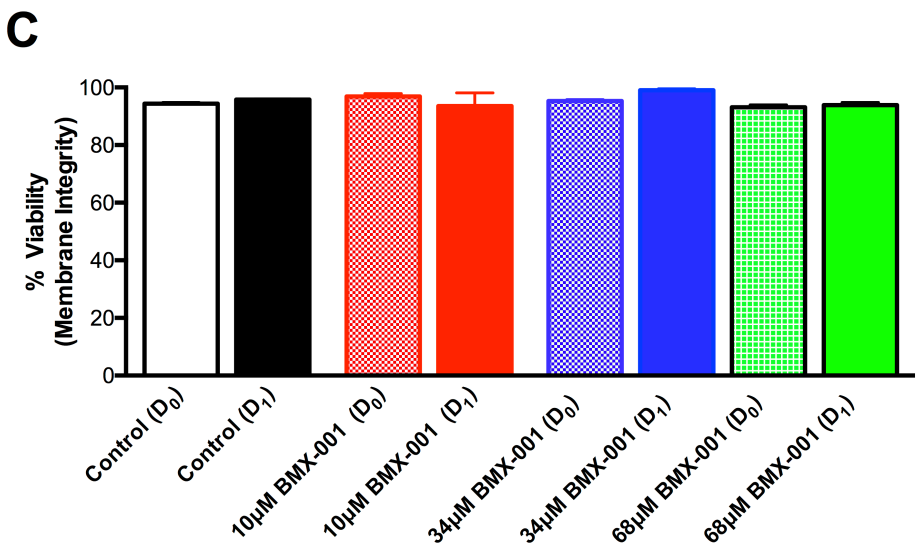
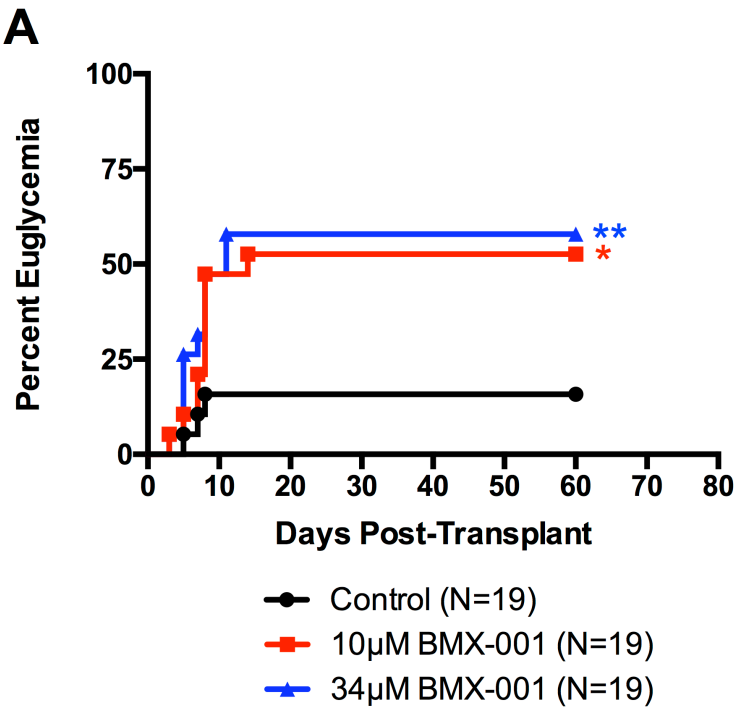

C
% Viability
(Membrane Integrity)
100
80
60
40
20
0
Control (D0)
Control (D1)
10μM BMX-001 (D0)
10μM BMX-001 (D1)
34μM BMX-001 (D0)
34μM BMX-001 (D1)
68μM BMX-001 (D0)
68μM BMX-001 (D1)

Figure 2.4. Evaluation of islet yield, islet recovery and membrane integrity.

(**A**) Islets isolated in the presence of 0 (control; black), 10 (red) and 34μM (blue) BMX-001 exhibited no discernable difference in islet yield per pancreas ($p>0.05$, one-way ANOVA). (**B**) Percent islet recovery, the number of surviving islets 24 hours post-culture relative to number of islets isolated, was similar between groups ($p>0.05$, one-way ANOVA). (**C**) Percent viability, as assessed by dual-fluorescence membrane integrity staining, reveals no discernable difference between groups on the day of isolation (D_0) or 24 hours post-culture (D_1) ($p>0.05$, one-way ANOVA). Data is from three isolations with triplicate samples per isolation. Data represented as mean ± SEM.


A
Percent Euglycemia
100
75
50
25
0
0
10
20
30
40
50
60
70
80
Days Post-Transplant
**
*
Control (N=19)
10μM BMX-001 (N=19)
34μM BMX-001 (N=19)

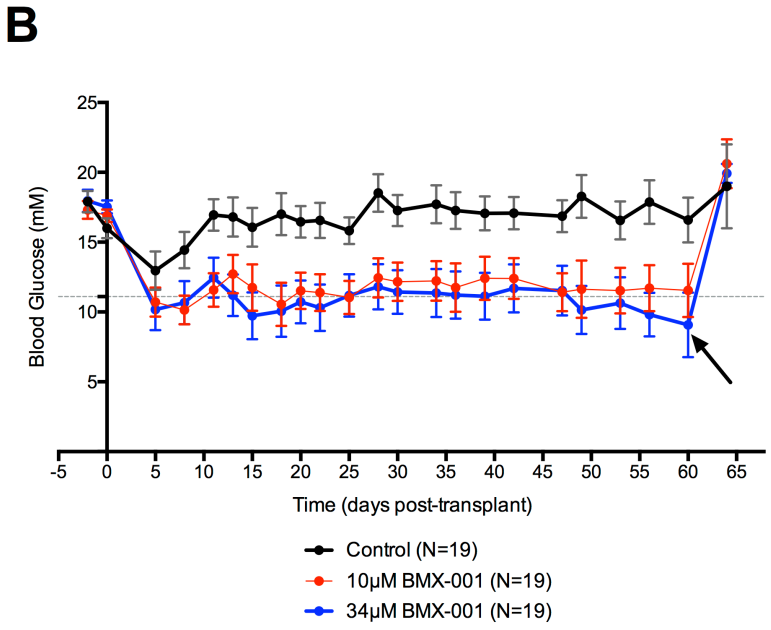

B
Blood Glucose (mM)
25
20
15
10
5
-5
0
5
10
15
20
25
30
35
40
45
50
55
60
65
Time (days post-transplant)
Control (N=19)
10μM BMX-001 (N=19)
34μM BMX-001 (N=19)

Figure 2.5. BMX-001 improves marginal, syngeneic murine islet mass engraftment. (A) Percent euglycemia of syngeneic, marginal transplant recipients receiving islets supplemented with 10μM BMX-001 (red, n=19) and 34μM BMX-001 (blue, n=19) were significantly higher than control transplant recipients (black, n=19) recipients 60 days post-transplant (*$p<0.05$, **$p<0.01$, log-rank). (B) Non-fasting blood glucose measurements of marginal, islet recipients post-transplant. Euglycemic recipients maintained glycemic control throughout the duration of engraftment until graft retrieval (arrow). Dashed line exhibits range of normoglycemia (≤11.1 mmol/L). Data represented as mean ± SEM per group.

2.4.6 – Glucose tolerance testing

Intraperitoneal glucose tolerance testing (IPGTT) was performed on euglycemic syngeneic islet transplant recipients 60 days post-transplant. Recipients transplanted with islets pre-treated with 0 (n=3), 10 (n=10) or 34μM BMX-001 (n=11) exhibited varying responses to dextrose bolus. Notably, 10μM and 34μM BMX-001-treated islet recipients exhibited a dose-dependent return to euglycemia at 120 minutes post-dextrose infusion subsequent to glucose challenge (**Figure 2.6A**). Blood glucose mean area under the curve (AUC) for control and 10μM BMX-001-treated islet recipients was elevated relative to non-diabetic mice, albeit non-significantly (AUC Control: 1777 ± 201.5 mmol/L/120 min) relative to 10μM BMX-001 (AUC: 1801 ± 123.7 mmol/L/120 min), 34μM BMX-001 (AUC: 1571 ± 118.6 mmol/L/120 min) and non-diabetic naïve mice (AUC: 1049 ± 95.7 mmol/L/120 min) (One-way ANOVA, $p>0.05$) (**Figure 2.6B**).

2.4.7 – BMX-001 does not alter pro-Inflammatory cytokine or caspase-3 activation 24 hours post-transplant

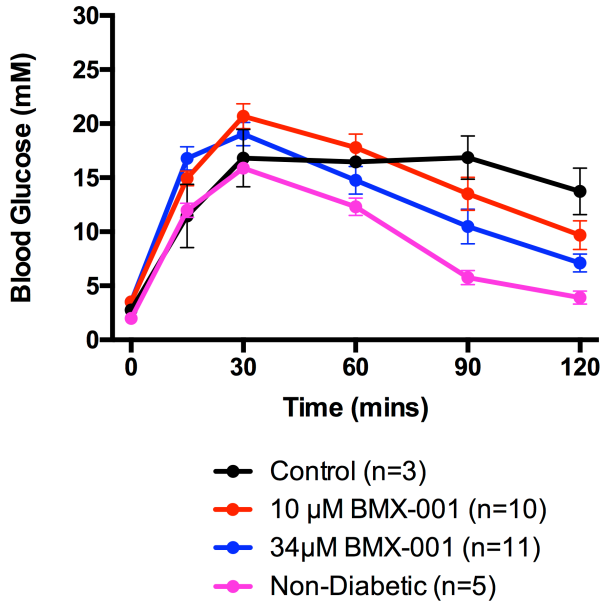
To determine whether pre-treatment of murine islets with BMX-001 reduced pro-inflammatory and apoptotic events post-transplant, islet-bearing grafts from all groups (n=3 grafts per group) were harvested from diabetic recipients 24 hours post-transplant and assessed for pro-inflammatory cytokines and caspase-3 activation. Among the three groups, 34μM BMX-001 islet-bearing grafts exhibited a trend towards reduced levels of inflammatory cytokines which did not reach statistical significance; notably, IFN-γ (Control: 0.97 ± 0.07 vs. 10μM BMX-001: 0.97 ± 0.05 vs. 34μM BMX-001: 0.80 ± 0.03) and IL-1β (Control: 1.83 ± 0.11 vs. 10μM BMX-001: 1.41 ± 0.19 vs.

34μM BMX-001: 1.68 ± 0.07) (**Figure 2.7A,B**). There was no discernable difference in cytokine levels for IL-2, IL-4, IL-5, IL-6, IL-10, IL-12p70, keratinocyte chemoattractant, and tumor necrosis factor (TNF)-α between groups. Similarly, caspase-3 activation in grafts harvested 24 hours post-transplant revealed non-significant differences among transplant groups (Control: 1.83 ± 0.11 vs. 10μM BMX-001: 1.41 ± 0.19 vs. 34μM BMX-001: 1.68 ± 0.07, $p>0.05$, one-way ANOVA) (**Figure 2.7C**).

2.4.8 – Evaluation of long-term human islet engraftment

To further evaluate whether BMX-001 could improve engraftment outcomes with human islets supplemented with or without BMX-001 in immunecompromised mice, recipients were transplanted with a marginal transplant dose of 750 IE under the KC. The average human islet purity for the transplant preparations utilized in this study was 48.8% (n=5 pancreata). Euglycemia was achieved in 8 of 14 (57.1%) control islet recipients. Conversely, a greater, albeit insignificant proportion of recipients transplanted with islets cultured with 34μM BMX-001 (12 of 13; 92.3%) achieved euglycemia subsequent to transplantation (log-rank, $p=0.11$) (**Figure 2.8A**). Mean non-fasting blood glucose profiles for recipients receiving BMX-001-supplemented human islets exhibited reduced daily non-fasting blood glucose profile relative to their non-treated counterparts (**Figure 2.8B**). Islet-bearing recovery nephrectomy in euglycemic recipients revealed a return to hyperglycemia, thus confirming graft-dependent glycemic control post-transplant.

A


Blood Glucose (mM)
30
25
20
15
10
5
0
0
30
60
90
120
Time (mins)
Control (n=3)
10 μM BMX-001 (n=10)
34μM BMX-001 (n=11)
Non-Diabetic (n=5)

B

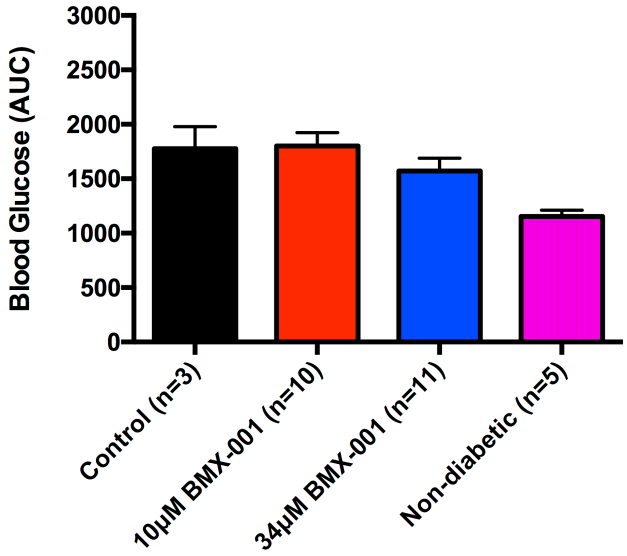
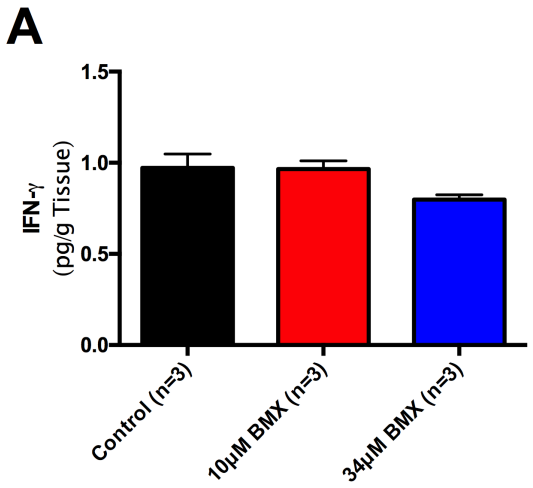
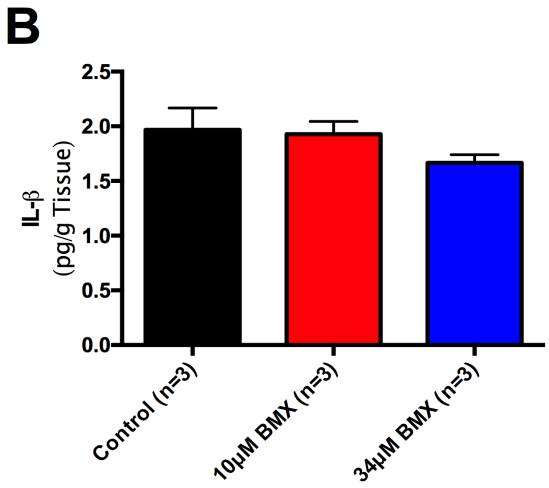

Blood Glucose (AUC)
3000
2500
2000
1500
1000
500
0
Control (n=3)
10μM BMX-001 (n=10)
34μM BMX-001 (n=11)
Non-diabetic (n=5)

Figure 2.6. Intraperitoneal glucose tolerance test (IPGTT) of syngeneic, marginal islet mass recipients transplanted with control, 10 or 34μM BMX-001-treated islets 60 days post-transplant.

(A) Blood glucose profile post-dextrose bolus of control (black, n=3), 10μM BMX-001 (red, n=10), 34μM BMX-001 (blue, n=11) islet recipients and non-diabetic, naïve mice (n=5). (B) IPGTT blood glucose AUC profiles of euglycemic recipients 60 days post-transplant. Mice were administered 3mg/kg 25%dextrose i.p. Blood glucose measurements were monitored at t=0, 15, 30, 60, 90 and 120 minutes. Data represented as mean ± SEM per group.


A
IFN-γ
(pg/g Tissue)
1.5
1.0
0.5
0.0
Control (n=3)
10μM BMX (n=3)
34μM BMX (n=3)


B
IL-β
(pg/g Tissue)
2.5
2.0
1.5
1.0
0.5
0.0
Control (n=3)
10μM BMX (n=3)
34μM BMX (n=3)

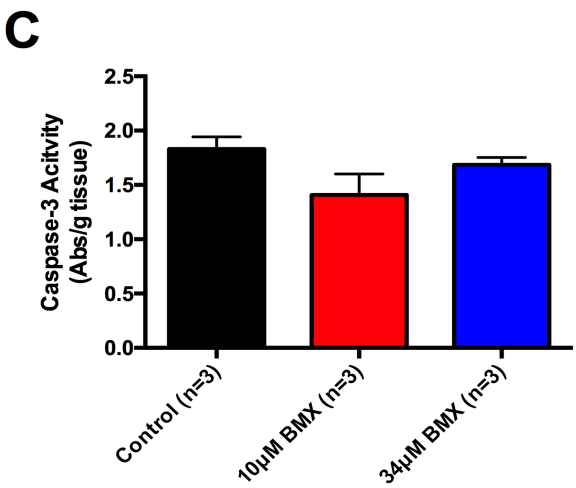

C
Caspase-3 Acitvity
(Abs/g tissue)
2.5
2.0
1.5
1.0
0.5
0.0
Control (n=3)
10μM BMX (n=3)
34μM BMX (n=3)

Figure 2.7. Pro-inflammatory cytokine profile and caspase-3 activation of islet-bearing kidney grafts harvested 24-hours post-transplant.

(A) IFN-γ cytokine profile of islet-bearing grafts harvested from control (black), 10 (red) and 34μM-treated islets (blue). (B) IL-1β cytokine profile of islet-bearing grafts harvested from control (black), 10 (red) and 34μM-treated islets (blue). (C) Activated caspase-3 profile of islet-bearing grafts harvested from control (black), 10 (red) and 34μM-treated islets (blue). No statistically significant difference is observed between groups. Data represented as mean ± SEM per group.

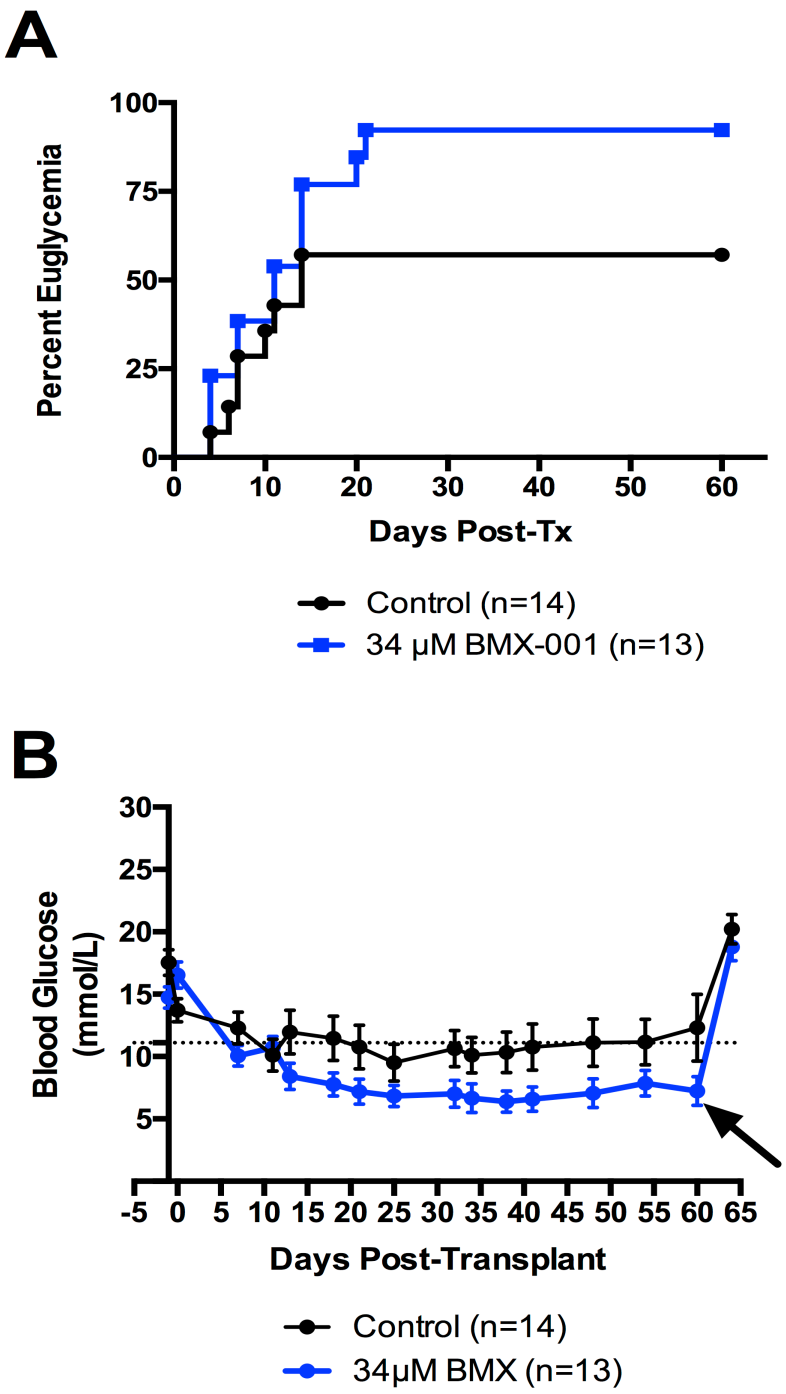

A
Percent Euglycemia
100
75
50
25
0
0
10
20
30
40
50
60
Days Post-Tx
Control (n=14)
34 μM BMX-001 (n=13)
B
Blood Glucose (mmol/L)
30
25
20
15
10
5
-5
0
5
10
15
20
25
30
35
40
45
50
55
60
65
Days Post-Transplant
Control (n=14)
34μM BMX (n=13)

Figure 2.8. Evaluation of human islets supplemented with BMX-001 in immunecompromised mice.

(A) Percent euglycemia of human marginal islet (750 IE) transplant recipients receiving islets supplemented with 34μM BMX-001 (blue, n=13) were modestly higher than control transplant recipients (black, n=14) recipients 60 days post-transplant ($p=0.08$). (B) Non-fasting blood glucose measurements of marginal, human islet recipients post-transplant. Euglycemic recipients maintained glycemic control throughout the duration of engraftment until graft retrieval (arrow). Dashed line indicates range of normoglycemia (≤11.1 mmol/L). Data represented as mean ± SEM per group.

2.5 – DISCUSSION

Endogenous antioxidant systems play a critical role in the redox modulation of free radicals. The imbalance between the generation of free radicals and native antioxidant expression, favoring the former, results in cytotoxic oxidative stress. Due to their inherently reduced expression of antioxidants, such as superoxide dismutase, catalase, and glutathione peroxidase, islets have increased susceptibility to the deleterious events associated with oxidative stress.[26-29] Islets experience various stresses during isolation and culture that results in cellular redox imbalance and subsequent cell death.[30] Incorporation of soluble reagents capable of increasing islet antioxidant defenses to ameliorate the consequences of oxidative stress, including the generation of ROS have been shown to have positive effects on islet survival and engraftment.[28] Early-generation metalloporphyrin MnSOD mimics have also been introduced in experimental islet transplantation as a means to abrogate these injurious events, promote survivability and augment islet engraftment.[19,27]

In the current study, we first investigated whether long-term culture of human islets in the presence of metalloporphyrin MnSOD mimics, BMX-010 or BMX-001 could confer cytoprotection *in vitro.* At the time of assessment, BMX-010 was under clinical investigation, and the development of the next generation MnSOD mimic, BMX-001, was made available with little pre-clinical efficacy evaluation. We utilized a dose of both drugs that mimics endogenous physiological SOD levels expressed in islets (34μM), which revealed long-term islets cultured in BMX-001 exhibited improved viability as assessed by TUNEL, while TUNEL levels were indiscernible between control and BMX-010-treated human islets.[19]

Given that BMX-001 conferred significant cytoprotection relative to BMX-010 in pre-clinical long-term human islet culture, we sought to further evaluate if the administration of BMX-001 during islet isolation and 24-hour culture could improve islet yield, recovery and *in vitro* function, as well as promote improved engraftment in a syngeneic, marginal transplantation model. Since BMX-001 was presumed to be more potent than earlier metalloporphyrin analogs as a result of lipid-soluble structural characteristics, we also sought to determine if a lower dose, 10μM, could confer adequate cytoprotection and improve *in vivo* outcomes. To simulate its prospective application in the clinical setting, the compound was introduced during murine pancreas enzymatic distension, digestion, cold storage and culture media supplementation but was not delivered to transplant recipients.[20]

Administration of either BMX-001 dose during islet isolation and culture did not alter yield or recovery 24 hours post-culture, respectively, or affect islet viability as assessed by membrane integrity. While no additional benefit was conferred, these results demonstrate that the drug is non-toxic at the prescribed doses, thus encouraging its utility in the clinical setting.

Conversely, our results demonstrate a dose-dependent relationship in islet function and viability when cultured with increasing doses of BMX-001 for 24 hours, suggesting that 34μM BMX-001 conferred significant cytoprotection from oxidative stress encountered in the acute culture period of the doses tested. Our previous study using BMX-001 in a murine islet isolation and culture model revealed that this agent is capable of minimizing the generation of ROS as exhibited by a significant reduction in extracellular ROS.[21] In a model of oxidative stress, Gandy et al. demonstrated that islets

pre-cultured with superoxide dismutase, followed by STZ treatment, exhibited preserved sGSIS responses to that of non-STZ treated islets.[29] In an *in vivo* model of STZ-induced free-radical damage, Sklavos and colleagues observed the islet-protective capacity of systemically delivered MnSOD in rodents.[19] In alignment with previous results established by Bottino et al., our study also revealed that islets cultured in the presence of the MnSOD BMX-001 exhibited reduced apoptotic activity.[11]

Preserved islet potency prior to transplant likely accounts for the improved engraftment outcomes observed in the current syngeneic, murine study. Islets isolated and cultured in the presence of 34μM BMX-001 exhibited significantly improved sGSIS responses and reduced TUNEL staining. It is possible that BMX-001 administration at the time of organ procurement and subsequent culture reduced downstream apoptotic events associated with oxidative stress, thus preserving viability and function.[28,31] While reduced apoptosis was observed in islets subsequent to culture, islet-bearing grafts harvested 24 hours post-transplant did not reveal significant differences in caspase-3 activation or in the pro-inflammatory cytokine profiles measured. Quite possibly, the time at which the grafts were harvested was insufficient in capturing transplant-induced islet injury or the assay itself may have insufficient sensitivity to detect differences in graft apoptosis. Alternatively, compromised *in vitro* islet function may be primarily responsible for the reduced islet engraftment observed in control islet recipients, with these effects mitigated in the presence of BMX-001.

Perhaps most notably, our results reveal that islets cultured in the presence of MnSOD could significantly restore euglycemia at a marginal islet dose in murine islet studies. IPGTT assessment of euglycemic recipients at 60 days post-transplant revealed

a trend towards a modest return to normoglycemia in 34μM BMX-001-treated islet recipients, while control and 10μM BMX-001-treated islet recipients exhibited a delayed response to glucose bolus, albeit non-significantly. These results are particularly encouraging, as the prospective islet-sparing potential of this drug could be of greater consequence in the clinical islet setting where multiple islet infusions are typically required to achieve long-term insulin-independence.[32,33] However, one should heed caution when evaluating *in vivo* engraftment efficacy using glucose tolerance testing in rodent models. Korsgren and Korsgren recently revealed that diabetic rodents were capable of rapidly normalizing blood glucose subsequent to dextrose bolus, despite lacking insulin-producing cells or any evidence of circulating serum c-peptide.[34] Indeed, our study could have measured circulating serum C-peptide subsequent to dextrose bolus during IPGTT assessment to dispel any prospective misinterpretations of engraftment efficacy.

Our group previously evaluated the utility of BMX-010 in human clinical islet isolation and transplantation.[20] The results reported by Gala-Lopez et al. revealed that BMX-010 did not significantly enhance islet viability and function.[20] Our comparative human islet data presented herein revealed greater cytoprotection *in vitro* with BMX-001 than BMX-010, so the next conceivable step is to evaluate its use in the clinical islet isolation and transplantation setting. Our efforts to delineate whether human islets cultured for 24 hours with 34μM BMX-001 in an immunocompromised transplantation model revealed a greater percentage of recipients achieving euglycemia relative to non-treated control islet recipients, albeit not significantly. Since human islets are released for research several hours post-isolation, we were unable to administer BMX-001 during

collagenase pancreatic perfusion. It may be possible that improved human islet engraftment may be further enhanced when pancreases are supplemented with BMX-001 during pancreatic distension. Given that murine pancreases were distended and islets subsequently cultured in the presence of BMX-001 revealed improved *in vivo* outcomes, an identical model using human pancreases distended with collagenase supplemented with BMX-001 may be required to determine the full therapeutic effect of BMX-001. Since all organs are susceptible to ischemic damage and oxidative stress, systemic delivery of BMX-001 to the donor at the time of organ procurement may broadly ameliorate such consequences, expanding the clinical utility of this SOD-mimetic. Clearly, this study has revealed the therapeutic benefit of BMX-001 administration *in vitro* and its ability to augment murine marginal islet engraftment in a renal subcapsular, syngeneic transplant model. Preliminary evaluation of this drug in a murine, syngeneic portal vein islet infusion model in our laboratory suggests that administration of BMX-001 to the recipient may be required to confer significant benefit, however further evaluation is warranted. Moreover, since earlier generation SOD mimics have demonstrated immunosuppressive potential[19,35,36], an allograft transplantation model is a conceivable next step in supporting the utility of this therapeutic agent in clinical islet transplantation.

2.6 – REFERENCES

1. Bruni A, Gala-Lopez B, Pepper AR, Abualhassan NS, Shapiro AJ. Islet cell transplantation for the treatment of type 1 diabetes: recent advances and future challenges. *Diabetes Metab Syndr Obes.* 2014;7:211-223.
2. Shapiro AM, Lakey JR, Ryan EA, et al. Islet transplantation in seven patients with type 1 diabetes mellitus using a glucocorticoid-free immunosuppressive regimen. *N Engl J Med.* 2000;343(4):230-238.
3. Ryan EA, Paty BW, Senior PA, et al. Five-year follow-up after clinical islet transplantation. *Diabetes.* 2005;54(7):2060-2069.
4. Gala-Lopez B, Pepper AR, Shapiro AM. Biologic agents in islet transplantation. *Curr Diab Rep.* 2013;13(5):713-722.
5. Shapiro AM. Immune antibody monitoring predicts outcome in islet transplantation. *Diabetes.* 2013;62(5):1377-1378.
6. Shapiro AM, Pokrywczynska M, Ricordi C. Clinical pancreatic islet transplantation. *Nat Rev Endocrinol.* 2016.
7. Hering BJ, Clarke WR, Bridges ND, et al. Phase 3 Trial of Transplantation of Human Islets in Type 1 Diabetes Complicated by Severe Hypoglycemia. *Diabetes Care.* 2016;39(7):1230-1240.
8. Hanley SC, Paraskevas S, Rosenberg L. Donor and isolation variables predicting human islet isolation success. *Transplantation.* 2008;85(7):950-955.
9. 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(11):1029-1035.

10. Paraskevas S, Maysinger D, Wang R, Duguid TP, Rosenberg L. Cell loss in isolated human islets occurs by apoptosis. *Pancreas.* 2000;20(3):270-276.

11. Bottino R, Balamurugan AN, Tse H, et al. Response of human islets to isolation stress and the effect of antioxidant treatment. *Diabetes.* 2004;53(10):2559-2568.

12. Lenzen S, Drinkgern J, Tiedge M. Low antioxidant enzyme gene expression in pancreatic islets compared with various other mouse tissues. *Free Radic Biol Med.* 1996;20(3):463-466.

13. Grankvist K, Marklund SL, Taljedal IB. CuZn-superoxide dismutase, Mn-superoxide dismutase, catalase and glutathione peroxidase in pancreatic islets and other tissues in the mouse. *Biochem J.* 1981;199(2):393-398.

14. Cai H, Yang B, Xu Z, et al. Cyanidin-3-O-glucoside enhanced the function of syngeneic mouse islets transplanted under the kidney capsule or into the portal vein. *Transplantation.* 2015;99(3):508-514.

15. Kim JS, Jang HJ, Kim SS, et al. Red Ginseng Administration Before Islet Isolation Attenuates Apoptosis and Improves Islet Function and Transplant Outcome in a Syngeneic Mouse Marginal Islet Mass Model. *Transplant Proc.* 2016;48(4):1258-1265.

16. Tanaka T, Fujita M, Bottino R, et al. Endoscopic biopsy of islet transplants in the gastric submucosal space provides evidence of islet graft rejection in diabetic pigs. *Islets.* 2016;8(1):1-12.

17. Delmastro-Greenwood MM, Tse HM, Piganelli JD. Effects of metalloporphyrins on reducing inflammation and autoimmunity. *Antioxid Redox Signal.* 2014;20(15):2465-2477.

18. Bottino R, Balamurugan AN, Bertera S, Pietropaolo M, Trucco M, Piganelli JD. Preservation of human islet cell functional mass by anti-oxidative action of a novel SOD mimic compound. *Diabetes.* 2002;51(8):2561-2567.

19. Sklavos MM, Bertera S, Tse HM, et al. Redox modulation protects islets from transplant-related injury. *Diabetes.* 2010;59(7):1731-1738.

20. Gala-Lopez B, Kin T, O'Gorman D, et al. The metalloporphyrin BMX-010 in human islet isolation and clinical transplantation *CellR4.* 2016;4(3):8.

21. Bruni A, Pepper AR, Gala-Lopez B, 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(4):e1190058.

22. Kin T. Islet isolation for clinical transplantation. *Adv Exp Med Biol.* 2010;654:683-710.

23. Ricordi C, Lacy PE, Finke EH, Olack BJ, Scharp DW. Automated method for isolation of human pancreatic islets. *Diabetes.* 1988;37(4):413-420.

24. Cabrera O, Jacques-Silva MC, Berman DM, et al. Automated, high-throughput assays for evaluation of human pancreatic islet function. *Cell Transplant.* 2008;16(10):1039-1048.

25. McCall M, Pawlick R, Kin T, Shapiro AM. Anakinra potentiates the protective effects of etanercept in transplantation of marginal mass human islets in immunodeficient mice. *Am J Transplant.* 2012;12(2):322-329.

26. Bao J, Cai Y, Sun M, Wang G, Corke H. Anthocyanins, flavonols, and free radical scavenging activity of Chinese bayberry (Myrica rubra) extracts and their color properties and stability. *J Agric Food Chem.* 2005;53(6):2327-2332.

27. Bertera S, Crawford ML, Alexander AM, et al. Gene transfer of manganese superoxide dismutase extends islet graft function in a mouse model of autoimmune diabetes. *Diabetes.* 2003;52(2):387-393.

28. do Amaral AS, Pawlick RL, Rodrigues E, 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(2):e55288.

29. Gandy SE, Buse MG, Crouch RK. Protective role of superoxide dismutase against diabetogenic drugs. *J Clin Invest.* 1982;70(3):650-658.

30. Armann B, Hanson MS, Hatch E, Steffen A, Fernandez LA. Quantification of basal and stimulated ROS levels as predictors of islet potency and function. *Am J Transplant.* 2007;7(1):38-47.

31. Pepper AR, Bruni A, Pawlick R, et al. Engraftment Site and Effectiveness of the Pan-Caspase Inhibitor F573 to Improve Engraftment in Mouse and Human Islet Transplantation in Mice. *Transplantation.* 2017.

32. Contreras JL, Eckstein C, Smyth CA, et al. Brain death significantly reduces isolated pancreatic islet yields and functionality in vitro and in vivo after transplantation in rats. *Diabetes.* 2003;52(12):2935-2942.

33. Frank A, Deng S, Huang X, et al. Transplantation for type I diabetes: comparison of vascularized whole-organ pancreas with isolated pancreatic islets. *Ann Surg.* 2004;240(4):631-640; discussion 640-633.

34. Korsgren E, Korsgren O. Glucose Effectiveness: The Mouse Trap in the Development of Novel ss-Cell Replacement Therapies. *Transplantation.* 2016;100(1):111-115.

35. Tse HM, Milton MJ, Piganelli JD. Mechanistic analysis of the immunomodulatory effects of a catalytic antioxidant on antigen-presenting cells: implication for their use in targeting oxidation-reduction reactions in innate immunity. *Free Radic Biol Med.* 2004;36(2):233-247.

36. Piganelli JD, Flores SC, Cruz C, et al. A metalloporphyrin-based superoxide dismutase mimic inhibits adoptive transfer of autoimmune diabetes by a diabetogenic T-cell clone. *Diabetes.* 2002;51(2):347-355.

CHAPTER 3.

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

CHAPTER 3. 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

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RESEARCH PAPER

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

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ABSTRACT

Islet transplantation is a highly effective treatment for stabilizing glycemic control for select patients with type-1 diabetes. Despite improvements to clinical transplantation, single-donor transplant success has been hard to achieve routinely, necessitating increasing demands on viable organ availability. Donation after circulatory death (DCD) may be an alternative option to increase organ availability however, these organs tend to be more compromised. The use of metalloporphyrin anti-inflammatory and antioxidant (MnP) compounds previously demonstrated improved *in vivo* islet function in preclinical islet transplantation. However, the administration of MnP (BMX-001) in a DCD islet isolation and transplantation model has yet to be established. In this study, murine donors were subjected to a 15-min warm ischemic (WI) period prior to isolation and culture with or without MnP. Subsequent to one-hour culture, islets were assessed for *in vitro* viability and *in vivo* function. A 15-minute WI period significantly reduced islet yield, regardless of MnP-treatment relative to yields from standard isolation. MnP-treated islets did not improve islet viability compared to DCD islets alone. MnP-treatment did significantly reduce the presence of extracellular reactive oxygen species (ROS) ($p < 0.05$). Marginal, syngeneic islets (200 islets) transplanted under the renal capsule exhibited similar *in vivo* outcomes regardless of WI or MnP-treatment. DCD islet grafts harvested 7 d post-transplant exhibited sustained TNF-α and IL-10, while MnP-treated islet-bearing grafts demonstrated reduced IL-10 levels. Taken together, 15-minute WI in murine islet isolation significantly impairs islet yield. DCD islets do indeed demonstrate *in vivo* function, though MnP therapy was unable to improve viability and engraftment outcomes.

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Introduction

Islet transplantation has become a well-established treatment therapy for a subset of patients with type 1 diabetes mellitus.[1] The establishment of the "Edmonton Protocol" by Shapiro and colleagues demonstrated a high rate of insulin independence up to 1 y post-transplant.[2] Follow-up of these patients revealed a decline in graft function, with some patients returning to modest amounts of exogenous insulin, though still maintaining the benefit of the absence of hypoglycemic unawareness.[3] To date, considerable improvements in clinical islet transplantation outcomes have been observed, revealing insulin independence in at least 50% of recipients at 5 y post-transplant, matching outcomes achieved by whole organ pancreas transplantation.[4]

Despite numerous advances in clinical islet transplantation, most recipients require more than one intraportal islet infusion to establish and maintain periods of insulin independence.[5] Cadaveric donor pancreata are currently the sole, scarce source of islets. As such, to meet clinical demand, transplant centers routinely process extended criteria donor organs to expand the donor pool. Recently, donation after circulatory death (DCD) donors have been identified as a potential source of extended donors.[5] In whole pancreas transplantation, DCD and

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ORIGINAL ARTICLE

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

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Running Title: Redox-active metalloporphyrin in DCD islet transplantation

3.1 – ABSTRACT

Islet transplantation is a highly effective treatment for stabilizing glycemic control for select patients with type-1 diabetes. Despite improvements to clinical transplantation, single-donor transplant success has been hard to achieve routinely, necessitating increasing demands on viable organ availability. Donation after circulatory death (DCD) may be an alternative option to increase organ availability however, these organs tend to be more compromised. The use of metalloporphyrin anti-inflammatory and antioxidant (MnP) compounds previously demonstrated improved *in vivo* islet function in preclinical islet transplantation. However, the administration of MnP (BMX-001) in a DCD islet isolation and transplantation model has yet to be established. In this study, murine donors were subjected to a 15-minute warm ischemic (WI) period prior to isolation and culture with or without MnP. Subsequent to one-hour culture, islets were assessed for *in vitro* viability and *in vivo* function. A 15-minute WI period significantly reduced islet yield, regardless of MnP-treatment relative to yields from standard isolation. MnP-treated islets did not improve islet viability compared to DCD islets alone. MnP-treatment did significantly reduce the presence of extracellular reactive oxygen species (ROS) ($p<0.05$). Marginal, syngeneic islets (200 islets) transplanted under the renal capsule exhibited similar *in vivo* outcomes regardless of WI or MnP-treatment. DCD islet grafts harvested 7 days post-transplant exhibited sustained TNF-α and IL-10, while MnP-treated islet-bearing grafts demonstrated reduced IL-10 levels. Taken together, 15-minute WI in murine islet isolation significantly impairs islet yield. DCD islets do indeed demonstrate *in vivo* function, though MnP therapy was unable to improve viability and engraftment outcomes.

3.2 – INTRODUCTION

Islet transplantation has become a well-established treatment therapy for a subset of patients with type 1 diabetes mellitus.[1] The establishment of the 'Edmonton Protocol' by Shapiro and colleagues demonstrated a high rate of insulin independence up to one year post-transplant.[2] Follow-up of these patients revealed a decline in graft function, with some patients returning to modest amounts of exogenous insulin, though still maintaining the benefit of protection from hypoglycemia.[3] To date, considerable improvements in clinical islet transplantation outcomes have been observed, revealing insulin independence in at least 50% of recipients at 5 years post-transplant, matching outcomes achieved by whole organ pancreas transplantation.[4]

Despite numerous advances in clinical islet transplantation, most recipients require more than one intraportal islet infusion to establish and maintain periods of insulin independence.[5] Cadaveric donor pancreata are currently the sole, scarce source of islets. As such, to meet clinical demand, transplant centers routinely process extended criteria donor organs to expand the donor pool. Recently, donation after circulatory death (DCD) donors have been identified as a potential source of extended donors.[5] In whole pancreas transplantation, DCD and neurological determination of death (NDD) organ recipients have demonstrated similar rates of patient survival and graft function.[6] Alternatively, DCD donation is associated with poor graft function in liver transplantation.[7,8]

Prior to transplantation, islet loss and impaired β-cell metabolic function are the result of cellular insults during organ procurement and islet isolation.[9] The isolation procedure itself contributes to islet injury as a result of mechanical, ischemic and

oxidative stress.[10,11] With reduced endogenous antioxidant capacity, islets are highly susceptible to oxidative stress and subsequent over-production of reactive oxygen species (ROS).[9,12] The generation of ROS during islet isolation has been linked to the up-regulation of the transcription factor NF-κB, generation of pro-inflammatory cytokines and subsequent cell death.[9] Evidence suggests that redox modulation of islets through treatment with metalloporphyrin anti-inflammatory and antioxidant (MnP) compounds in culture can abrogate the deleterious consequences of oxidative stress, thus preserving islet mass *in vitro.*[9] Moreover, administration of MnP to islets prior to transplantation demonstrated improved graft function in a murine, marginal syngeneic model, as well as delayed allograft rejection in an MHC-mismatched islet transplant model.[12] Within the context of human islets, MnP administration has demonstrated beneficial viability and engraftment outcomes in rodent transplant models.[9,10,12]

To expand on the growing utility of DCD organs in islet transplantation, herein, we sought to establish a murine DCD islet isolation model and assess whether the reduction of ROS through acute redox modulation via a novel MnP-agent, BMX-001, could improve *in vitro* and *in vivo* islet function in a syngeneic, marginal transplantation model.

3.3 – MATERIALS AND METHODS

3.3.1 – Murine donation after circulatory death model

Mouse care was in accordance with the guidelines approved by the Canadian Council on Animal Care. Animals were housed under conventional conditions having access to food and water *ad libitum*. 8 to 12 week old male BALB/c mice (Jackson Laboratories, Canada) were placed under anesthetic with 5% isoflurane and euthanized

via cervical dislocation. Animals were confirmed deceased by cardiac palpation and were maintained under a heat lamp to maintain an internal body temperature of 37°C, as measured by a rectal thermometer, for a total warm ischemic (WI) period of 15 minutes. DCD BALB/c mice were randomly assigned as non-treated (DCD) or MnP donors (DCD+MnP-treated) and subsequently were administered 1 ml of cold histadine-tryptophan-ketoglutarate (HTK) with 1U/μl heparin (Sandoz Canada Inc., Boucherville, QC, CA) supplemented with or without 30 μmol/L MnP through the abdominal aorta in DCD donors.

3.3.2 – Mouse pancreatectomy and islet isolation

Pancreatic islets were isolated from standard (Control) or DCD BALB/c mice (Jackson Laboratories, Canada). Prior to pancreatectomy, the common bile duct was cannulated with a 27-gauge needle and the pancreas was distended with 0.125 mg/mL cold Liberase TL Research Grade enzyme (Roche Diagnostics, Laval, QC, CA) in Hank's Balanced Salt Solution (HBSS, Sigma-Aldrich Canada Co., Oakville, ON, CA). Islets were isolated by digesting the pancreases in a 50 ml Falcon tube placed in a 37°C water bath for 14 minutes with light agitation. Following the pancreatic digestion phase, islets were purified using histopaque-density gradient centrifugation (1.108, 1.083 and 1.069 g/mL, Sigma-Aldrich Canada Co., Oakville, ON, Canada).

3.3.3 – Administration of MnP

BMX-001 (MnTnBuOE-2-PhP^{5+} MN(III) *meso*-tetrakis(N-b-butoxyethylpyridinium-2-yl)porphyrin) was provided by BioMimetix JV LLC. BMX-

001 is one of the most potent metalloporphyrins with regard to anti-inflammatory and catalytic antioxidant function.[23] BMX-001 was administered during organ procurement, islet isolation, as well as during brief islet culture. DCD BALB/c mice were randomly assigned as non-treated (DCD) or MnP donors (DCD+MnP-treated). During mouse pancreatectomy and islet isolation, MnP (concentration 30 μmol/L) was delivered to pancreatic tissue with Liberase, as described above. In the non-treated group, Liberase with vehicle was delivered. DCD+MnP-treated and DCD pancreata were maintained in cold HBSS supplemented with or without MnP, respectively, until islet isolation (as described above).

3.3.4 – Islet culture

Connaught Medical Research Laboratories (CMRL-1066) medium supplemented with 10% fetal bovine serum, L-glutamine (2mM), penicillin (50 000 units), streptomycin (50 mg), HEPES (5mM), nicotinamide (10mM) and sodium pyruvate (5mM) at 37°C/5%CO_2 for one hour. DCD+MnP-treated islets were cultured in CMRL with 30 μM MnP.

3.3.5 – Reactive oxygen species analysis

Subsequent to one hour culture, cell-free supernatant samples from the study groups were assayed for ROS released into the culture media by Acridan Lumigen PS-3 assay (Amersham ECL Plus Kit; Fisher Scientific Inc., Ottawa, ON, Canada).[24] Acridan Lumigen PS-3 is excited by ROS and reactive nitrogen species in the presence of hydrogen peroxide, producing chemiluminescense at 430 nm. Media samples were

stored at -20°C until time of analysis. CMRL and CMRL+MnP culture medium served as controls for each group, and results were expressed as fold-change increase compared to each respective control.

3.3.6 – Assessment of islet yield and viability

One hour post-culture, islets were handpicked and counted to determine yield, and represented as islets per pancreas. Islet viability was determined by simultaneous staining of live and dead cells using a two-color fluorescence assay (SytoGreen 13 and ethidium bromide, Invitrogen, Oregon, USA). The percentage of viable and dead cells was determined for DCD and DCD+MnP-treated islets.

3.3.7 – Static glucose-stimulated insulin secretion (s-GSIS)

Handpicked islets from DCD and DCD+MnP-treated groups were subjected to s-GSIS. Islets were 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. Subsequent to glucose challenge, cell-free supernatants were harvested and insulin levels were measured by enzyme-linked immunosorbent assay (ELISA) (Mercodia, Uppsala, Sweden).

3.3.8 – Diabetes induction and marginal islet mass transplantation

One week prior to transplantation, recipient BALB/c mice were rendered diabetic by chemical induction with intraperitoneal streptozotocin (STZ) (Sigma-Aldrich Canada Co., Oakville, ON, Canada), at 185 mg/kg in acetate phosphate buffer, pH 4.5.

Diabetes was confirmed when non-fasting blood glucose levels exceeded 15 mmol/L for 2 consecutive daily readings. One hour post-islet culture, marginal mass islets from Control, DCD or DCD+MnP-treated islets (200 islets ± 10% per diabetic recipient) with purity of 90 ± 5%, were aspirated into polyethylene (PE-50) tubing using a micro-syringe, and centrifuged into a pellet suitable for transplantation. A left lateral paralumbar incision was made and the left kidney delivered. The renal capsule was incised and the islets were infused.

3.3.9 – Evaluation of islet graft function

Transplant efficacy was assessed three times per week in recipients through non-fasting blood glucose measurements (mmol/L), using a portable glucometer (OneTouch Ultra 2, LifeScan, Canada) in all groups tested. Graft function and reversal of diabetes was defined as two consecutive readings ≤ 11.1 mmol/L and maintained until study completion. To assess metabolic capacity of the islet graft, intraperitoneal glucose tolerance tests (IPGTTs) were conducted on euglycemic mice 60 days post-transplant. Mice were fasted overnight prior to receiving an intraperitoneal glucose bolus (3g/kg). Blood glucose levels were evaluated at baseline (time 0), 15, 30, 60, 90 and 120 minutes post-injection. Blood glucose area under the curve (AUC-blood glucose) was calculated and analyzed between transplant groups.

3.3.10 – Islet graft retrieval

In order to corroborate graft-dependent euglycemia, islet transplants were retrieved by nephrectomy. Islet transplant recipients were placed under anesthesia, and

their graft-bearing kidney was exposed. Using a LT200 Ligaclip (Johnson & Johnson, Inc., Ville St-Laurent, QC, CA), the renal vessels and ureter were ligated and the islet graft-bearing kidney was removed. Non-fasting blood glucose measurements were monitored up to 7 days post-graft removal to confirm hyperglycemia and thus post-transplant graft function.

3.3.11 – Pro-inflammatory cytokine assessment

Pro-inflammatory cytokines were analyzed from islet-bearing kidney grafts harvested 24 hours and 7 days post-transplant. Three mice per group underwent recovery nephrectomy at the aforementioned time points, and grafts were assessed for mouse tumor necrosis factor (TNF)-α, KC-GRO, interferon (IFN)-γ, interleukin (IL)-1β, IL-10, IL-6 and IL-12p70. Pro-inflammatory levels were measured using a Mouse ProInflammatory 7-Plex Tissue Culture Kit according to manufacturer instructions (Meso Scale Diagnostics, K15012B-1). The plate was loaded into an MSD-SECTOR® instrument for analysis where a voltage was applied and the bound label emitted a quantitative (0 - 1.0 x 10^6 pg/mL) measure of light. Values were normalized to weight of tissue homogenized.

3.3.12 – Statistical analysis

All data are represented as the mean ± standard error of mean (s.e.m.). *In vitro* islet viability data comparisons between DCD and DCD+MnP-treated islets were conducted through unpaired Student's t-test. Blood glucose AUC analysis for glucose tolerance test data was conducted through parametric one-way ANOVA using GraphPad

Prism (GraphPad Software, La Jolla, CA, USA). Tukey's post-hoc tests were used following the analysis of variances for multiple comparisons between study groups. Kaplan-Meyer survival function curves were compared using the log-rank statistical method (Mantel-Cox). $P<0.05$ was considered significant.

3.4 – RESULTS

3.4.1.1 – DCD mouse islets cultured in the presence of MnP exhibit reduced extracellular ROS

Supernatants from DCD islets cultured in media alone exhibited a greater fold-increase in extracellular ROS production, which was ameliorated in the presence of MnP (DCD: 2.68 ± 0.11vs. DCD+MnP-treated: 1.65 ± 0.15) (n=3 isolations, $P<0.001$) (**Figure 3.1A**).

3.4.1.2 – 15-minute warm ischemia significantly impairs islet yield in a murine DCD model

Subsequent to islet isolation and culture, islets were handpicked and quantified to determine islet yield. For standard control conditions, an islet yield of 192.6 ± 24.2 islets/pancreas was achieved. In contrast, islets harvested from DCD and DCD+MnP-treated donors exhibited a significantly reduced islet yield (77 ± 12.9 islets/pancreas vs. 79.1 ± 17.3 islets/pancreas, respectively, $p<0.05$). Notably, MnP-treatment during organ procurement and one hour culture did not improve islet yield outcomes in DCD donors ($p>0.05$) (**Figure 3.1B**).

3.4.1.3 – MnP administration does not improve in vitro islet viability and function

Dual-fluorescence staining assessing islet viability in DCD and DCD+MnP-treated islets revealed no discernable difference between groups when assessed one hour post-culture (DCD: 77.23 ± 4.9 vs. DCD+MnP-treated: 75.32 ±5.3) (**Figure 3.1C**). Similarly, glucose static challenge revealed that murine DCD islets secreted insulin in response to glucose (Stimulation index: 1.38 ± 0.33) to a similar degree to that of DCD+MnP-treated islets (Stimulation index: 1.29 ± 0.24, $p>0.05$) (**Figure 3.1D**).

3.4.2 – Efficacy of DCD islets pre-treated with MnP

Islet engraftment efficacy of islets isolated from DCD donors pre-treated with or without MnP was evaluated in a marginal islet transplant mass model (200 islets per recipient, n=14 per group). As a means to compare engraftment efficiency, an additional group of diabetic recipients were transplanted with a marginal dose under the kidney capsule from standard control donors (Control: n=7). Recipients of control islet transplants became euglycemic, 5 of 7 (71%), on average 14.5 ± 5.7 days post-syngeneic transplant (red). DCD islet recipient mice became euglycemic, 11 of 14 (78.6%) by 24.5 ± 6.4 days post-transplant, while 10 of 14 (71.4%) DCD+MnP-treated islet recipients became euglycemic in 12.10 ± 3.7 days (data non-significant) (**Figure 3.2A**). Daily non-fasting blood glucose monitoring of euglycemic transplant recipients revealed no difference between control, DCD or DCD+MnP-treated islet recipients (**Figure 3.2B**).

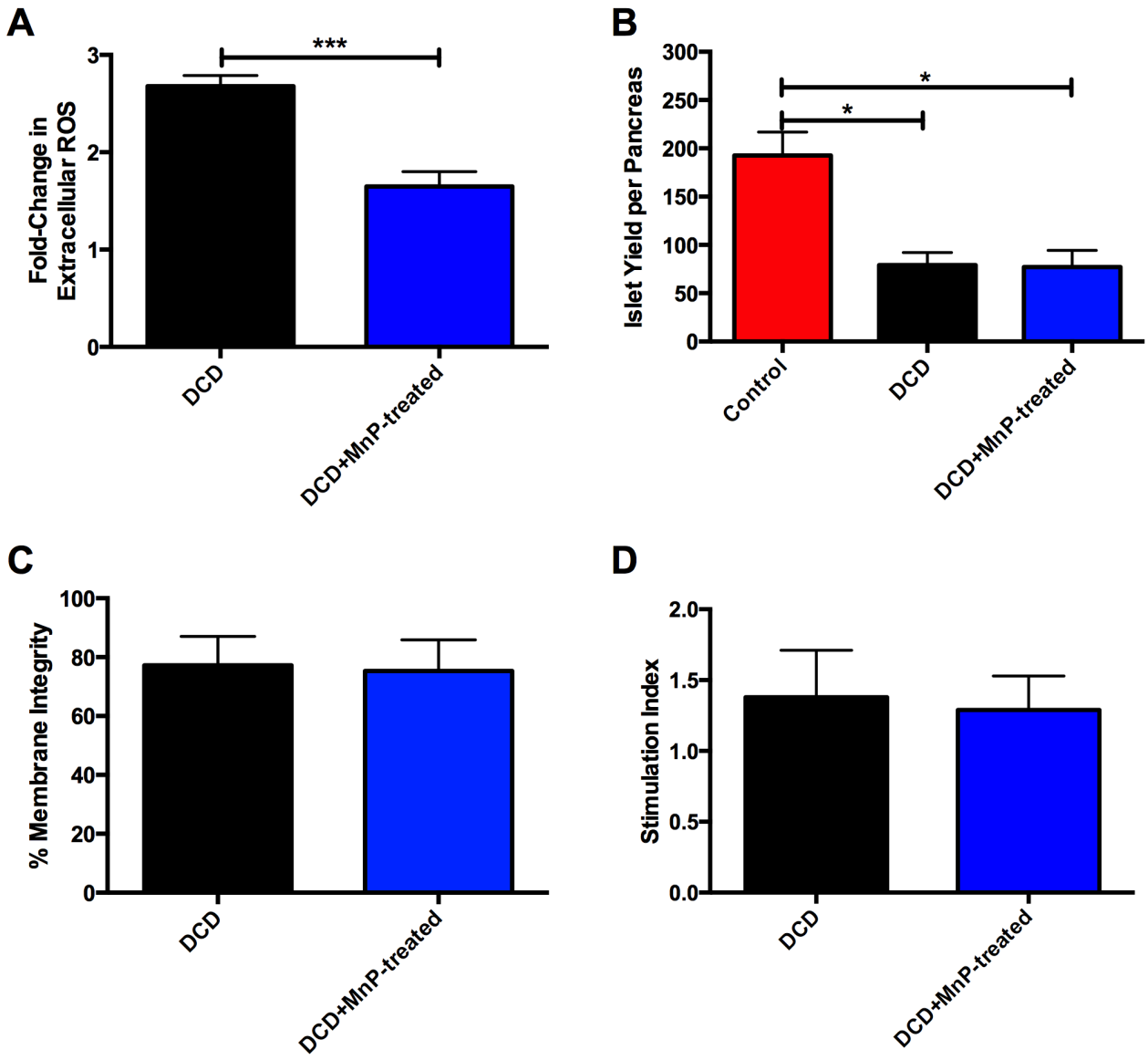

A
Fold-Change in Extracellular ROS
3
2
1
0
***
DCD
DCD+MnP-treated
B
Islet Yield per Pancreas
300
250
200
150
100
50
0
*
*
Control
DCD
DCD+MnP-treated
C
% Membrane Integrity
100
80
60
40
20
0
DCD
DCD+MnP-treated
D
Stimulation Index
2.0
1.5
1.0
0.5
0.0
DCD
DCD+MnP-treated

Figure 3.1. *In vitro* assessment of control, DCD and DCA+MnP-treated islets. (**A**) Fold-change in extracellular ROS assessed from cell-free supernatants was significantly reduced in DCD+MnP-treated (blue) islets than DCD islets (black) ($p<0.001$, t-test). (**B**) Control islet yield per pancreas (red) was significantly greater than DCD and DCD+MnP-treated islets (blue) ($p<0.05$, ANOVA). In contrast, supplementation with MnP did not improve islet isolation yields in DCD donors. (**C**) DCD and DCD+MnP-treated islets exhibited similar islet viability as assessed by dual-fluorescence staining ($p>0.05$, t-test). (**D**) Islet function, as assessed by static glucose stimulated insulin secretion, demonstrated no significant difference between DCD and DCD+MnP-treated islets ($p>0.05$, t-test).

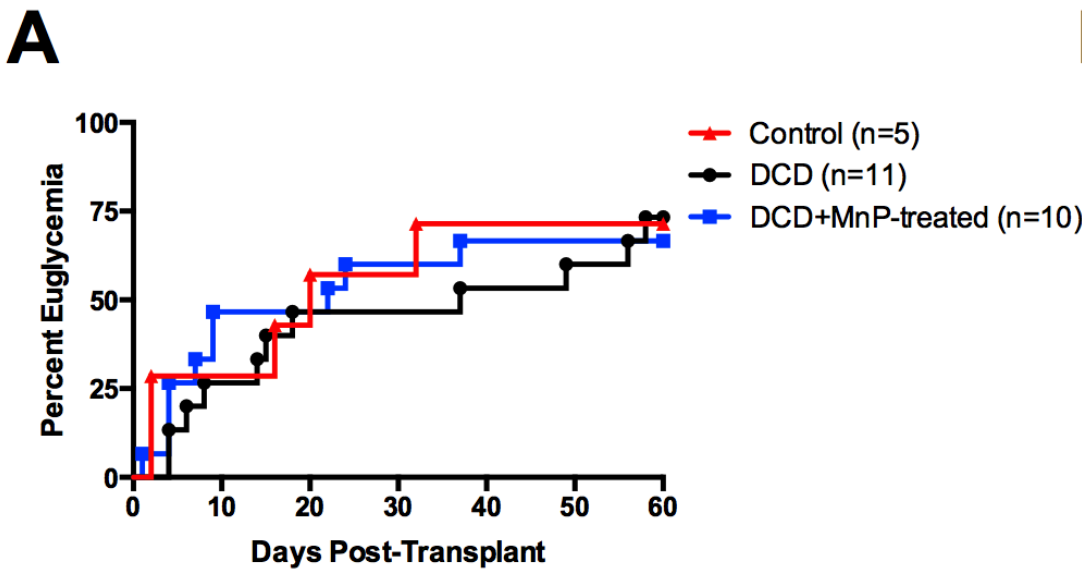
3.4.3 – Glucose tolerance testing

Intraperitoneal glucose tolerance tests (IPGTTs) were performed on all euglycemic recipients 60 days post-transplant. Mice in all transplant groups exhibited a physiological response to glucose bolus, with a prompt restoration of normoglycemia up to 120 minutes post-dextrose infusion (**Figure 3.3A**). Furthermore, there was no significant difference in mean area under the curve (AUC) ± s.e.m. (AUC Control: 1773 ± 93.4 mmol/L/120min vs. DCD: 2090 ± 177.6 mmol/L/120min vs. DCD+MnP-treated: 1753 ± 105.9 mmol/L/120min, $p>0.05$, ANOVA, **Figure 3.3B**).

3.4.4 – Pro-inflammatory cytokine profile

Islet-bearing kidney grafts from control, DCD and DCD+MnP-treated islet recipients were assessed for the pro-inflammatory cytokines TNF-α, KC-GRO, IFN-γ, IL-1β, IL-10, IL-6 and IL-12p70, 24 hours and 7 days post-transplant. Control islet-bearing grafts harvested 7 days post-transplant exhibited a significant reduction in IL-10, IL-1β and TNF-α cytokine levels compared to grafts harvested 24 hours post-transplant ($p<0.05$, $p<0.05$, and $p<0.001$, respectively) (**Figures 3.4A – C**). In contrast, DCD islet-bearing grafts exhibited indistinguishable IL-10 and TNF-α levels at 24 hours and 7 days post-transplant, but reduced IL-1β levels at 7 days post-transplant ($p<0.05$) (**Figure 3.4D – F**). With the exception of TNF-α, DCD+MnP-treated islets exhibited significantly reduced IL-10 and IL-1β cytokine levels for grafts harvested at 7 days post-transplant in comparison to grafts harvested at 24 hours post-transplant ($p<0.05$ and $p<0.01$, respectively) (**Figure 3.4G – I**). Inflammatory levels for cytokines KC-GRO, IFN-γ, IL-

6 and IL-12p70 were non-detectable in grafts harvested at the aforementioned time points (data not shown).


A
100
75
50
25
0
Percent Euglycemia
0
10
20
30
40
50
60
Days Post-Transplant
Control (n=5)
DCD (n=11)
DCD+MnP-treated (n=10)

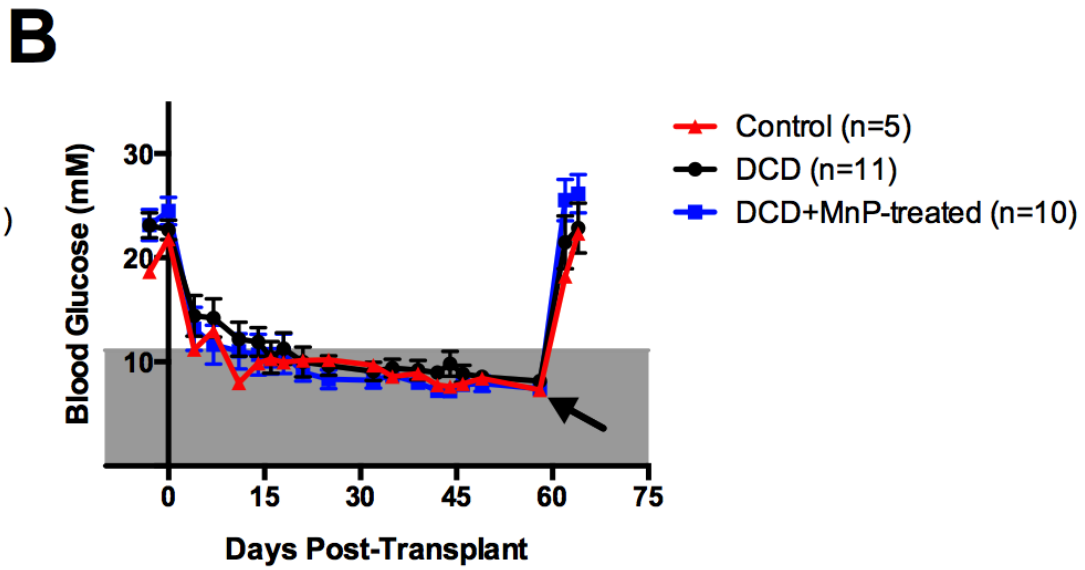
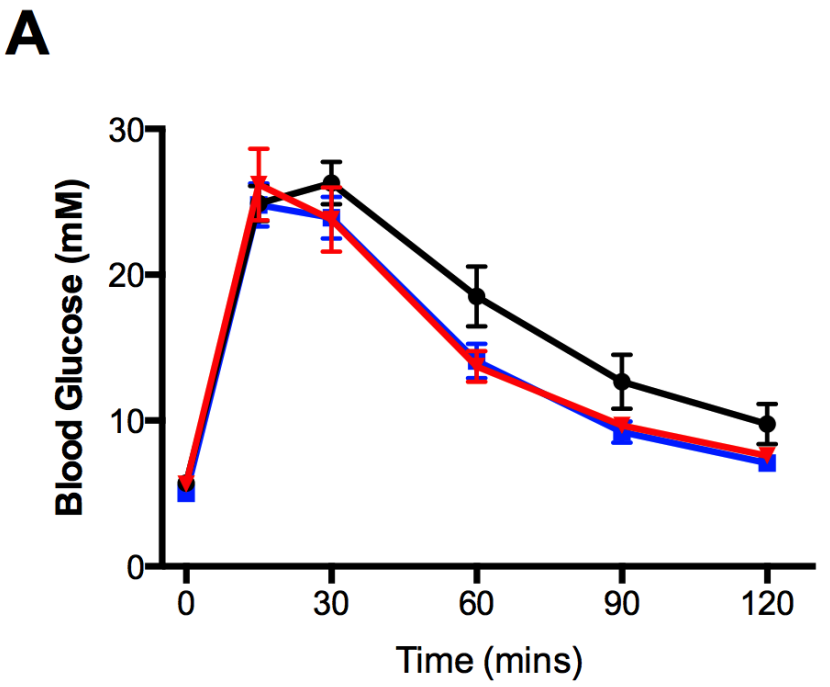

B
30
20
10
Blood Glucose (mM)
0
15
30
45
60
75
Days Post-Transplant
Control (n=5)
DCD (n=11)
DCD+MnP-treated (n=10)

Figure 3.2. Efficacy of syngeneic, marginal islet transplants under the renal capsule of BALB/c recipients.

Percent euglycemia of syngeneic, marginal islet transplant recipients was indistinguishable between control (red, n=5 of 7), DCD (black, 11 of 14) and DCD+MnP-treated (blue, n=11 of 14) recipients ($p>0.05$, Mantel-Cox). (**B**) Non-fasting blood glucose measurements of euglycemic recipients post-transplant. Recipients of control, DCD or DCD+MnP-treated marginal islets exhibited robust glycemic control until graft retrieval (arrow).


A
Blood Glucose (mM)
30
20
10
0
0
30
60
90
120
Time (mins)

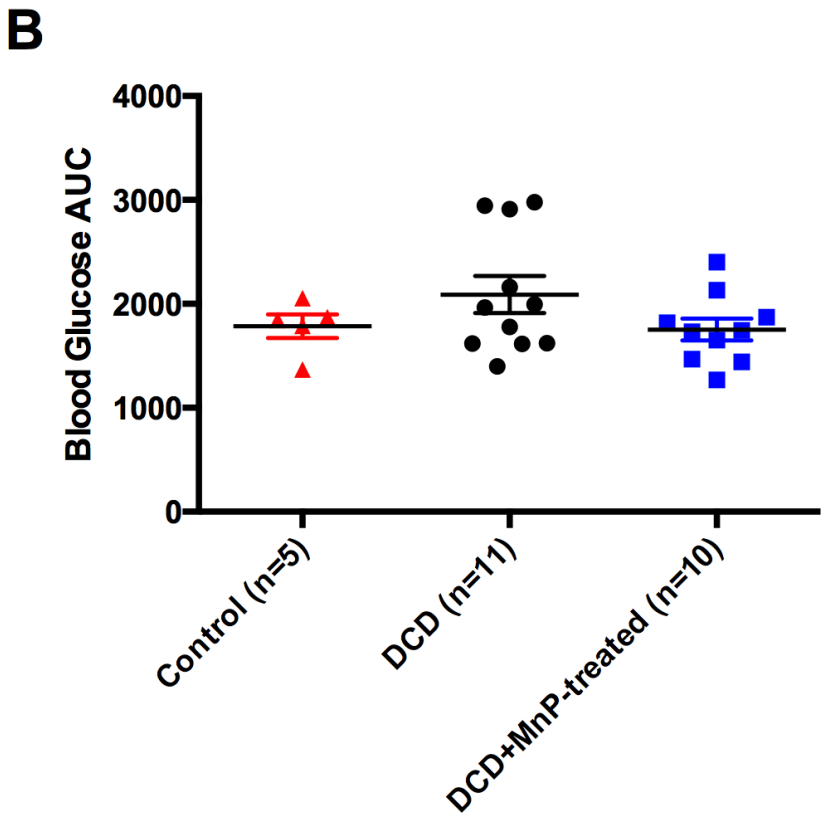

B
Blood Glucose AUC
4000
3000
2000
1000
0
Control (n=5)
DCD (n=11)
DCD+MnP-treated (n=10)

Figure 3.3. Intraperitoneal glucose tolerance test (IPGTT) of syngeneic, marginal islet mass recipients transplanted with control, DCD or DCD+MnP-treated islets 60 days post-transplant.

(**A**) Blood glucose prolife post-dextrose bolus of control (red, n=5), DCD (black, n=11) and DCD+MnP-treated islets (blue, n=10) (**B**) Blood glucose area under the curve (AUC) analysis did not differ between control, DCD and DCD+MnP-treated islet recipients ($p>0.05$, ANOVA). Mice were administered 3 mg/kg 25% dextrose i.p. Blood glucose measurements were monitored at t=0, 15, 30, 60, 90 and 120 minutes.

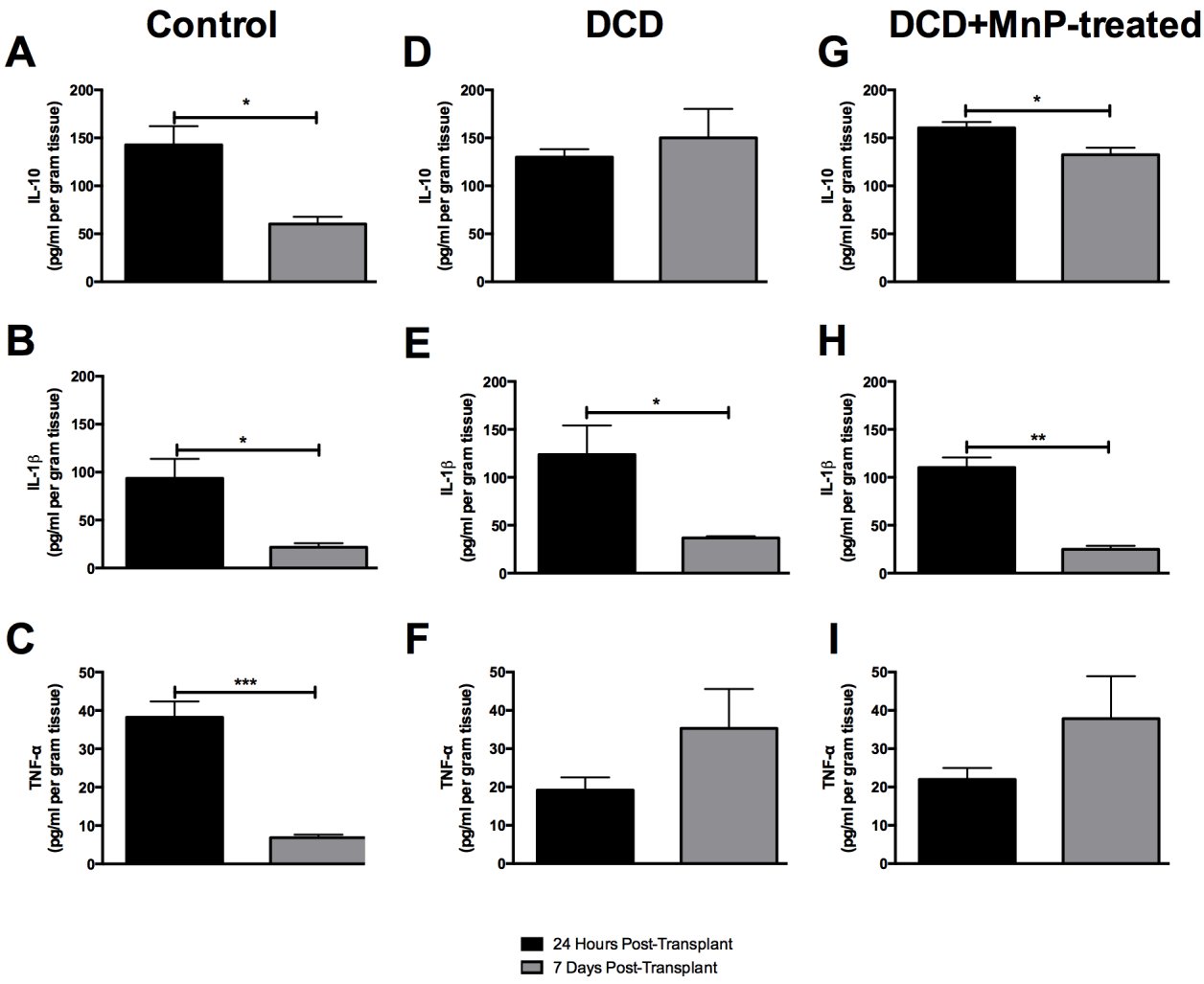

Control
DCD
DCD+MnP-treated
A
B
C
D
E
F
G
H
I
IL-10 (pg/ml per gram tissue)
IL-1β (pg/ml per gram tissue)
TNF-α (pg/ml per gram tissue)
*
**
***
24 Hours Post-Transplant
7 Days Post-Transplant

Figure 3.4. Pro-Inflammatory profile of islet-bearing kidney grafts harvested 24 hours and 7 days post-transplant.

(**A - C**) Control islet-bearing kidney grafts exhibited significantly reduced pro-inflammatory profiles for IL-10, IL-1β and TNF-α at 7 days post-transplant (grey, n=3) relative to 24 hours post-transplant (black, n=3). (**D - F**) DCD islet-bearing kidney grafts exhibited significantly reduced IL-1 β ($p<0.05$), but persistent IL-10 and TNF-α levels at 7 days post-transplant (grey, n=3) compared to 24 hours post-transplant (black, n=3). (**G - I**) DCD+MnP-treated islet-bearing kidney grafts exhibited significantly reduced pro-inflammatory profiles for IL-10 and IL-1β ($p<0.05$), but sustained TNF-α cytokine levels at 7 days post-transplant (grey, n=3) relative to 24 hours post-transplant (black, n=3) ($p<0.05$).

3.5 – DISCUSSION

Islet transplantation is limited, in part, by availability of cadaveric donor pancreata. Expansion of the donor pool to include extended criteria and DCD donors would considerably enhance availability of clinical islet transplants. To date, the success of DCD islets in the context of clinical islet transplantation has been evaluated at few single centers worldwide, with limited long-term successful outcomes[13-15] The necessity to establish preclinical models evaluating the utility of DCD islets could provide insights into their clinical application. Herein, we sought to establish a murine DCD model and evaluate its efficacy in a marginal, syngeneic islet transplant model, as well as determine if supplementation with a novel MnP during organ procurement, islet isolation and brief culture could improve DCD islet transplant function.

In the present study, we determined that a 15-minute warm ischemic (WI) model in mice significantly impaired islet yield per pancreas relative to standard islet donors without WI. A study by Giraud and colleagues also established that mice exposed to WI exhibited a significantly reduced islet yield relative to islets not exposed to periods of WI.[16] The authors reported that 30 minutes of WI in humans is equivalent to 3.5 minutes of WI in mice based on metabolic differences between the two species, like oxygen consumption and resting heart rate.[16] Our 15-minute WI model greatly exceeds this equivalence rate, and though MnP administration could reduce extracellular ROS, it could not improve islet yield or *in vitro* viability outcomes.

Despite our *in vitro* observations, DCD islets exhibited engraftment outcomes similar to standard procured islets. Similar to our findings, in a porcine islet isolation model, pancreata subjected to 30 minutes of warm ischemia exhibited a significantly

reduced islet yield in comparison to non-warm ischemic pancreata. In contrast, ischemic porcine islets exhibited reduced graft function compared to their non-warm ischemic counterparts in a diabetic nude mouse transplant model.[17] Within the clinical setting, a recent report by Andres and colleagues revealed that clinical islet isolations from 15 human DCD pancreata experiencing a maximal warm ischemia limit of <30 minutes exhibited no discernable difference in islet yield in comparison to standard neurological determination of death (NDD) pancreata. Moreover, examination of insulin requirement one month post-transplant in recipients of islets from NDD or DCD pancreata revealed no significant difference between groups.[5] WI significantly impaired islet yield in our murine model, but DCD islets exhibited similar *in vivo* islet function to standard control islet recipients, as evidenced by the restoration of euglycemia and IPGTT responses at 60 days post-transplant. Though MnP administration did not enhance islet function, the findings in the present study demonstrate that MnP supplementation is safe and non-toxic to islets.

Control islet recipients exhibited significantly reduced pro-inflammatory cytokine levels at 7 days post-transplant. To the contrary, DCD islets exhibited a sustained pro-inflammatory cytokines IL-10 and TNF-α up to 7 days post-transplant relative to standard control islet recipients. Short-term culture of DCD+MnP islets did not significantly improve TNF-α profiles, though a significant reduction was observed in IL-1β and IL-10. Though heightened pro-inflammatory cytokines in DCD islet recipients did not impact the long-term engraftment outcomes in this syngeneic model, the consequences in allotransplantation may prove detrimental. The interplay between the innate immune system, pro-inflammatory cytokines and the adaptive immune system

is critical in transplantation.[18] Within the context of clinical islet transplantation, Bellin and colleagues observed significantly improved long-term insulin independence rates in recipients administered a TNF-α inhibitor in the peri-transplant period along with T-cell depleting antibody.[19] Tse et al. demonstrated that redox modulation through MnP administration greatly diminished ROS production and subsequently ameliorated the synergism between the innate and adaptive immune response, and subsequent inflammatory cytokine production.[20] In parallel to measuring pro-inflammatory cytokine markers, it may have been of added benefit to examine the levels of monocyte chemoattractant protein-1 (MCP-1/CCL2) in the acute transplant period to determine whether MnP administration impacted these levels. Previous bodies of work demonstrated that increased MCP-1 levels negatively impacted islet engraftment outcomes.[21,22] These events may account for the observation that 25% of DCD-islet recipients were euglycemic 10 days post-transplant, as compared to 50% of DCD+MnP-treated islet recipients. Furthermore, our observation that MnP treatment reduced some pro-inflammatory cytokines, exploring the affect of MnP administration paired with an effective immunosuppressive regimen in a preclinical allograft model could improve islet engraftment outcomes.

The results from the current study demonstrated that short-term administration of a novel MnP, BMX-001, was capable of significantly reducing extracellular ROS production in a DCD islet isolation model. Work in our laboratory has demonstrated that human islets cultured with 30 μM BMX-001 improves islet recovery up to 7 days in culture, and exhibited cytoprotection in the presence of tacrolimus, relative to islets cultured without MnP (data not published). These results provided the rationale for

utilizing this dose in our DCD model. Numerous studies have utilized and observed cytoprotection and improved engraftment outcomes when human or murine islets were cultured in the presence of early-generation MnPs for 24 hours or more.[9,10,12] It may be possible that short-term culture with MnP in our model cannot feasibly confer protection and that longer islet culture may be required to observe a significant benefit of MnP treatment. Due to an initial low islet yield, a prolonged islet culture period was not incorporated into the study to avoid further islet loss in culture. We are in the process of utilizing this novel MnP treatment in a standard murine transplant model to determine if MnP administration can confer improved engraftment outcomes, which may translate to improved clinical islet transplantation success. Despite these observations, our data reveal that DCD mouse islets are capable of restoring euglycemia comparable to control islets which is an encouraging finding, strengthening their utility in the islet transplant setting. It is clear that the DCD WI model is especially challenging for islet isolation in mice, and likely does not parallel a similar process in human islet isolation. Therefore, we cannot extrapolate from the current studies how protective MnP therapies will be in human islet isolation and transplantation. However, the impact upon inflammatory markers and ROS is strongly positive. Further studies in large animals and human islet isolation are now required to fully understand the potential benefit of this approach.

3.6 – REFERENCES

1. Bruni A, Gala-Lopez B, Pepper AR, Abualhassan NS, Shapiro AJ. Islet cell transplantation for the treatment of type 1 diabetes: recent advances and future challenges. *Diabetes Metab Syndr Obes.* 2014;7:211-223.
2. Shapiro AM, Lakey JR, Ryan EA, et al. Islet transplantation in seven patients with type 1 diabetes mellitus using a glucocorticoid-free immunosuppressive regimen. *N Engl J Med.* 2000;343(4):230-238.
3. Ryan EA, Paty BW, Senior PA, et al. Five-year follow-up after clinical islet transplantation. *Diabetes.* 2005;54(7):2060-2069.
4. Shapiro AM. Immune antibody monitoring predicts outcome in islet transplantation. *Diabetes.* 2013;62(5):1377-1378.
5. Andres A, Kin T, O'Gorman D, et al. Clinical islet isolation and transplantation outcomes with deceased cardiac death donors are similar to neurological determination of death donors. *Transpl Int.* 2016;29(1):34-40.
6. Siskind E, Maloney C, Akerman M, et al. An analysis of pancreas transplantation outcomes based on age groupings--an update of the UNOS database. *Clin Transplant.* 2014;28(9):990-994.
7. D'Alessandro A M, Hoffmann RM, Knechtle SJ, et al. Liver transplantation from controlled non-heart-beating donors. *Surgery.* 2000;128(4):579-588.
8. Selck FW, Grossman EB, Ratner LE, Renz JF. Utilization, outcomes, and retransplantation of liver allografts from donation after cardiac death: implications for further expansion of the deceased-donor pool. *Ann Surg.* 2008;248(4):599-607.

9. Bottino R, Balamurugan AN, Tse H, et al. Response of human islets to isolation stress and the effect of antioxidant treatment. *Diabetes.* 2004;53(10):2559-2568.

10. Bottino R, Balamurugan AN, Bertera S, Pietropaolo M, Trucco M, Piganelli JD. Preservation of human islet cell functional mass by anti-oxidative action of a novel SOD mimic compound. *Diabetes.* 2002;51(8):2561-2567.

11. Rosenberg L, Wang R, Paraskevas S, Maysinger D. Structural and functional changes resulting from islet isolation lead to islet cell death. *Surgery.* 1999;126(2):393-398.

12. Sklavos MM, Bertera S, Tse HM, et al. Redox modulation protects islets from transplant-related injury. *Diabetes.* 2010;59(7):1731-1738.

13. Saito T, Gotoh M, Satomi S, et al. Islet transplantation using donors after cardiac death: report of the Japan Islet Transplantation Registry. *Transplantation.* 2010;90(7):740-747.

14. Anazawa T, Saito T, Goto M, et al. Long-term outcomes of clinical transplantation of pancreatic islets with uncontrolled donors after cardiac death: a multicenter experience in Japan. *Transplant Proc.* 2014;46(6):1980-1984.

15. Markmann JF, Deng S, Desai NM, et al. The use of non-heart-beating donors for isolated pancreatic islet transplantation. *Transplantation.* 2003;75(9):1423-1429.

16. Giraud S, Hauet T, Eugene M, Mauco G, Barrou B. A new preservation solution (SCOT 15) Improves the islet isolation process from pancreata of non-heart-beating donors: a Murine model. *Transplant Proc.* 2009;41(8):3293-3295.

17. Brandhorst D, Iken M, Bretzel RG, Brandhorst H. Pancreas storage in oxygenated perfluorodecalin does not restore post-transplant function of isolated

pig islets pre-damaged by warm ischemia. *Xenotransplantation.* 2006;13(5):465-470.

18. Otterbein LE, Fan Z, Koulmanda M, Thronley T, Strom TB. Innate immunity for better or worse govern the allograft response. *Curr Opin Organ Transplant.* 2015;20(1):8-12.

19. Bellin MD, Barton FB, Heitman A, et al. Potent induction immunotherapy promotes long-term insulin independence after islet transplantation in type 1 diabetes. *Am J Transplant.* 2012;12(6):1576-1583.

20. Tse HM, Milton MJ, Schreiner S, Profozich JL, Trucco M, Piganelli JD. Disruption of innate-mediated proinflammatory cytokine and reactive oxygen species third signal leads to antigen-specific hyporesponsiveness. *J Immunol.* 2007;178(2):908-917.

21. Melzi R, Mercalli A, Sordi V, et al. Role of CCL2/MCP-1 in islet transplantation. *Cell Transplant.* 2010;19(8):1031-1046.

22. Piemonti L, Leone BE, Nano R, et al. Human pancreatic islets produce and secrete MCP-1/CCL2: relevance in human islet transplantation. *Diabetes.* 2002;51(1):55-65.

23. Batinic-Haberle I, Tovmasyan A, Roberts ER, Vujaskovic Z, Leong KW, Spasojevic I. SOD therapeutics: latest insights into their structure-activity relationships and impact on the cellular redox-based signaling pathways. *Antioxid Redox Signal.* 2014;20(15):2372-2415.

24. Uy B, McGlashan SR, Shaikh SB. Measurement of reactive oxygen species in the culture media using Acridan Lumigen PS-3 assay. *J Biomol Tech.* 2011;22(3):95-107.

CHAPTER 4.

ENGRAFTMENT SITE AND EFFECTIVENESS OF THE PAN-CASPASE INHIBITOR F573 TO IMPROVE ENGRAFTMENT IN MURINE AND HUMAN ISLET TRANSPLANTATION IN MICE

CHAPTER 4. ENGRAFTMENT SITE AND EFFECTIVENESS OF THE PAN-CASPASE INHIBITOR F573 TO IMPROVE ENGRAFTMENT IN MURINE AND HUMAN ISLET TRANSPLANTATION IN MICE

Original Basic Science—General

Engraftment Site and Effectiveness of the Pan-Caspase Inhibitor F573 to Improve Engraftment in Mouse and Human Islet Transplantation in Mice

Andrew R. Pepper, PhD,[1,2] Antonio Bruni, BSc,[1,2] Rena Pawlick, BSc,[1] John Wink, BSc,[1] Yasmin Rafiei,[1] Boris Gala-Lopez, MD, PhD,[1,2] Mariusz Bral, MD,[1,2] Nasser Abualhassan, MD, MSc,[1,2] Tatsuya Kin, MD, PhD,[1] and A.M. James Shapiro, MD, PhD[1,2]

Background. Islet transplantation is an effective therapy in type 1 diabetes and recalcitrant hypoglycemia. However, there is an ongoing need to circumvent islet loss posttransplant. We explore herein the potential of the pan-caspase inhibitor F573 to mitigate early apoptosis-mediated islet death within portal and extrahepatic portal sites in mice. **Methods.** Mouse or human islets were cultured in standard media ±100 μM F573 and subsequently assessed for viability and apoptosis via terminal deoxynucleotidyl transferase dUTP nick end labeling staining and caspase-3 activation. Diabetic mice were transplanted with syngeneic islets placed under the kidney capsule (KC) or into the subcutaneous devicelless (DL) site at a marginal islet dose (150 islets), or into the portal vein (PV) at a full dose (500 islets). Human islets were transplanted under the KC of diabetic immunodeficient mice at a marginal dose (500 islet equivalents). Islets were cultured in the presence of F573, and F573 was administered subcutaneously on days 0 to 5 posttransplant. Control mice were transplanted with nontreated islets and were injected with saline. Graft function was measured by nonfasting blood glucose and glucose tolerance testing. **Results.** F573 markedly reduced human and mouse islet apoptosis after in vitro culture ($P < 0.05$ and $P < 0.05$, respectively). Furthermore, F573 improved human islet function when transplanted under the KC ($P < 0.05$); whereas F573 did not enhance murine islet marginal KC transplants. Conversely, F573 significantly improved mouse islet engraftment in the PV and DL site ($P < 0.05$ and $P < 0.05$, respectively). **Conclusions.** The pan-caspase inhibitor F573 markedly reduces human and mouse islet apoptosis and improves engraftment most effectively in the portal and DL subcutaneous sites.

(*Transplantation* 2017;101: 2321–2329)

Islet transplantation has recently been proven to stabilize glycemic control in select patients with type 1 diabetes complicated by hypoglycemia in a phase 3 trial,[1] extending the initial success of the Edmonton Protocol popularized by Shapiro and colleagues in 2000.[2] Within the last 16 years, islet transplantation success rates have improved substantially, with insulin-independence rates in at least 50% of recipients achieved out to 5 years post transplant in 6 International Centers.[3] Yet despite these clear advances in clinical islet transplantation, achievement of single-donor engraftment success has been difficult to establish routinely, with most recipients requiring at least 2 donors to achieve insulin independence.[4,5] Numerous factors contribute to islet loss in the acute and peritransplant period which results in an estimated 70% loss of transplanted β-cell mass.[6] The instant blood-mediated inflammatory reaction is largely responsible for immediate cell loss especially in the intraportal hepatic site, and associated with platelet activation and triggering of biochemical cell death pathways including both apoptosis and necrosis.[7,8] Potent cell death inhibitors, and exploration of

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A.R.P. participated in the research design, performance of the research, data analysis and writing of the article. A.B., R.P., B.G.L., M.B., N.A., J.W., Y.R. participated in the performance of the research and writing of the article. T.K. provided human islets and review of the article. A.M.J.S. participated in research design and writing of the article.

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ORIGINAL ARTICLE

Engraftment Site and Effectiveness of the Pan-Caspase Inhibitor F573 to Improve Engraftment in Mouse and Human Islet Transplantation in Mice

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Note: The current chapter is the result of combined efforts of our entire team where Dr. Andrew Pepper and I had leading roles in the design of the experiments, execution of transplant procedures and the final writing of this manuscript, under the supervision of Dr. James Shapiro. Despite being second author in this publication, the experiments associated with this project became an important component of my PhD work.

4.1 – ABSTRACT

Background. Islet transplantation is an effective therapy in type 1 diabetes and recalcitrant hypoglycemia. However, there is an ongoing need to circumvent islet loss post-transplant. We explore herein the potential of the pan-caspase inhibitor F573, to mitigate early apoptosis-mediated islet death within portal and extrahepatic portal sites in mice. **Methods.** Mouse or human islets were cultured in standard media ±100 μM F573 and subsequently assessed for viability and apoptosis via TUNEL staining and caspase-3 activation. Diabetic mice were transplanted with syngeneic islets placed under the kidney capsule (KC) or into the subcutaneous device-less (DL) site at a marginal islet dose (150 islets), or into the portal vein (PV) at a full dose (500 islets). Human islets were transplanted under the KC of diabetic immunodeficient mice at a marginal dose (500 islet equivalents). Islets were cultured in the presence of F573, and F573 was administered subcutaneously on days 0-5 post-transplant. Control mice were transplanted with non-treated islets and were injected with saline. Graft function was measured by non-fasting blood glucose and glucose tolerance testing. **Results.** F573 markedly reduced human and mouse islet apoptosis after in vitro culture ($p<0.05$ and $p<0.05$, respectively). Furthermore, F573 improved human islet function when transplanted under the KC ($p<0.05$); whereas F573 did not enhance murine islet marginal KC transplants. Conversely, F573 significantly improved mouse islet engraftment in the PV and DL site ($p<0.05$ and $p<0.05$, respectively). **Conclusions.** The pan-caspase inhibitor F573 markedly reduces human and mouse islet apoptosis and improves engraftment most effectively in the portal and DL subcutaneous sites.

4.2 – INTRODUCTION

Islet transplantation has recently been proven to stabilize glycemic control in select patients with type-1 diabetes complicated by hypoglycemia in a phase 3 trial[1], extending the initial success of the Edmonton Protocol popularized by Shapiro and colleagues in 2000.[2] Within the last sixteen years, islet transplantation success rates have improved substantially, with insulin-independence rates in at least 50% of recipients achieved out to 5 years post transplant in 6 International Centers.[3] Yet despite these clear advances in clinical islet transplantation, achievement of single-donor engraftment success has been difficult to establish routinely, with most recipients requiring at least two donors to achieve insulin-independence. [4,5] Numerous factors contribute to islet loss in the acute and peri-transplant period which results in an estimated 70% loss of transplanted β-cell mass.[6] The instant blood-mediated inflammatory reaction (IBMIR) is largely responsible for immediate cell loss especially in the intraportal hepatic site, and associated with platelet activation and triggering of biochemical cell death pathways including both apoptosis and necrosis.[7,8] Potent cell death inhibitors, and exploration of non-blood exposed extrahepatic sites, are being actively explored to improve islet viability and engraftment outcomes in the early transplant period.

The use of early generation anti-apoptotic agents have previously demonstrated improved in vitro islet viability outcomes.[9] Further studies revealed marked improvements in islet engraftment in pre-clinical experimental models. For example, using short course zVAD-FMK therapy in islet culture and within the acute transplant period, Emamaullee et al demonstrated enhanced long-term in vivo function up to one year post-transplant in mice transplanted with a marginal islet dose under the renal

capsule and via the intraportal route.[10] The next generation pan-caspase inhibitors, including EP1013 and IDN-6556, demonstrated augmented long-term engraftment using a marginal islet dose capable of effectively restoring euglycemia in transplant recipients in both small and large animal models.[11]

In an alternative approach to avoid the intravascular site, recent studies in our laboratory have also examined extra-hepatic transplant sites that could permit the engraftment of islets and alternative β-cell sources, including insulin-producing stem cells or xenogeneic sources. Alternative transplant sites with potential clinical feasibility, should accommodate a sufficient transplant mass, and be readily retrievable if they are to be considered as prospective sites.[12] The device-less (DL) transplant technique, which modifies the subcutaneous space through temporary implantation of a commercially-approved angiocatheter, in routine clinical use for other indications, has demonstrated successful restoration of euglycemia using mouse and human islets in a pre-clinical animal model.[13] Moreover, the DL technique was also effective in reversing hyperglycemia when transplanted with a marginal islet dose in a syngeneic mouse model.[14] Thus far, incorporating therapeutic strategies to augment islet engraftment in this alternative transplant site have yet to be elucidated.

Herein, we sought to determine whether the potent pan-caspase inhibitor, F573, could effectively reduce apoptosis in murine and human islets. Furthermore, we evaluated whether F573 treatment could differentially augment islet engraftment in standard and alternative transplant sites using full and marginal islet transplant doses. We reasoned that alternative sites would have differential susceptibility to oxygen

delivery and metabolic exchange, and therefore initially non-vascularized implanted cells may have increased susceptibility to hypoxia-mediated cell death signaling.

4.3 – MATERIALS AND METHODS

4.3.1 – Caspase inhibitor F573

The pan-caspase inhibitor F573 (Molecular weight: 382.38 g/mol) was obtained from Shanghai Genomics Inc. (Lot: 20141203, Shanghai, China). Stock preparations of F573 were prepared by dissolving 30 mg (lyophilized white powder) in 1ml DMSO. For in vitro and in vivo studies, stock solutions were diluted with sterile saline to a final working concentration of 1 mg/ml.

4.3.2 – Human islets

Human islets were prepared by the Clinical Islet Laboratory at Alberta Health Services. Deceased donor pancreata were processed for islet isolation with appropriate ethical approval and consent obtained from next-of-kin of the donor. Islets were isolated from two donor pancreata, implementing a modified Ricordi technique.[15,16] Permission for these studies was granted by the Health Research Ethics Board of the University of Alberta, Edmonton, Alberta, Canada. Upon receiving the human islet preparation, islet aliquots (± 10%) were counted and randomly distributed into standard culture media ± 100 μM F573 supplementation for 24 hours at 37°C and 5% CO_2 prior to transplantation. Standard culture media (CMRL-1066, Mediatech, Manassas, VA, USA) contains fetal bovine serum (10%), L-glutamine (100 mg/l), penicillin (112 kU/l), streptomycin (112

mg/l) and HEPES (25 mmol/l) at pH 7.4. The average human islet purity for the transplant preparations utilized in this study was 43.8% (n=2).

4.3.3 – Mouse islet isolation and culture

Pancreatic islets were isolated from 8 to 12 week old male C57BL/6 mice (Jackson Laboratories, Canada). Animals were housed under conventional conditions having access to food and water ad libitum. Mouse care was in accordance with the guidelines approved by the Canadian Council on Animal Care. Prior to pancreatectomy, the common bile duct was cannulated with a 30G needle and the pancreas was distended with 0.125 mg/ml cold Liberase TL Research Grade enzyme (Roche Diagnostics, Laval, QC, CA) in Hank's Balanced Salt Solution (Sigma-Aldrich Canada Co., Oakville, ON, CA). Islets were isolated by digesting the pancreases in a 50 ml tube placed in a 37°C water bath for 14 minutes with light agitation. Following the pancreatic digestion phase, islets were purified using histopaque-density gradient centrifugation (1.108, 1.083 and 1.069 g/ml, Sigma-Aldrich Canada Co., Oakville, ON, Canada). Isolated mouse islet aliquots (± 10%) were then counted and randomly distributed into standard culture media ± 100 μM F573 supplementation for 2 hours at 37°C and 5% CO_2 prior to transplantation. Standard culture media (CMRL-1066, Mediatech, Manassas, VA, USA) contains fetal bovine serum (10%), L-glutamine (100 mg/l), penicillin (112 kU/l), streptomycin (112 mg/l) and HEPES (25 mmol/l) at pH 7.4.

4.3.4 – Apoptosis analysis

Mouse and human islet apoptosis was measured prior to transplantation and subsequent to culture (2 and 24 hours, respectively) ± F573 media supplementation. Apoptosis was assayed in all islets groups using terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining (DeadEnd Apoptosis Detection System, Promega, Madison, WI), following formalin fixation (10%), agar embedding, processing, paraffinizing and sectioning. In addition to TUNEL staining, islets were co-stained with insulin and DAPI. Subsequent to deparaffinization and antigen heat retrieval, islet sections were washed with phosphate buffered saline (PBS) supplemented with 1% goat serum, followed by blocking with 20% goat serum in PBS for 30 minutes. The sections were treated with a primary antibody of guinea pig anti-pig insulin (Dako A0564) diluted 1:100 (PBS with 1% goat serum) for 2 hours at 4ºC. Samples were rinsed with PBS with 1% goat serum followed by secondary antibody treatment consisting of goat anti-guinea pig (Alexa 568) diluted 1:500 (PBS with 1% goat serum) for 1 hour at room temperature. Samples were rinsed with PBS and counter-stained with DAPI in anti-fade mounting medium (ProLong®, LifeTechnologies). Using a fluorescent microscope, the resulting microphotographs were taken using the appropriate filter with AxioVision imaging software.

Islet apoptosis was quantified as a percentage of positive TUNEL staining nuclei per islet (+TUNEL/Total Nuclei) using ImageJ software (freeware ImageJ v1.33 and Cell Counter plug-in, both downloaded from the NIH website [http://rsb.info.nih.gov/ij]).

To further evaluate the degree of apoptosis in both mouse and human islet preparations prior to transplantation, cytosolic cleaved caspase-3 activation was quantified from the lysates of frozen islet samples (3 x 100-300 islets per group, n=2 isolations). Briefly, islet lysates from both control and F573 cultured mouse and human islets were tested, according to manufactures specifications, for protease activity by the addition of a caspase-specific peptide that is conjugated to the color reporter substrate p-nitroaniline (BF3100: R & D Systems, Minneapolis, MN). Caspase activity was quantified spectrophotometrically at 405nm. Results are expressed as absorbance normalized to 100 islets.

4.3.5 - *In Vitro* islet viability assessment

Human and mouse islet viability was assessed post-culture ± F573 media supplementation, (24 and 2 hours, respectively) at the time of transplantation, Islet viability was determined by simultaneous staining of live and dead cells using a membrane integrity fluorescence assay (SytoGreen 13 and ethidium bromide, Invitrogen, Oregon, USA). The percentage of viable and dead cells was determined for both control and F573 treated islets.

Concurrently, static glucose-stimulated insulin secretion (s-GSIS) assay was performed on both human and mouse islets post-culture (3 x 50 islets per group, n=2 isolations). Islets were 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. Cell-free supernatants were harvested post-glucose incubation and insulin levels were measured by enzyme-linked immunosorbent assay (ELISA) (Mercodia, Uppsala, Sweden). Human

insulin data is represented as U/L and as a stimulation index (insulin secreted high glucose/low glucose). Mouse insulin data is represented as μg/L and as a stimulation index.

4.3.6 – Diabetes induction and islet transplantation

One week prior to transplantation, recipient mice either C57BL/6 (syngeneic studies) or B6-RAG$^{-/-}$ (B6.129S7-Rag1^{tm1Mom}/J) (human islet studies) were rendered diabetic by chemical induction with intraperitoneal streptozotocin (Sigma-Aldrich Canada Co., Oakville, ON, Canada), at 180 mg/kg in acetate phosphate buffer, pH 4.5. Diabetes was confirmed when blood glucose levels exceeded 15 mmol/l for 2 consecutive daily readings.

For human islet transplants, post-culture ± F573 media supplementation, islets were counted and transplanted under KC at a marginal islet dose of 500 islet equivalents per diabetic recipient. Syngeneic, mouse islets, post-culture ± F573 media supplementation were transplanted into 3 groups: 1) under kidney capsule (KC)[17] at a marginal islet dose (150 islets), 2) intrahepatic portal vein (PV) infusion[11] at a full dose (500 islets), and 3) into the prevascularized subcutaneous device-less (DL) site[13,14] at a marginal islet dose (150 islets). Non-supplemented F573 islet transplants served as controls for each transplant group. Prior to recovery, all recipients received a 0.1 mg/kg subcutaneous bolus of buprenorphine.

Transplant recipients were administered a subcutaneous injection of either F573 (3 mg/kg: KC, PV, DL groups and 10 mg/kg: PV group) for the treatment groups or vehicle (saline) for the control recipients on the day of transplant and for 5 days thereafter.

4.3.7 – Evaluation of islet graft function

Transplant efficacy was assessed three times per week in recipients through non-fasting blood glucose measurements, using a portable glucometer (OneTouch Ultra 2, LifeScan, Canada) in all groups tested. Graft function and reversal of diabetes was defined as two consecutive readings <11.1 mmol/l and maintained until study completion. In addition, intraperitoneal glucose tolerance tests (IPGTTs) were conducted post-transplant to further assess metabolic capacity by mimicking postprandial stimulation. Mice were fasted overnight prior to receiving an intraperitoneal glucose bolus (3 g/kg). Blood glucose levels were evaluated at baseline (Time 0), 15, 30, 60, 90 and 120 minutes post-injection. Blood glucose area under the curve (AUC-blood glucose) was calculated and analyzed between transplant groups.

Both KC and DL islet transplants were retrieved by nephrectomy or subcutaneous graft excision to confirm graft dependent euglycemia, as previously described[14]. Non-fasting blood glucose measurements were monitored for 7 days following graft removal to confirm hyperglycemia and thus post-transplant graft function.

4.3.8 – Statistical analysis

Non-fasting blood glucose, AUC-blood glucose, mouse and human insulin, caspase-3 activation, and percent apoptosis data are represented as the mean ± standard error of mean (s.e.m.). In vitro and in vivo data analysis between treatment groups were conducted by unpaired two-tailed t-test. Kaplan-Meier survival function curves were

compared using the log-rank statistical method (Mantel-Cox). P<0.05 was considered significant.

4.4 – RESULTS

4.4.1 – Pan-caspase inhibitor F573 abrogates human islet apoptosis post-culture

Human islets were cultured in media supplemented with or without F573. Following in vitro culture, control islets exhibited a significantly higher rate of apoptosis compared to F573-treated islets (control islets: 3.06 ± 1.10% vs. F573 islets: 0.60 ± 0.19%, p<0.05, unpaired two-tailed t-test) (**Figure 4.1A,B**). Furthermore, F573-treated human islets displayed significantly less activated caspase-3 compared to controls (F573 islets: 15.02 ± 1.18 vs. control islets: 20.15 ± 2.23 Abs.405nm/100Islets, p<0.05, unpaired two-tailed t-test) (**Figure 4.1C).**

4.4.2 – F573 culture supplementation improves human islet in vitro viability and function

Dual-fluorescence membrane integrating staining (SytoEB) of human islets revealed a significant increase in viability in islets cultured for 24 hours in the presence of F573 compared to controls (F573: 96.3 ± 2.2% vs. control: 86.0 ± 4.2%, p<0.05, unpaired t-test, n=2 isolations) (**Figure 4.2A**). Similarly, glucose static challenge revealed that F573-treated human islets had a greater insulin secretory capacity in response to glucose (Stimulation index: 1.87 ± 0.05) compared to control islets (Stimulation index: 1.66 ± 0.03, p<0.05, unpaired t-test, n=2 human islet preparations tested in triplicate) (**Figure 4.2B,C)**.

4.4.3 – F573 enhances diabetes reversal in marginal mass transplantation of human islets in immunodeficient mice

A marginal human islet mass (500 islet equivalents per recipient) transplanted beneath the murine KC was evaluated in the presence or absence of F573 (3 mg/kg). Of the diabetic mice recipients transplanted with control human islets, 2 of 8 (25%) became euglycemic. In contrast, F573 supplementation in culture plus subcutaneous F573 therapy significantly improved human islet engraftment efficacy and diabetes reversal in 6 of 8 (75%) recipients ($p<0.05$, long-rank, compared to control transplants) (**Figure 4.3A**). As such, F573 recipients presented with an overall reduced daily non-fasting blood glucose profile compared to controls (**Figure 4.3B**). Euglycemic mice in both transplant cohorts maintained glucose homeostasis until islet-bearing kidney grafts were retrieved; reverting to hyperglycemia thus proving graft-dependent function (**Figure 4.3B**). Prior to graft retrieval, mice in the F573 groups (n=7) displayed a superior physiological response to IPGTT 30 days post-transplant compared to control mice (n=7) (**Figure 4.3C**), as demonstrated by a significantly lower mean AUC-blood glucose ± s.e.m. (F573: 1998 ± 237 mmol/l/120min vs. control: 2678 ± 290 mmol/l/120min, $p<0.05$, unpaired two-tailed t-test) (**Figure 4.3D).**

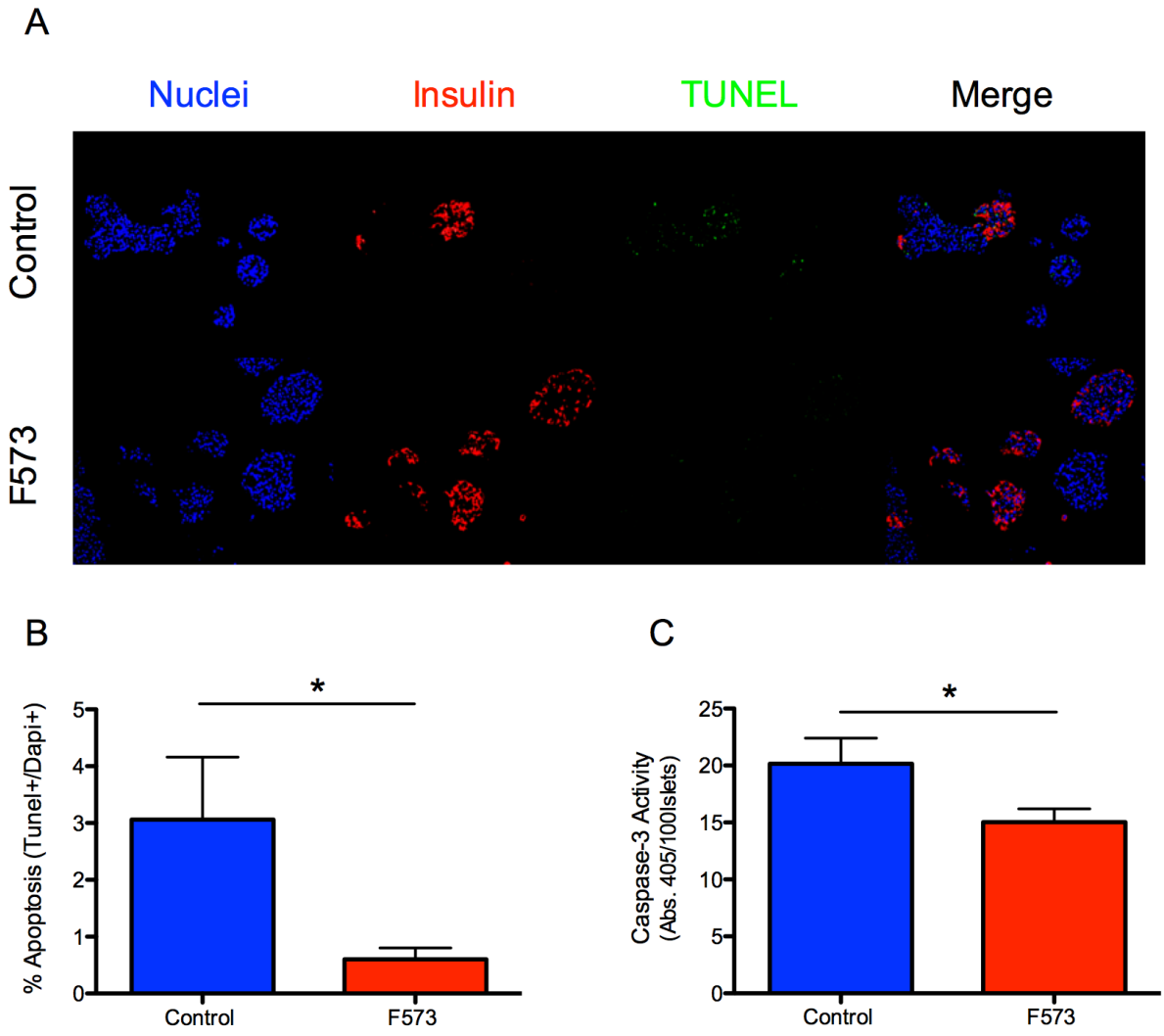


Figure 4.1. Assessment of human islet apoptosis prior to transplantation and subsequent to culture with or without F573 supplementation.

(**A**) Representative fluorescent microphotographs of human islets stained for insulin (red), TUNEL (apoptosis) (green) and nuclei (blue) ±F573. (**B**) Percentage of TUNEL positive cells in both groups as an expression of apoptosis post-culture. (**C**) Concentration of cleaved caspase-3 expressed in islets post-culture ± F573 supplementation. Data points represent mean ± SEM, n=2 donor, *$p<0.05$, unpaired two-tail t-test.

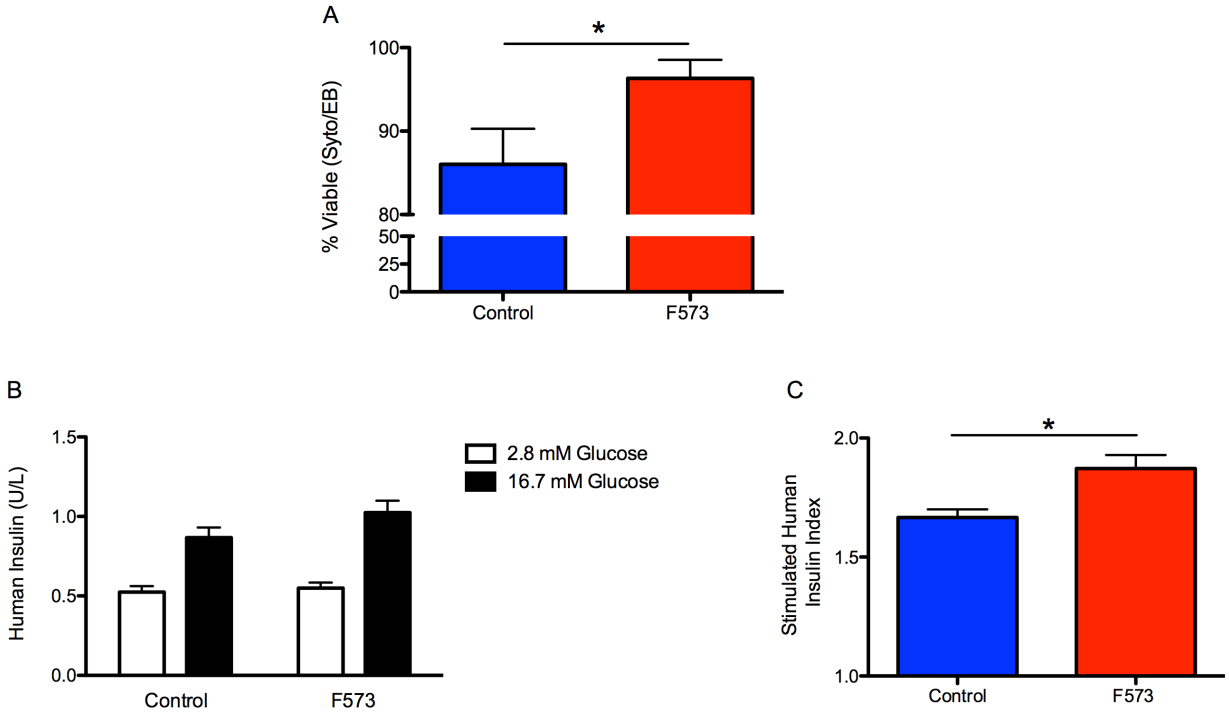
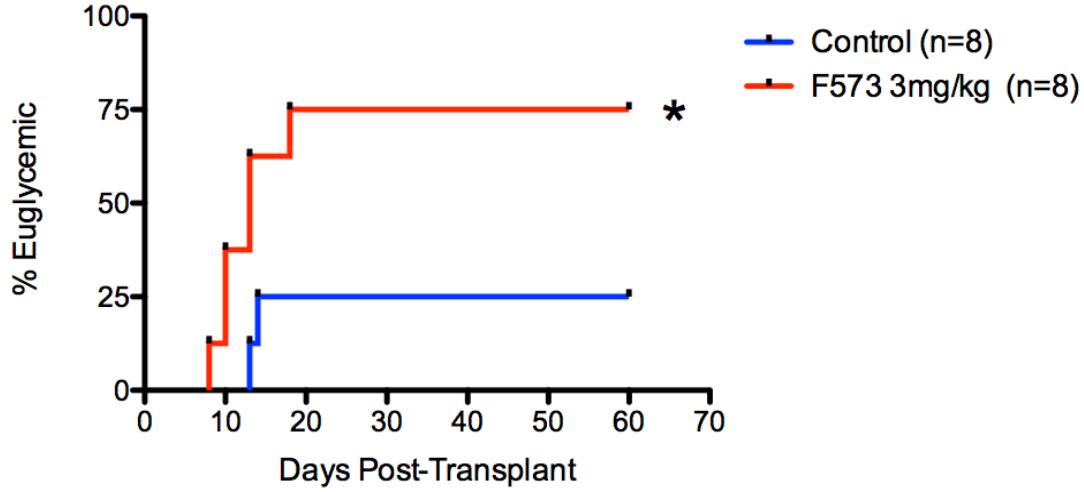


Figure 4.2. *In vitro* viability assessment of control and F573-treated human islets 24 hours post-culture.

(**A**) F573-treated islets exhibited improved islet viability as assessed by dual-fluorescence staining compared to control islets (*p<0.05, t-test). (**B,C**) Islet function, as assessed by static glucose stimulated insulin secretion, demonstrated that F573-treated islets have an improved insulin secretory capacity compared to control (*p<0.05, t-test)(n=2 isolations, triplicate samples per isolation).

A


100
75
50
25
0
% Euglycemic
0
10
20
30
40
50
60
70
Days Post-Transplant
*
Control (n=8)
F573 3mg/kg (n=8)

B

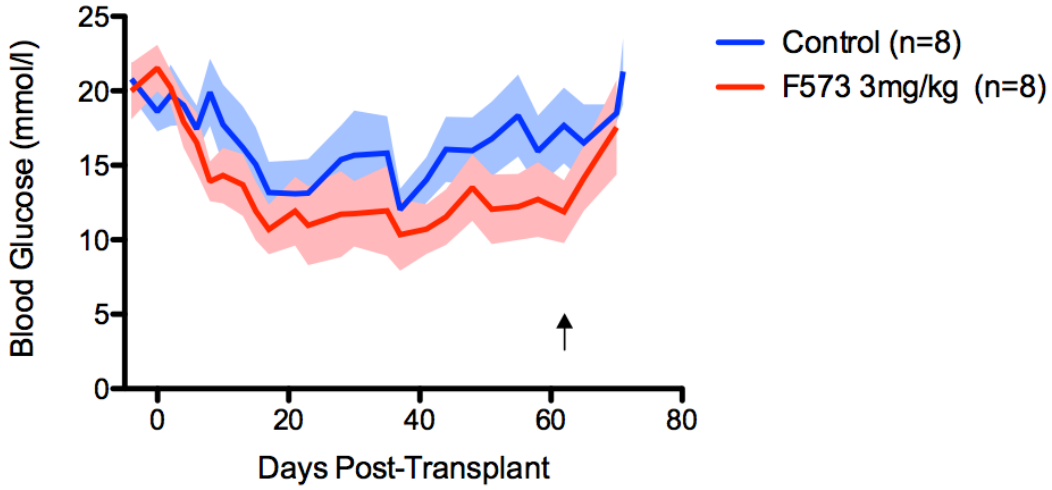
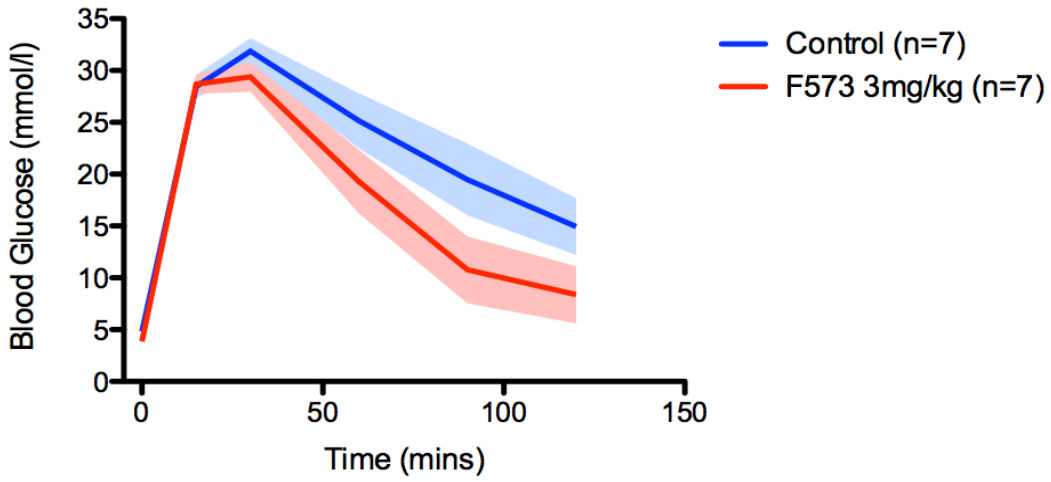

25
20
15
10
5
0
Blood Glucose (mmol/l)
0
20
40
60
80
Days Post-Transplant
Control (n=8)
F573 3mg/kg (n=8)

Figure 4.3 (A – B). F573 therapy improves marginal human islet mass engraftment transplanted under the kidney capsule (KC) of mice.

(**A**) Reversal of diabetes rates, percent euglycemia, in the F573 3 mg/kg recipients group (red, n=8) were higher than the control transplant recipients (blue, n=8) recipients 60 days post-transplant (*$p<0.001$, log-rank). (**B**) Non-fasting blood glucose measurements of recipients post-transplant. All euglycemic recipient of marginal human islet grafts maintained glycemic control until graft retrieval (arrow).

C


Blood Glucose (mmol/l)
35
30
25
20
15
10
5
0
0
50
100
150
Time (mins)
Control (n=7)
F573 3mg/kg (n=7)

D

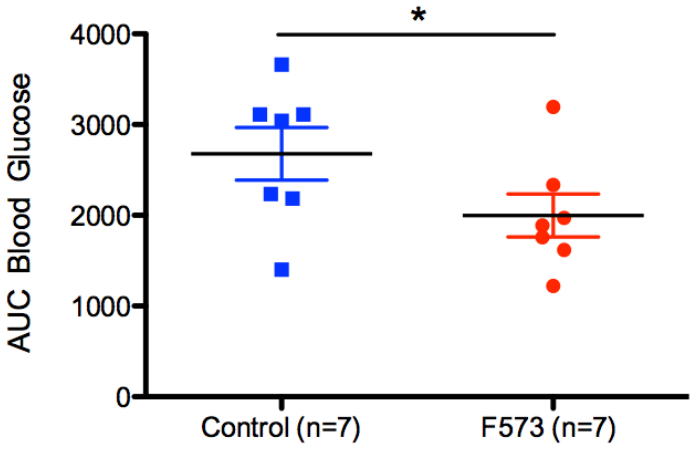

AUC Blood Glucose
4000
3000
2000
1000
0
*
Control (n=7)
F573 (n=7)

Figure 4.3 (C – D). F573 therapy improves marginal human islet mass engraftment transplanted under the kidney capsule (KC) of mice.

(**C**) Blood glucose profile during IPGTT of F573 recipients (red, n=7) and control recipients (blue, n=7), 30 days post-transplant. (**D**) Mean AUC-blood glucose was significantly lower in the F573 treatment group (*$p<0.05$, unpaired two-tail t-test). Mice were administered 3 mg/kg 50% dextrose i.p. Blood glucose measurements were monitored at t=0, 15, 30, 60, 90 and 120 minutes.

4.4.4 – F573 inhibits mouse islet apoptosis in vitro

Immediately post-isolation and prior to syngeneic islet transplantation, murine islets were cultured with or without F573 media supplementation. The percentage of apoptotic cells in the control cells was significantly greater than the F573-treated islets when assessed by TUNEL assay (F573: 0.49 ± 0.18% vs. control: 2.34 ± 1.08%, $p<0.05$, unpaired two-tailed t-test) (**Figure 4.4A,B**). Likewise, the quantity of active caspase-3 was significantly elevated post-culture in control mouse islets compared to F573-treated islets. (Control islets: 10.20 ± 1.17 vs. F573 islets: 7.60 ± 0.37 Abs. 405nm/100 Islets, $p<0.05$, unpaired two-tailed t-test) (**Figure 4.4C).**

4.4.5 – F573 culture supplementation does not improve mouse islet in vitro viability

Membrane integrity dual-florescence staining did not differ between control and F573-treated mouse islets, 2 hours post-culture (Control: 93.4 ± 0.8% vs. F573: 93.7 ± 0.6%, $p>0.05$, unpaired t-test, n=2 isolations) (**Figure 4.5A**). Similarly, glucose static challenge revealed that F573-treated and control mouse islets did not differ in their insulin secretory capacity in response to glucose post-culture (F573 stimulation index: 1.56 ± 0.16 vs. control stimulation index 1.87 ± 0.16, $p>0.05$, unpaired t-test, n=2 mouse islet preparations tested in triplicate) (**Figure 4.5B,C)**.

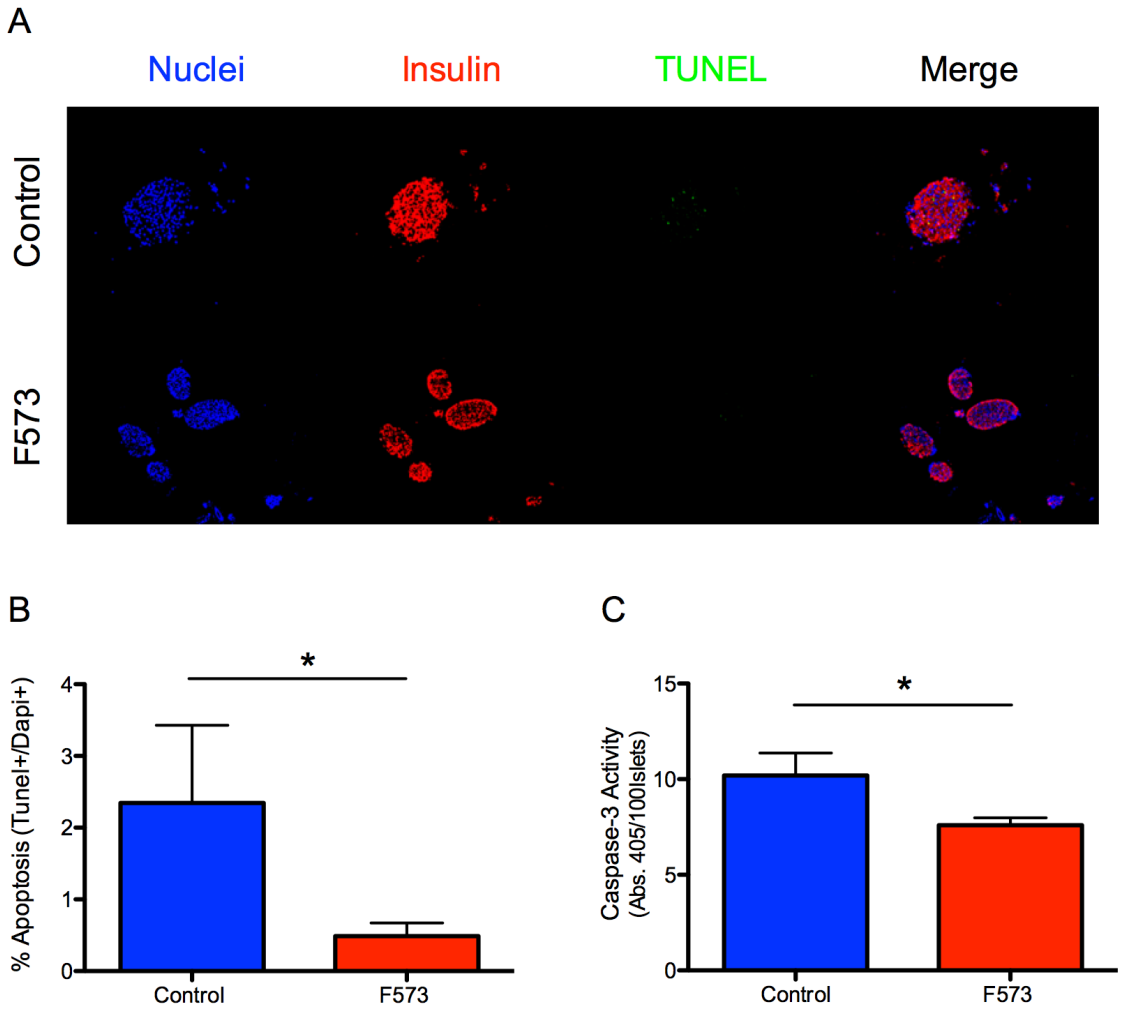


Figure 4.4. Evaluation of mouse islet apoptosis prior to transplantation and subsequent to culture with or without F573 supplementation.

(**A**) Representative fluorescent microphotographs of mouse islets stained for insulin (red), TUNEL (apoptosis) (green) and nuclei (blue) ± F573. (**B**) Percentage of TUNEL positive cells in both groups as an expression of apoptosis post-culture. (**C**) Caspase-3 activation expressed in islets post-culture ± F573 supplementation. Data points represent mean ± SEM, n=7/group, *p<0.05, unpaired two-tail t-test.

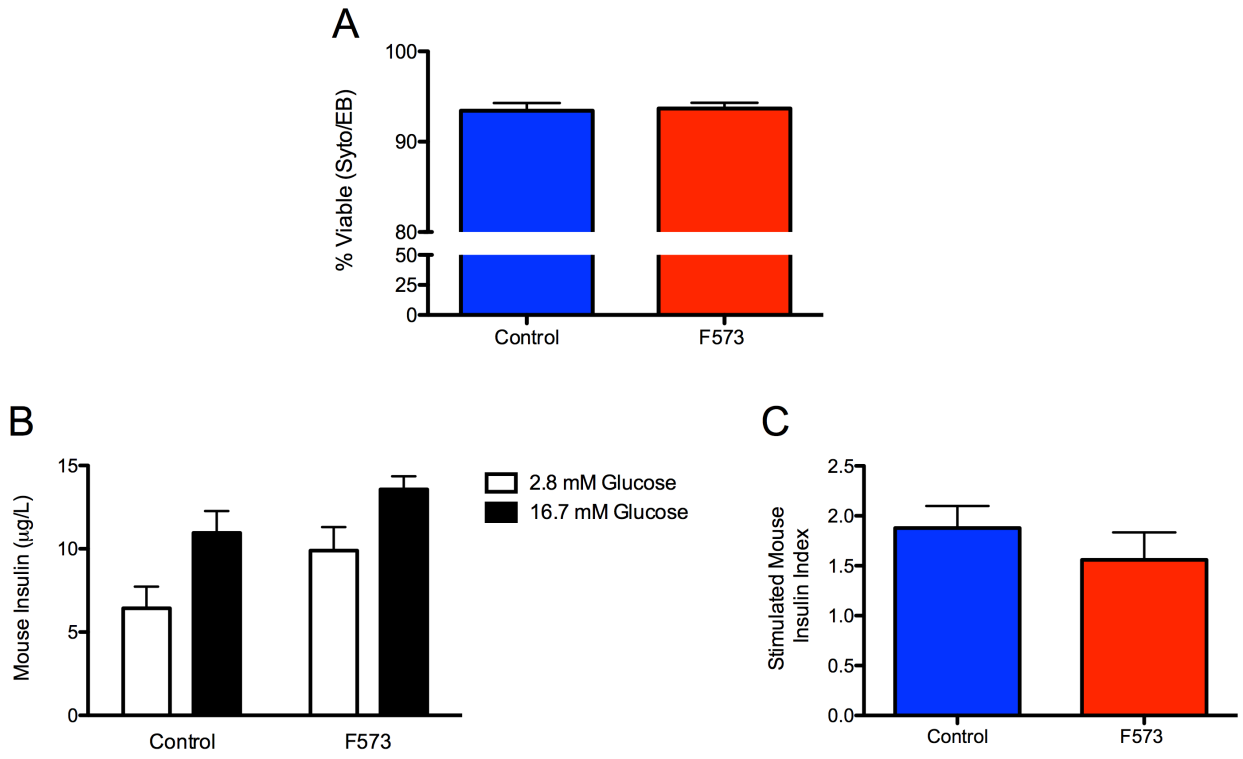


Figure 4.5. *In vitro* viability assessment of control and F573-treated mouse islets 2 hours post-culture.

(**A**) Comparison of membrane integrity of F573-treated islets to control islets as assessed by dual-fluorescence staining ($p>0.05$, t-test). (**B,C**) Islet function, as assessed by static glucose stimulated insulin secretion, demonstrated that F573-treated islets exhibit no difference in insulin secretory capacity compared to control ($p>0.05$, t-test)(n=2 isolations, triplicate samples per isolation).

4.4.6.1 – F573 does not improve efficacy of mouse marginal islet mass grafts transplanted under the KC

The ability of F573 to enhance islet engraftment efficacy was evaluated using the KC site (F573: 3 mg/kg: n=11), in a marginal islet transplant mass model (150 islets per recipient). As a means to compare engraftment efficiency, a control group (no F573 supplementation or therapy) of diabetic recipients were also transplanted with 150 islets, under the KC (control: n=11). F573 therapy did not enhance rates of euglycemia post-marginal islet engraftment compared to controls [F573 3 mg/kg: 64% (7 of 11) vs. control: 55% (6 of 11), $p>0.05$, log-rank] (**Figure 4.6A**). IPGTTs were performed on recipients 45 days post-transplant. Mice from both F573 (n=7) and control (n=6) groups demonstrated a robust physiological response to the glucose challenge with a prompt restoration of normoglycemia (**Figure 4.6B**). Furthermore, there was no difference in mean AUC ± s.e.m. (F573: 1932 ± 238 mmol/l/120min vs. control: 1890 ± 141 mmol/l/120min, $p>0.05$, unpaired two-tailed t-test) (**Figure 4.6C)**.

4.4.6.2 – F573 enhances the rate of diabetes reversal post-full mouse islet mass transplanted into PV

Full islet mass (500 islets per recipient) engraftment efficacy post-transplant into the PV was evaluated ± F573 therapy at 3 and 10 mg/kg. Post-transplant intervention with 3 mg/kg of F573 in a small cohort of recipients had no beneficial effect on engraftment (data not shown). Subsequently, a dose of 10 mg/kg of F573 was examined. Of the recipients transplanted with control islets into the PV, 4 of 13 (31%) became euglycemic. In contrast, 8 of 11 (72%) recipients receiving F573 supplemented islets

and exogenous F573 (10 mg/kg) therapy post-PV transplant, reversed diabetes; a significant improvement compared to control recipients ($p<0.05$, long-rank) (**Figure 4.6D**). Recipients in the F573 transplant groups (n=6) demonstrated an improved glucose clearance in response to an IPGTT 45 days post-transplant compared to control mice (n=8) (**Figure 4.6E**), as corroborated by a markedly reduced mean AUC-blood glucose ± s.e.m. (F573: 1980 ± 242 mmol/l/120min vs. control: 2617 ± 199 mmol/l/120min, $p<0.05$, unpaired two-tailed t-test) (**Figure 4.6F)**.

4.4.6.3 – F573 improves and accelerates marginal islet mass engraftment into a prevascularized subcutaneous Device-less site

The effect of F573 islet supplementation and recipient administration (3mg/kg) was evaluated using a prevascularized subcutaneous site with a marginal islet transplant dose (150 islet per recipient). Of the recipients transplanted with control islets into the DL site 16 of 22 (72%) became euglycemic, whereas in the F573 experimental group, a significantly higher rate of diabetes reversal was observed, as 10 of 12 (83%) became euglycemic post-transplant ($p<0.05$, log-rank) (**Figure 4.6G**). Furthermore, F573 significantly reduced the time to euglycemia from 41.1 ± 4.3 days post-transplant in control recipients to 20.1 ± 5.4 days post-transplant in the F573 experimental group ($p<0.001$, unpaired two-tailed t-test). As a means to assess long-term function of mice post-transplant with or without F573 therapy, IPGTTs were conducted 100 days post-transplant. Recipients in the F573 group (n=9) rapidly became normoglycemic following glucose challenge, demonstrating superior glucose clearance profiles compared to control DL transplants (n=14) (**Figure 4.6H**). As a result, blood glucose

AUCs ± s.e.m. for glucose clearance were significantly elevated in the control group compared to the F573 DL islet transplant recipient (Control DL: 2861 ± 119 mmol/L/120min vs. F573 DL: 2012 ± 137 mmol/L/120min, $p>0.001$, unpaired two-tailed t-test) (**Figure 4.6I**).

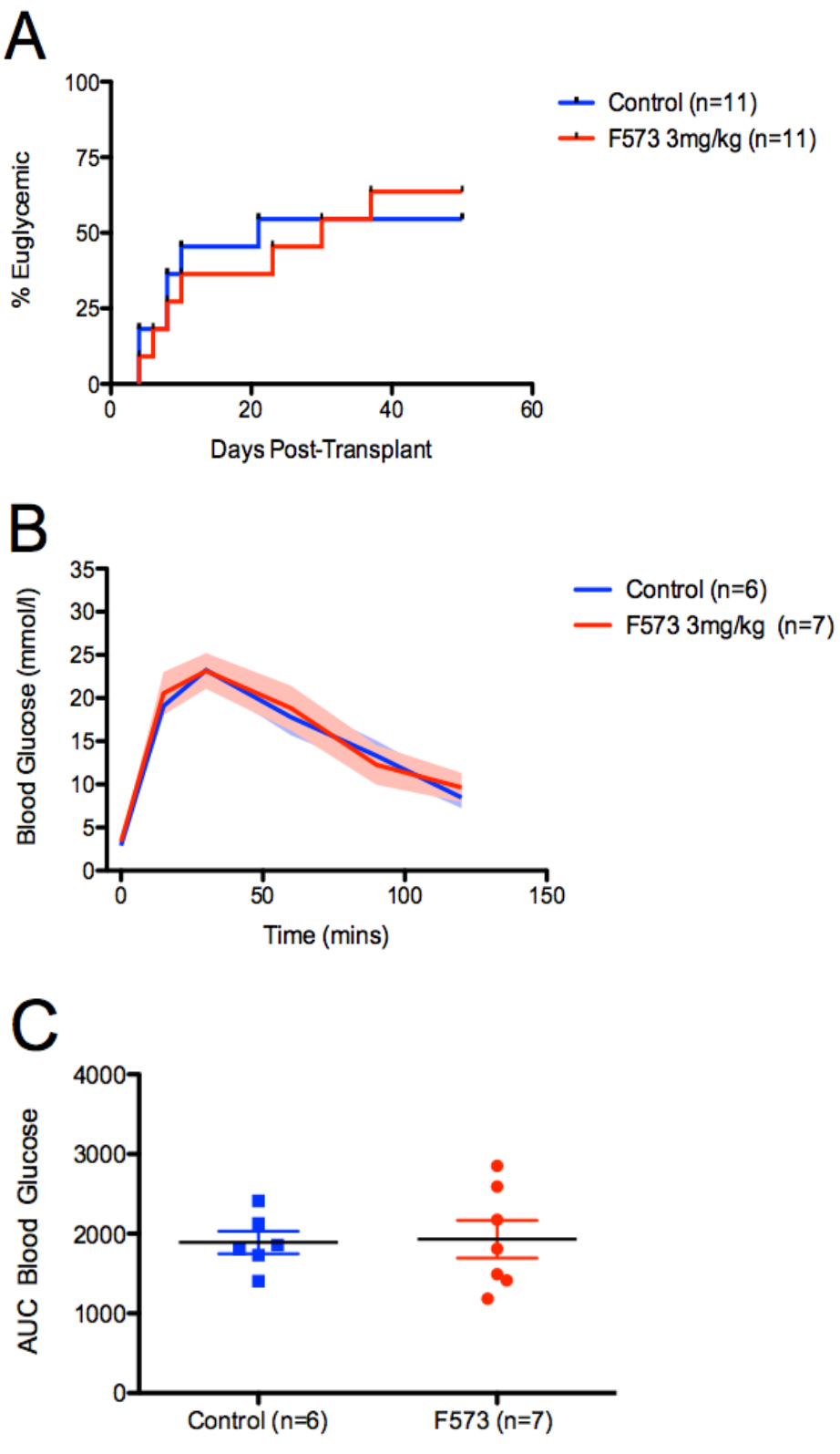
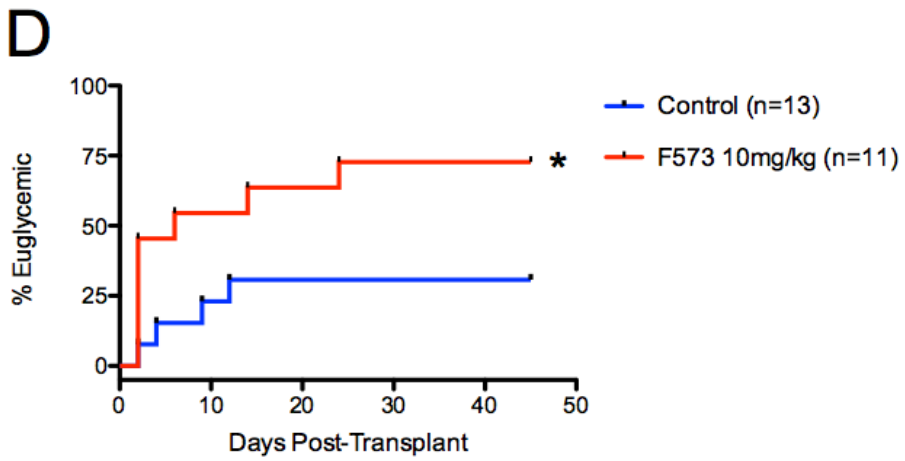
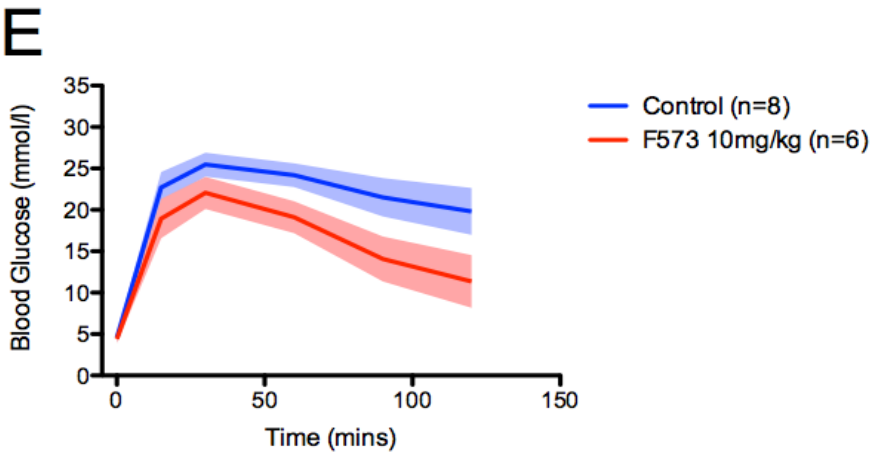

A
100
75
50
25
0
% Euglycemic
0
20
40
60
Days Post-Transplant
Control (n=11)
F573 3mg/kg (n=11)
B
35
30
25
20
15
10
5
0
Blood Glucose (mmol/l)
0
50
100
150
Time (mins)
Control (n=6)
F573 3mg/kg (n=7)
C
4000
3000
2000
1000
0
AUC Blood Glucose
Control (n=6)
F573 (n=7)

Figure 4.6 (A – C). Post-transplant efficacy of mouse syngeneic islet transplants ± F573 therapy utilizing standard and alternative engraftment sites.

(**A**) F573 therapy did not improve marginal mouse islet engraftment efficacy (control: n=11, 3 mg/kg F573: n=11)($p>0.05$ log-rank), (**B**) glucose clearance in response to IPGTT and (**C**) AUC-blood glucose ($p>0.05$, unpaired two-tailed t-test), when islet were transplanted under the KC.


D
100
75
50
25
0
% Euglycemic
*
Control (n=13)
F573 10mg/kg (n=11)
0
10
20
30
40
50
Days Post-Transplant


E
35
30
25
20
15
10
5
0
Blood Glucose (mmol/l)
Control (n=8)
F573 10mg/kg (n=6)
0
50
100
150
Time (mins)

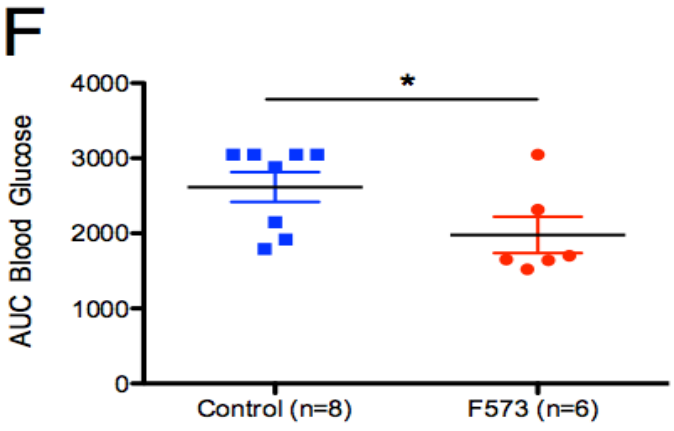
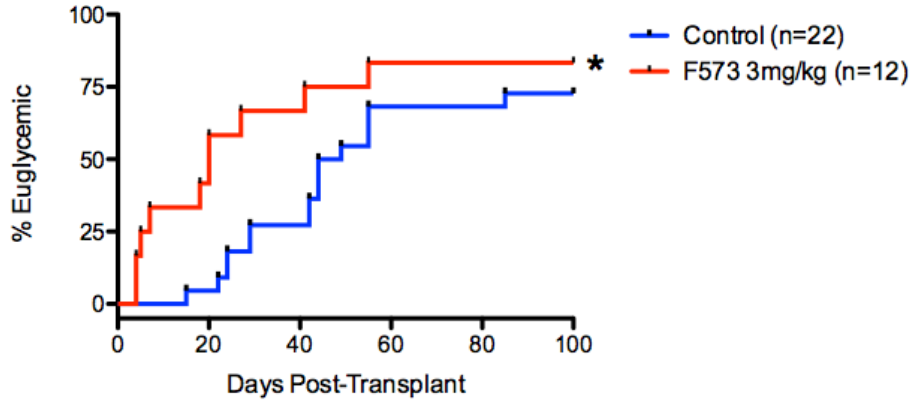

F
4000
3000
2000
1000
0
AUC Blood Glucose
*
Control (n=8)
F573 (n=6)

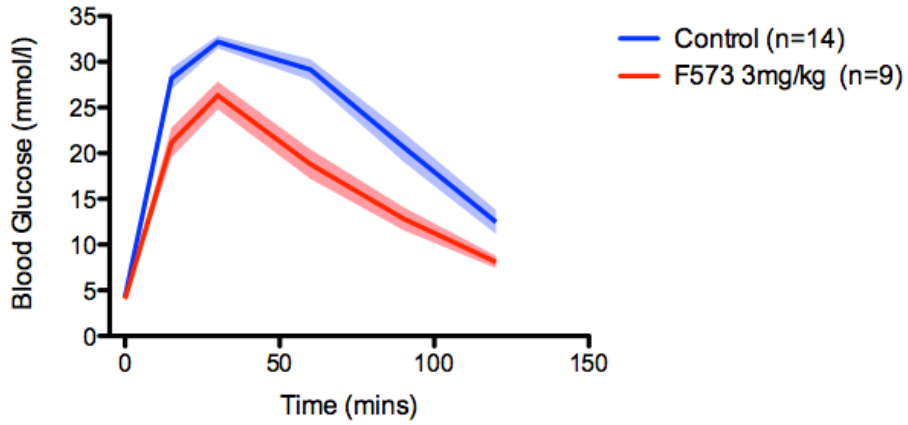
Figure 4.6 (D – F). Post-transplant efficacy of mouse syngeneic islet transplants ± F573 therapy utilizing standard and alternative engraftment sites.

(**D**) Reversal of diabetes rates were significantly greater in 10 mg/kg F573 therapy recipients (red, n=11) compared to control transplants (blue, n=13) (*$p<0.01$, log-rank), when islets were transplanted into the PV at a full islet dose. (**E**) Blood glucose profiles and (**F**) AUC-blood glucose in response to an IPGTT was improved in the F573 transplant group compared to controls (*$p<0.05$, unpaired two-tailed t-test).

G


100
75
50
25
0
% Euglycemic
0
20
40
60
80
100
Days Post-Transplant
*
Control (n=22)
F573 3mg/kg (n=12)

H


35
30
25
20
15
10
5
0
Blood Glucose (mmol/l)
0
50
100
150
Time (mins)
Control (n=14)
F573 3mg/kg (n=9)

I

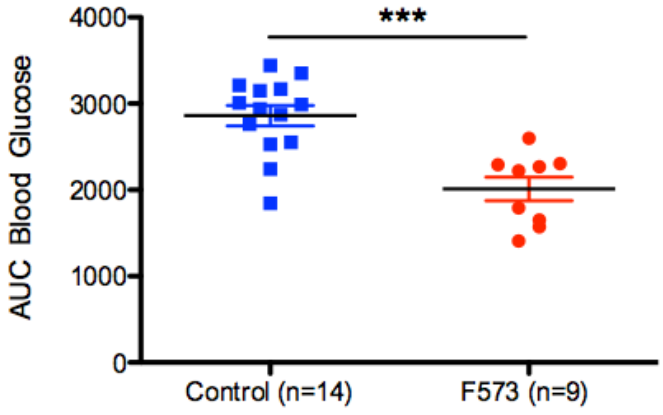

4000
3000
2000
1000
0
AUC Blood Glucose
***
Control (n=14)
F573 (n=9)

Figure 4.6 (G – I). Post-transplant efficacy of mouse syngeneic islet transplants ± F573 therapy utilizing standard and alternative engraftment sites.

(**G**) Therapeutic intervention with F573 (red, n=12) improved the rate of euglycemia post-marginal islet mass transplants into the DL site compared to control islet recipients (blue, n=22)(*$p<0.05$). (**H**) Blood glucose profile during IPGTTs of F573 recipients (red, n=9) and control recipients (blue, n=14), 100 days post-transplant. (**I**) F573 recipients demonstrated a significantly improved response to glucose challenge compared to control recipients as demonstrated by markedly reduced AUC-blood glucose (***$p<0.001$, unpaired, two-tailed t-test). Mice were administered 3 mg/kg 50% dextrose i.p. Blood glucose measurements were monitored at t=0, 15, 30, 60, 90 and 120 minutes.

4.5 – DISCUSSION

Islet viability is compromised in the acute and peri-transplant period, which accounts for substantial cell death, leading to failed engraftment and impaired islet function. Strategies aimed to reduce islet death and promote engraftment have included the utility of therapeutic agents capable of reducing apoptosis through pan-caspase inhibition, and alternatively through exploration of alternative transplant sites of potential clinical relevance, that do not involve direct introduction of freshly transplanted cells within the vascular space. One such strategy was recently described by Giovannoni et al, who demonstrated that Toll-like receptor 4 blockage was efficacious in reducing islet apoptosis and improving both syngeneic and allogeneic islet transplant outcomes in mice. [18] In the current study, we sought to evaluate whether the administration of a pan-caspase inhibitor, F573, in culture and in the acute post-transplant period could reduce islet death and enhance engraftment in various transplant sites in pre-clinical rodent models.

The culture of murine and human islets with F573 resulted in reduced TUNEL-positive nuclei and caspase-3 activity when compared to islets in standard culture media alone. These findings demonstrate F573's caspase-specific inhibition of islet death, confirming previously established findings from our laboratory with earlier generations of caspase inhibitors.[7,8,10,11] Our in vivo results demonstrated varying degrees of efficacy between murine and human islets transplanted beneath the renal capsule, as well as murine islets transplanted in alternative transplant sites. Notably, we found that a marginal mass of human islets transplanted beneath the kidney capsule of immunodeficient mice were highly protected by F573 treatment, but there was less

measurable difference with murine islets. It is routine for us to culture human islets for periods exceeding 24 hours, but we routinely transplant mouse islets within 2 hours of isolation. Therefore, the window of F573 exposure for the human islet studies (24 hours) was considerably longer than that of the mouse islet experiments (2 hours). Nonetheless we see significant protection from apoptosis in both settings, and of greater magnitude in human vs murine islets. Furthermore, by the nature of human organ procurement, long cold ischemic transport times, a more intense isolation, purification and culture conditions, by necessity human islets are exposed to more cumulative stressing events than the murine islets. This likely also explains the differential susceptibility of human islets to apoptosis and protection from F573 in the kidney capsule setting. In vitro viability data within the present study supports this hypothesis as the distressed human islets demonstrated improved function when cultured in the presence of F573 compared to the robust freshly isolated mouse islets. The cytoprotective effect of F573 in mouse islets was not apparent until a more inhospitable transplant site was implemented.

Further accounting for the in vivo difference is the impurity of human islet preparations in comparison to murine islets, with the former containing a greater percentage of acinar tissue. When contained in the renal subcapsular space and placed in close proximity to one another, the release of acinar enzymes may inflict significant death on neighboring islets.[19] Drognitz and colleagues previously demonstrated that cold ischemia and reperfusion of the pancreas induces acinar apoptosis whereas endocrine tissue was less susceptible.[20] It is plausible that acinar tissue-specific apoptosis was preserved in the presence of F573, thus protecting neighboring islets from enzymatic lysis and improving engraftment outcomes.

Early generations of pan-caspase inhibitors have demonstrated improved PV islet engraftment at doses of 10 mg/kg.[10] Initially we sought to evaluate if a lower dose (3 mg/kg) would yield improved islet engraftment; however, this therapeutic strategy did not prove to be efficacious as it did when human islets were transplanted under the KC. Therefore, we increased the dose of F573 to 10 mg/kg for the PV transplant recipients. When administered via the intraportal route, murine islets transplanted at a full therapeutic dose exhibited significant engraftment in F573-treated (10 mg/kg) recipients as compared to control islet recipients. Robust islet loss occurs in the early transplant period, culture and administration of F573 likely preserved a sufficient islet mass subsequent to transplant, allowing for reversal of diabetes in a greater number of recipients. This has been confirmed in previous studies using earlier generation pan-caspase inhibitors in which islets were pre-incubated with the inhibitor and recipients were treated up to 5 days post-transplant.[11] The inherent inflammatory cascades evoked by this route of islet delivery, such as IBMIR[21], may indeed explain, in part, the requirement of a larger therapeutic dose of F573 in order to demonstrate improved transplant efficacy. Several antioxidants have been shown to optimize islet engraftment in mice such as the antioxidant cyaniding-3-O-glucoside.[22] Our results support these findings, further suggesting the therapeutic benefit of this novel inhibitor, F573, in the clinical islet transplant setting.

Additional sources of insulin-producing cells are becoming available for potential future β-cell replacement therapy, including insulin-producing stem cells and xenogeneic islet sources. The establishment of an optimal alternative transplant site should consider its ability to accommodate a sufficient transplant mass, its clinically

feasibility and whether it can be easily retrieved should complications arise.[13] We previously reported that modification of the subcutaneous space using the DL technique could adequately support islet engraftment and restore euglycemia to a greater extent than the unmodified space while having the capacity to safely retrieve the graft.[13] Moreover, the DL technique was shown to be efficacious in restoring euglycemia when transplanted with a marginal islet mass in a murine diabetes model.[14] Our data in the current study demonstrates that F573 administration augments islet engraftment outcomes relative to control recipients in the DL site suggesting that this technique may exert some degree of apoptosis on the transplanted graft. Similarly, Espes et al demonstrated induced apoptosis in intramuscular islet grafts that could be reduced with the co-transplantation of low dose polymerized hemoglobin. [23] Furthermore, our observations reflect the efficiency of F573 to improve prevascularized subcutaneous DL engraftment while expediting the restoration of glycemic control in a regulated physiological manner. Should this transplant technique be translated to the clinical setting, it may be of benefit to incorporate pan-caspase treatment in vitro and in the acute transplant period as an added therapy to promote early engraftment.

This study supports the utility of the pan-caspase inhibitor, F573, in islet transplantation. The differences in engraftment efficacy observed in murine and human islets transplanted under the renal capsule strongly endorse the potential benefit of this therapy in clinical transplantation. Should extrahepatic transplant sites be incorporated in the clinical setting, pan-caspase therapy may also prove beneficial in the acute post-transplant period to expedite engraftment outcomes. The ability of F573 to restore

euglycemia with sub-therapeutic, marginal islet doses is of added benefit, as donor availability impedes the number of potential patients that may be treated.

4.6 – REFERENCES

1. Ricordi C, Goldstein JS, Balamurugan AN, et al. NIH-sponsored Clinical Islet Transplantation Consortium Phase 3 Trial: Manufacture of a Complex Cellular Product at Eight Processing Facilities. Diabetes. 2016.
2. Bruni A, Gala-Lopez B, Pepper AR, Abualhassan NS, Shapiro AJ. Islet cell transplantation for the treatment of type 1 diabetes: recent advances and future challenges. Diabetes Metab Syndr Obes. 2014;7:211-223.
3. Shapiro AMJ, Ricordi, C. Islet Cell Transplantation Procedure and Surgical Technique. In: A. D. Kirk SJK, C. P. Larsen, J. C. Madsen, T. C. Pearson and S. A. Webber, ed. Textbook of Organ Transplantation Oxford, UK: John Wiley & Sons; 2014.
4. Hering BJ, Kandaswamy R, Ansite JD, et al. Single-donor, marginal-dose islet transplantation in patients with type 1 diabetes. JAMA. 2005;293(7):830-835.
5. Hering BJ, Kandaswamy R, Harmon JV, et al. Transplantation of cultured islets from two-layer preserved pancreases in type 1 diabetes with anti-CD3 antibody. Am J Transplant. 2004;4(3):390-401.
6. 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(26):2754-2755.
7. McCall M, Toso C, Emamaullee J, et al. The caspase inhibitor IDN-6556 (PF3491390) improves marginal mass engraftment after islet transplantation in mice. Surgery. 2011;150(1):48-55.

8. McCall MD, Maciver AM, Kin T, et al. Caspase inhibitor IDN6556 facilitates marginal mass islet engraftment in a porcine islet autotransplant model. Transplantation. 2012;94(1):30-35.

9. Montolio M, Biarnes M, Tellez N, Escoriza J, Soler J, Montanya E. Interleukin-1beta and inducible form of nitric oxide synthase expression in early syngeneic islet transplantation. J Endocrinol. 2007;192(1):169-177.

10. Emamaullee JA, Stanton L, Schur C, Shapiro AM. Caspase inhibitor therapy enhances marginal mass islet graft survival and preserves long-term function in islet transplantation. Diabetes. 2007;56(5):1289-1298.

11. Emamaullee JA, Davis J, Pawlick R, et al. The caspase selective inhibitor EP1013 augments human islet graft function and longevity in marginal mass islet transplantation in mice. Diabetes. 2008;57(6):1556-1566.

12. Pepper AR, Gala-Lopez B, Ziff O, Shapiro AM. Revascularization of transplanted pancreatic islets and role of the transplantation site. Clin Dev Immunol. 2013;2013:352315.

13. 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(5):518-523.

14. Pepper AR, Pawlick R, Bruni A, et al. Harnessing the Foreign Body Reaction in Marginal Mass Device-less Subcutaneous Islet Transplantation in Mice. Transplantation. 2016;100(7):1474-1479.

15. Kin T. Islet isolation for clinical transplantation. Adv Exp Med Biol. 2010;654:683-710.

16. Ricordi C, Lacy PE, Finke EH, Olack BJ, Scharp DW. Automated method for isolation of human pancreatic islets. Diabetes. 1988;37(4):413-420.

17. Toso C, McCall M, Emamaullee J, et al. Liraglutide, a long-acting human glucagon-like peptide 1 analogue, improves human islet survival in culture. Transpl Int. 2010;23(3):259-265.

18. Giovannoni L, Muller YD, Lacotte S, et al. Enhancement of islet engraftment and achievement of long-term islet allograft survival by Toll-like receptor 4 blockade. Transplantation. 2015;99(1):29-35.

19. Gray DW, Sutton R, McShane P, Peters M, Morris PJ. Exocrine contamination impairs implantation of pancreatic islets transplanted beneath the kidney capsule. J Surg Res. 1988;45(5):432-442.

20. Drognitz O, Obermaier R, Liu X, et al. Effects of organ preservation, ischemia time and caspase inhibition on apoptosis and microcirculation in rat pancreas transplantation. Am J Transplant. 2004;4(7):1042-1050.

21. 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(6):620-626.

22. Cai H, Yang B, Xu Z, et al. Cyanidin-3-O-glucoside enhanced the function of syngeneic mouse islets transplanted under the kidney capsule or into the portal vein. Transplantation. 2015;99(3):508-514.

23. Espes D, Lau J, Quach M, Banerjee U, Palmer AF, Carlsson PO. Cotransplantation of Polymerized Hemoglobin Reduces beta-Cell Hypoxia and

Improves beta-Cell Function in Intramuscular Islet Grafts. Transplantation. 2015;99(10):2077-2082.

CHAPTER 5.

FERROPTOSIS-INDUCING AGENTS COMPROMISE *IN VITRO* HUMAN ISLET VIABILITY BUT NOT *IN VIVO* FUNCTIONALITY IN MICE

A version of this Chapter has been submitted to *Cell Death and Disease* on November 2, 2017 and is currently under active peer-review for publication.

ORIGINAL ARTICLE

Ferroptosis-inducing agents compromise *in vitro* human islet viability but not *in vivo* functionality in mice

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Keywords: *Islet transplantation, cell death, ferroptosis, reactive oxygen species, erastin, ferrostatin-1*

Running Title*: Ferroptosis in human islets*

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5.1 – ABSTRACT

Human islet transplantation has been hampered by donor cell death associated with the islet preparation procedure before transplantation. Regulated necrosis pathways are biochemically and morphologically distinct from apoptosis. Recently, one such pathway, ferroptosis, was identified as an iron-dependent, non-apoptotic form of regulated necrosis and has been implicated in various pathological conditions. Key mediators of islet oxidative stress, including glutathione peroxidase-4 (GPX4), have been identified as targets to induce ferroptosis. Mechanisms that affect GPX4 function have been shown to impact islet function and viability. Ferroptosis has not been investigated directly in human islets, and the relevance of this pathway in islet transplantation remains unknown. Herein, we sought to determine whether *in vitro* human islet viability and function is compromised in the presence of two distinct ferroptosis-inducing agents (FIA), erastin or RSL3, and whether these effects could be rescued with an inhibitor of ferroptosis called ferrostatin-1 (Fer-1). Viability assessed through lactate dehydrogenase (LDH) release revealed significant death in erastin and RSL3 treated islets, 20.3% ± 3.8 and 24.4% ± 2.5, 24 hours post-culture, respectively. These effects were ameliorated in islets pre-treated with Fer-1. A significant reduction in stimulation index was observed in islets treated with erastin (control 1.97 ± 0.13 vs. 50 μM erastin 1.32 ± 0.1) ($p<0.05$). However, when transplanted under the kidney capsule of immunodeficient mice, pre-treatment with erastin or Fer-1 did not impact engraftment outcomes in the model and islet dose tested. Our data reveal that islets are indeed susceptible to ferroptosis *in vitro*, and induction of this novel cell death modality leads to compromised islet function, which can be recoverable in the presence of the

ferroptosis-specific inhibitor, Fer-1. The *in vivo* impact of this pathway in islet transplantation appears to be of minor importance, however, at least within the constrains of our testing, but further investigation is warranted.

5.2 – INTRODUCTION

The inception of the 'Edmonton Protocol' by Shapiro and colleagues, and more recent modifications and improvements were critical in establishing islet transplantation as a viable therapeutic option for select patients with type 1 diabetes mellitus.[1, 2] With complete insulin-independence up to 1 year post-transplant, 5 year follow-up of early transplant recipients demonstrated maintained graft function with presence of C-peptide, correction of hemoglobin A1C and stabilization of glycemic control, but the majority returned to modest exogenous insulin therapy over time.[3] Early insights into long-term success rates suggest that there are numerous limitations associated with engraftment outcomes, many of which occur during islet isolation and in the acute and peri-transplant period.

When transplanted into the portal vein, it is estimated that up to 70% of the transplanted islet mass is lost in the acute and peri-transplant period, resulting from numerous factors. Such factors include the instant blood-mediated inflammatory reaction, hypoxia, delayed revascularization and inflammatory cytokines.[4] These events stimulate the initiation of cell death cascades, apoptosis and necrosis, contributing to islet loss during the preparation procedure and within hours and days of transplant, long before the initiation of alloimmune or recurrent autoimmune responses.[5, 6, 7] In contrast to non-immunogenic apoptosis, necrosis is increasingly recognized as the most potent trigger of the immune system.[8] Likely, this event is further exaggerated by HLA- or

species-incompatibility.[9, 10, 11] Strategies to deter early cell death could critically augment islet engraftment thereby improving long-term graft function. Along these lines, strategies to prevent caspase-dependent islet death, including the administration of interleukin-1β receptor agonists,[12] withaferin A,[13] and caspase-specific inhibitors both *in vitro* and *in vivo* have been explored previously.[7, 14, 15, 16]

Non-apoptotic cell death has been identified in various pathological conditions, including myocardial infarction, stroke, ischemia-reperfusion injury and many others.[17] In contrast to unregulated necrosis, whereby cell death can occur through spontaneous, 'accidental' triggers like trauma, regulated necrosis occurs through distinct biochemical mediators that activate molecular machinery.[8] One particular subroutine of regulated necrosis, termed ferroptosis, is morphologically and biochemically distinct from other forms of cell death, that it is iron-dependent and non-apoptotic.[18, 19] Ferroptosis was first described in parallel to the identification ferrostatin-1 (Fer-1), an inhibitor of this cell death pathway that functioned to prevent erastin-induced cell death.[8, 9, 17, 18] Erastin, a small potent molecule capable of selectively inhibiting the X_c^-cystine/glutamate antiporter required for glutathione (GSH) biosynthesis, induces ferroptosis.[17, 18] Subsequent to intracellular GSH depletion, the GSH-dependent, lipid repair enzyme, glutathione peroxidase 4 (GPX4), lacks the ability to sufficiently repair aberrant downstream accumulation of reactive oxygen species (ROS).[17, 18, 20, 21, 22] RSL3, has recently been identified as a potent GPX4-specific inhibitor and known inducing agent of ferroptosis.[18, 21] Relative to other native tissues, islets exhibit reduced antioxidant defences, and as a result are susceptible to the dysregulation of free radical production and subsequent oxidative stress.[23, 24] Ferrostatins were previously demonstrated to

reduce ferroptosis-induced death in cellular models of Huntington's disease, periventricular leukomalacia, kidney tubular necrosis and acute kidney injury.[17, 25] Given that key mediators of islet survivability are also key targets that induce ferroptosis, it has yet to be elucidated if islets are susceptible to ferroptosis-induced cell death.

Herein, we sought to establish whether human islets exhibit reduced islet viability and function when challenged with ferroptosis-inducing agents (FIAs), erastin or RSL3, *in vitro.* We also sought to determine whether inhibitors of ferroptosis such as the small molecule Fer-1 could rescue human islets from the subsequent deleterious effects of these agents. Given the conceived cytoprotective effects of Fer-1 in other disease models, we evaluated whether pre-conditioning with Fer-1 could augment islet engraftment in an immunodeficient, marginal human islet transplant model, as well as assess whether *in vitro* challenge with erastin could compromise subsequent *in vivo* engraftment in a full-dose transplant model.

5.3 – MATERIALS AND METHODS

5.3.1 – Erastin and RSL3

Erastin (Sigma, Oakville, ON) was prepared by dissolving the drug in phosphate buffered saline (PBS) at a stock concentration of 10 mM. RSL3 was received by the Stockwell Laboratory (Columbia University, NY, NY) and was prepared by dissolving the drug in dimethyl sulfoxide (DMSO) at a stock concentration of 40 mM. For long-term storage, both reagents were stored at -20°C.

5.3.2 – Human islet isolation, purification, and culture

Human islet preparations were isolated after family consent to retrieve pancreas organs from deceased multi-organ donors, as previously described,[37] with intent for clinical transplantation and were only made available for research when the islet yield fell below that of the minimal mass required. Permission regarding the performance of these studies was granted by the Health Research Ethics Board at the University of Alberta (Edmonton, Alberta, Canada), after written permission from donor families. Human islets were cultured in clinical grade CMRL-1066 media (Media Tech, MT99-603-L) supplemented with insulin selenium-transferrin and insulin-like growth factor-1 at 22°C and were received 24 to 72 hours after isolation.

5.3.3 – Islet culture

Human islets were cultured in Connaught Medical Research Laboratories (CMRL-1066) medium supplemented with 10% fetal bovine serum, L-glutamine (2mM), penicillin (50 000 units), streptomycin (50 mg), HEPES (5mM), nicotinamide (10mM) and sodium pyruvate (5mM). For select erastin experiments, islets were maintained in CMRL ± 1, 5 or 10 μM of the ferroptosis inhibitor Fer-1 (Sigma, Oakville, ON) for 24 hours. Subsequently, islets were harvested, quantified and cultured in the above conditions ± 20 or 50 μM FIA, erastin (Sigma, Oakville, ON), for an additional 24 hours. For RSL3 experiments, islets were maintained in CMRL ± 1 μM Fer-1 for 24 hours and subsequently cultured in the aforementioned conditions ± 20 μM RSL3.

5.3.4 – Lactate dehydrogenase as a measure of cytotoxicity

Human islets were cultured in the aforementioned conditions in non-tissue treated 6-well plates (Costar, Corning, NY). Cell-free supernatants were subsequently harvested and assessed for LDH release with the Cytotoxicity Detection Kit (Roche). Percentage cytotoxicity was calculated as per the manufacturer's protocol using the formula: (test LDH release - spontaneous release) ÷ maximal release. Test LDH release is the LDH released after treatment with the various treatment conditions; spontaneous release is the baseline cell LDH release; and maximal LDH release is the release of LDH when cells are lysed with 5% Triton-X. The data are the means of at least three independent experiments ± SEM.

5.3.5 – Static glucose stimulated insulin secretion (sGSIS)

Subsequent to 24-hour culture, islets were harvested from control (CMRL only) and treatment groups and were subjected to static GSIS (sGSIS). For each experiment, 50 islet equivalents (IEQ) from each group were 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. Subsequent to glucose challenge, cell-free supernatants were harvested and insulin levels were measured by enzyme-linked immunosorbent assay (ELISA) (Mercodia, Uppsala, Sweden). Stimulation Index is represented as the ratio of insulin secreted in response to high glucose versus insulin secreted in response to low glucose.

5.3.6 – Diabetes induction and islet transplantation

One week prior to transplantation, immunodeficient C57BL/6 $RAG^{-/-}$ mice (Jackson Laboratories, Bar Harbor, ME, USA) 12-14 weeks of age were rendered diabetic by chemical induction with intraperitoneal streptozotocin (STZ) (Sigma-Aldrich Canada Co., Oakville, ON, Canada), at 185 mg/kg in acetate phosphate buffer, pH 4.5. Diabetes was confirmed when non-fasting blood glucose levels exceeded 15 mmol/L for 2 consecutive daily readings.

For marginal human islet transplants, 24 hours post-culture ± 1 μM Fer-1 islets were quantified and transplanted under the kidney capsule at a dose of 500 islet equivalents (IEQ) ± 10% per diabetic recipient. For full-dose human islet transplants, islets were cultured for 24 hours ± 1 μM Fer-1 and an additional 24 hours ± 50 μM erastin, were harvested, quantified and transplanted under the kidney capsule at a dose of 1500 islet equivalents (IEQ) ± 10% per diabetic recipient. From the time of islet isolation to full-dose islet transplantation, the median culture period was 96 hours.

For all transplants, human islets were aspirated into polyethylene (PE-90) tubing using a micro-syringe, and centrifuged into a pellet suitable for transplantation. A left lateral paralumbar incision was made and the left kidney delivered. The renal capsule was incised and the islets were infused.

5.3.7 – Evaluation of islet graft function

Non-fasting blood glucose measurements (mmol/L) were assessed three times weekly using a portable glucometer (FreeStyle InsuLinx, Abbott Diabetes Care Ltd., Oxon, UK)

in the three transplant groups tested. Graft function and reversal of diabetes was defined as two consecutive readings ≤11.1 mmol/L and maintained until study completion. Intraperitoneal glucose tolerance tests (IPGTTs) were conducted at study endpoint; 35 days post-transplant for marginal islet recipients and 40 days post-transplant in full-dose islet transplant recipients. Mice were fasted overnight prior to receiving an intraperitoneal 25% glucose bolus (3 g/kg). Blood glucose levels were evaluated at baseline (time 0), 15, 30, 60, 90 and 120 minutes post-injection. Blood glucose area under the curve (Blood glucose AUC) was calculated and analyzed between transplant groups.

5.3.8 – Islet graft retrieval

In order to corroborate graft-dependent euglycemia, islet transplants were retrieved by recovery nephrectomy. Islet transplant recipients were placed under anesthesia, and their graft-bearing kidney was exposed. Using a LT200 Ligaclip (Johnson & Johnson, Inc., Ville St-Laurent, QC, CA), the renal vessels and ureter were ligated and the islet graft-bearing kidney was removed. Non-fasting blood glucose measurements were monitored up to 7 days post-graft removal to confirm hyperglycemia and thus post-transplant graft function.

5.3.9 – Statistical Analysis

All data are represented as the mean ± standard error of mean (s.e.m.). Islet viability data comparisons between control and treatment groups were analyzed through parametric one-way ANOVA. Tukey's post-hoc tests were used following the analysis

of variances for multiple comparisons between study groups. IPGTT area under the curve (AUC) was also analyzed by parametric one-way ANOVA. Data was analyzed using GraphPad Prism (GraphPad Software, La Jolla, CA, USA). $P<0.05$ was considered significant.

5.4 – RESULTS

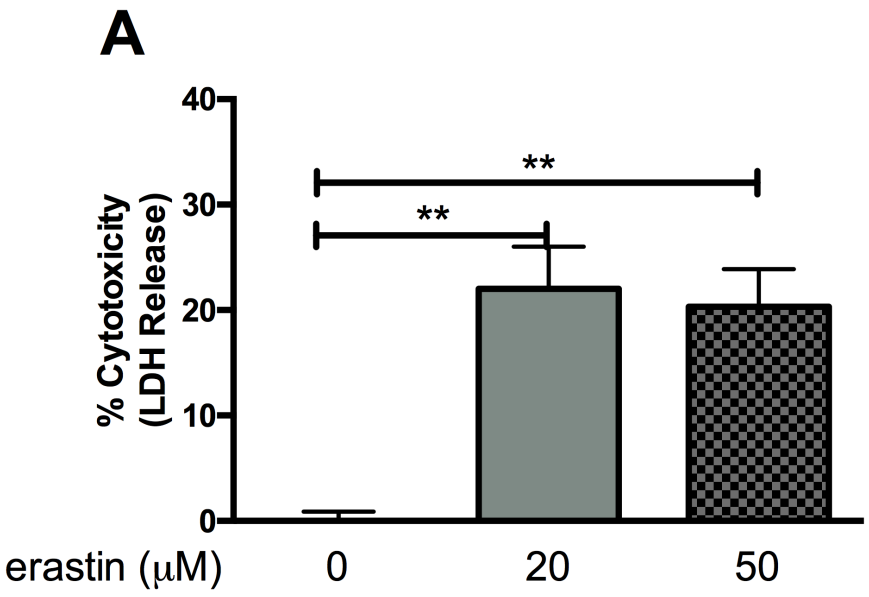
5.4.1 – Human islets cultured in the presence of erastin exhibit impaired function and viability

Cell free supernatants harvested 24 hours post-culture revealed significant levels of LDH from islets challenged with 20 μM or 50μM erastin compared to supernatants from islet cultured in CMRL alone (**Figure 5.1A**) (20 μM erastin: 22.0% ± 4.0; 50 μM erastin: 20.3% ± 3.8, $p<0.01$). When assessed for islet function, human islets exposed to 50 μM erastin exhibited significantly reduced insulin-secreting capacity in response to glucose versus control islets (Stimulation Index: control 1.97 ± 0.13 vs. 50 μM erastin 1.32 ± 0.1, $p<0.05$) (**Figure 5.1B**).

5.4.2 – Pre-treatment with 1μM Fer-1 rescues erastin-induced islet cell death

After 24 hours of 20 μM erastin treatment, human islets pre-treated with 1, 5 or 10 μM Fer-1 exhibited significantly reduced LDH levels as compared to islets treated with erastin alone ($p<0.01$, $p<0.001$ and $p<0.001$, respectively) (**Figure 5.2A-C**). Notably, in all cases, islets pre-treated with Fer-1 exhibited no significant difference in LDH release compared to their erastin-treated counterparts. Moreover, in the presence of 50 μM

erastin, pre-treatment with 1 μM Fer-1 exhibited significant cytoprotection compared to erastin-treatment alone ($p<0.01$) (**Figure 5.2D).** Similarly, islets pre-treated with 5 or 10 μM Fer-1 conferred significant cytoprotection in the presence of 50 μM erastin as exhibited by reduced LDH release.


A
40
30
20
10
0
% Cytotoxicity
(LDH Release)
**
**
erastin (μM)
0
20
50

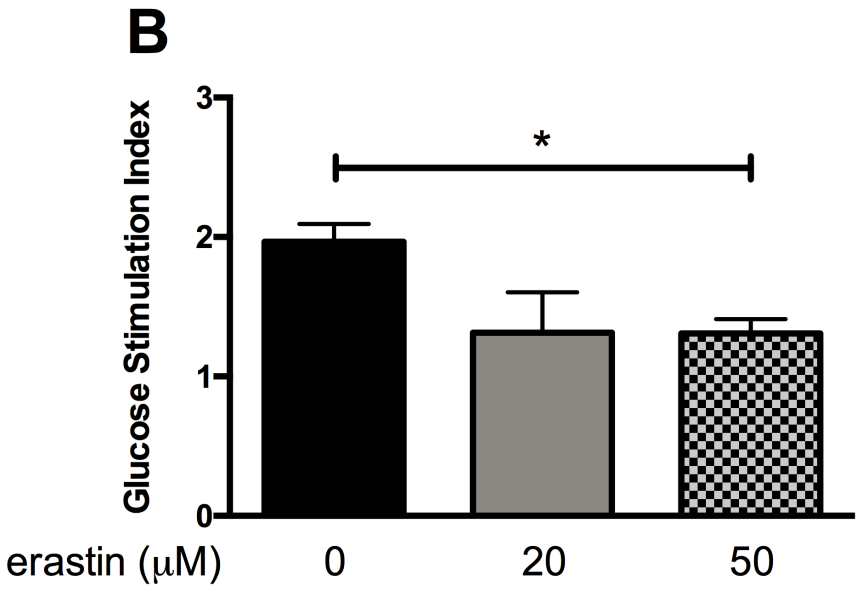

B
3
2
1
0
Glucose Stimulation Index
*
erastin (μM)
0
20
50

Figure 5.1. *In vitro* human islet viability and function is compromised in the presence of erastin.

(**A**) Human islets challenged with 20 and 50 μM erastin exhibit increased LDH release relative to non-treated control islets ($P<0.01$). Data represented as percent of control. (**B**) Erastin treatment impairs glucose stimulated insulin secretion in human islets. ($P<0.05$). Data represented as mean ± SEM. (one-way ANOVA followed by Tukey's multiple comparison test). Triplicate samples from 3 human pancreata.

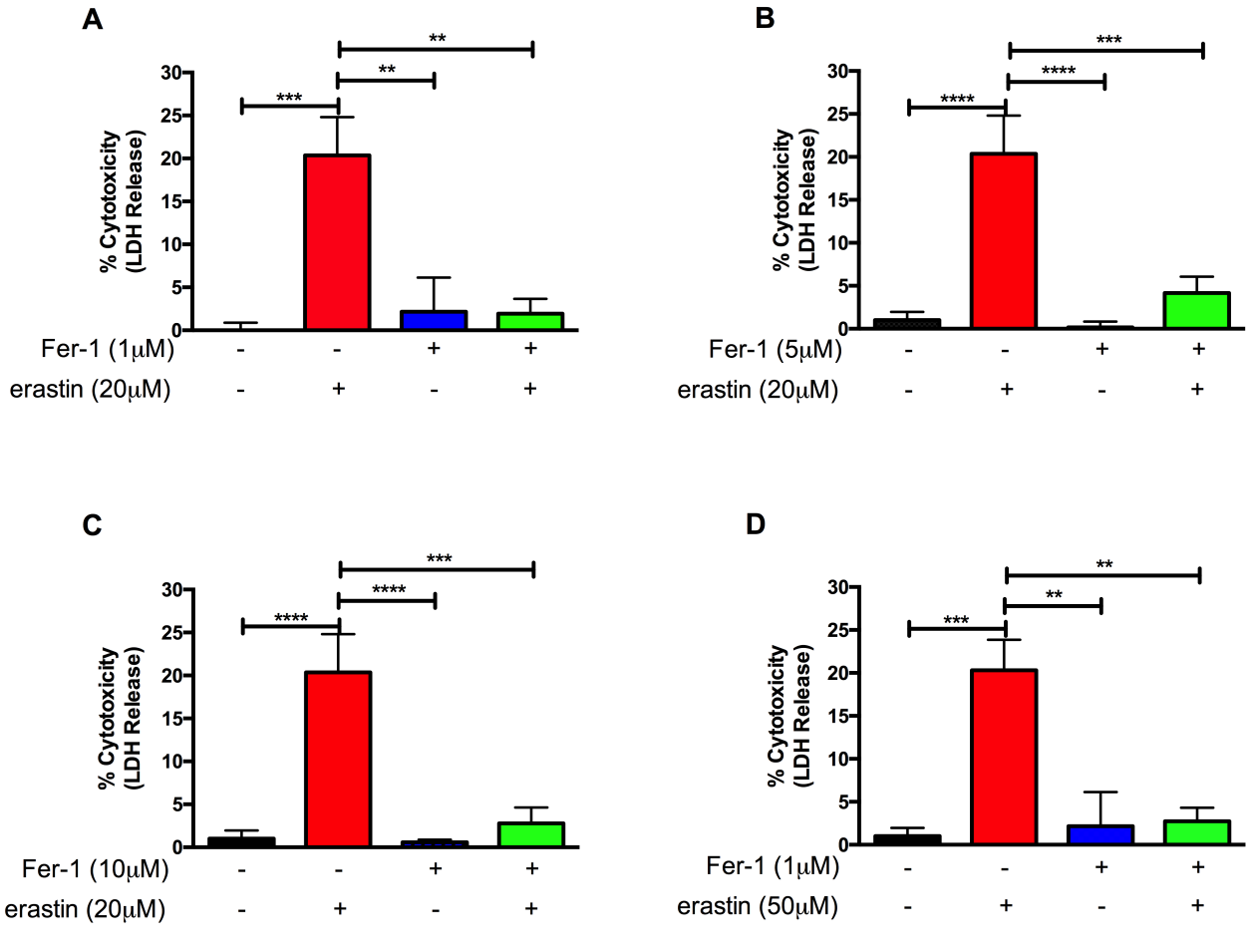

A
B
C
D
% Cytotoxicity (LDH Release)
Fer-1 (1μM)
Fer-1 (5μM)
Fer-1 (10μM)
erastin (20μM)
erastin (50μM)
**
***
****

Figure 5.2. Assessment of LDH release of human islets pre-treated with or without Fer-1 in the presence of erastin.

(**A-C**) Human islets exhibit significantly reduced islet cell death when pre-treated for 24 hours with 1, 5 or 10 μM Fer-1 and subsequently challenged with erastin for an additional 24 hours. **P<0.01, ***P<0.001, ****P<0.0001 (one-way ANOVA). (**D**) 1 μM Fer-1 exhibited reduced LDH release in the presence of 50 μM Fer-1 erastin relative to erastin-alone treated islets, **P<0.01. Data represented as mean ± SEM, Triplicate samples from three human pancreata.

5.4.3 – Fer-1 pre-treatment preserves sGSIS in the presence of erastin

Since human islets treated with 50 μM erastin exhibited significant impairment of glucose-stimulated insulin secretion relative to non-treated control islets, we evaluated whether pre-treatment with 1 μM Fer-1 could improve glucose stimulated insulin secretion in the presence of erastin. Human islets pre-treatment with 1 μM Fer-1 followed by erastin treatment preserved the insulin-secreting capacity of human islets (Stimulation Index: 2.29 ± 0.06), whereas islets treated with erastin alone exhibited reduced insulin-secreting capabilities (Stimulation Index: 1.32 ± 0.11) ($p<0.05$) (**Figure 5.3**). As anticipated, Fer-1 alone treated islets demonstrated similar insulin secretory capacity as control islets (Stimulation Index control: 1.97 ± 0.13 vs Fer-1: 2.10 ± 0.30, $P>0.05$).

5.4.4 – RSL3 compromises human islet viability and moderately impairs sGSIS

To further elucidate whether FIAs could compromise *in vitro* viability and function, human islets were cultured for 24 hours in the presence of an alternative known stimulator of ferroptosis, RSL3, at 20 μM concentration subsequent to 24 hours of pre-conditioning with or without 1 μM Fer-1. Cell free supernatants from islets cultured in RSL3 alone exhibited significantly elevated LDH in comparison to non-treated control islets alone (control: 1.7% ± 0.1 vs. 20 μM RSL3: 24.4% ± 2.5, $p<0.0001$). Islets pre-treated with 1 μM Fer-1 and subsequently challenged with 20 μM RSL3 for an additional 24 hours exhibited significantly reduced cell death in comparison to RSL3 treatment alone (20 μM RSL3: 24.4% ± 2.5 vs 1 μM Fer-1 ± 20 μM RSL3: 12.57% ± 1.85, $p<0.01$) (**Figure 5.4A**). When evaluating the insulin-secreting capacity of islets

through sGSIS, islets challenged with 20 μM RSL3 were not significantly different from control islets (control: 2.35 ± 0.07 vs. 20 μM RSL3: 1.80 ± 0.23, p>0.05) (**Figure 5.4B**). This experiment suggests that islets in general are sensitive to ferroptosis.

5.4.5 – Engraftment Efficacy of Fer-1-treated human islets transplanted under the renal capsule of immunodeficient mice.

Islet engraftment efficacy of human islets pre-treated with or without Fer-1 for 48 hours was evaluated in a marginal islet transplant mass model (500 IEQ per recipient, n=2 control, n=4 Fer-1). All recipients became euglycemic subsequent to transplant in both transplant groups. Daily non-fasting blood glucose monitoring of euglycemic transplant recipients revealed no difference between control and Fer-1-treated islet recipients (**Figure 5.5A**). Intraperitoneal glucose tolerance tests (IPGTTs) were performed on all marginal islet transplant, euglycemic recipients 35 days post-transplant. Mice in both transplant groups exhibited a physiological response to glucose bolus, with a prompt restoration of normoglycemia up to 120 minutes post-dextrose infusion (**Figure 5.5B**). Furthermore, there was no significant difference in mean area under the curve (AUC) ± s.e.m. (AUC control: 1557 ± 300.4 mmol/L/120min vs. Fer-1: 1335 ± 100.1 mmol/L/120min, p>0.05, unpaired t-test, **Figure 5.5C**). This experiment suggests that ferroptosis is not the primary cause of cell death in this model. However, it cannot be excluded that more stable second generation ferrostatins may still be beneficial.

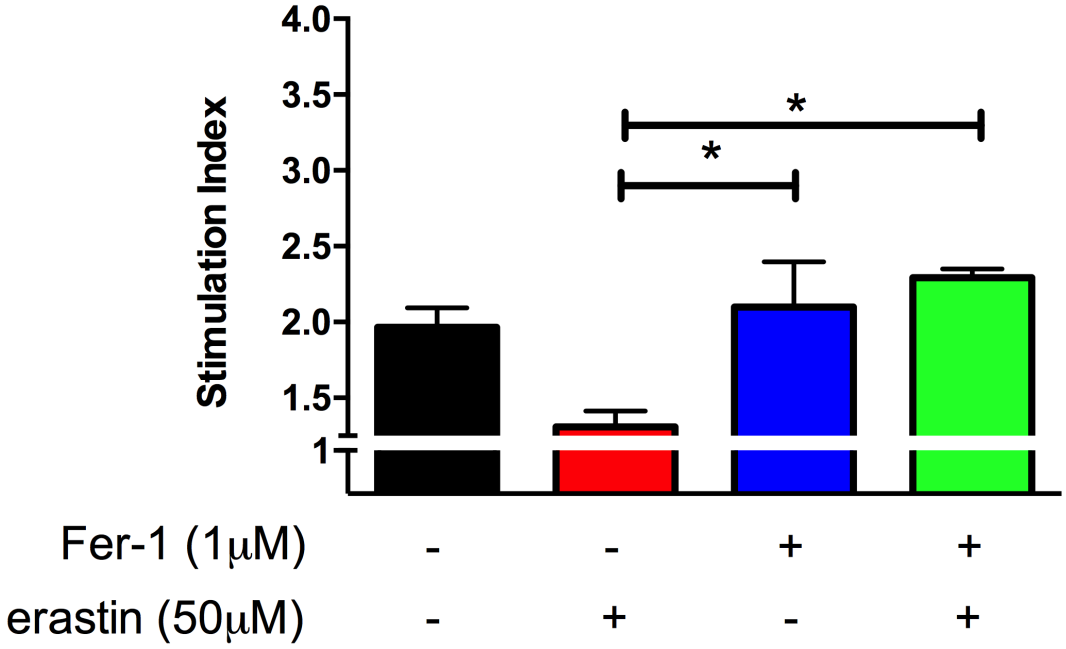
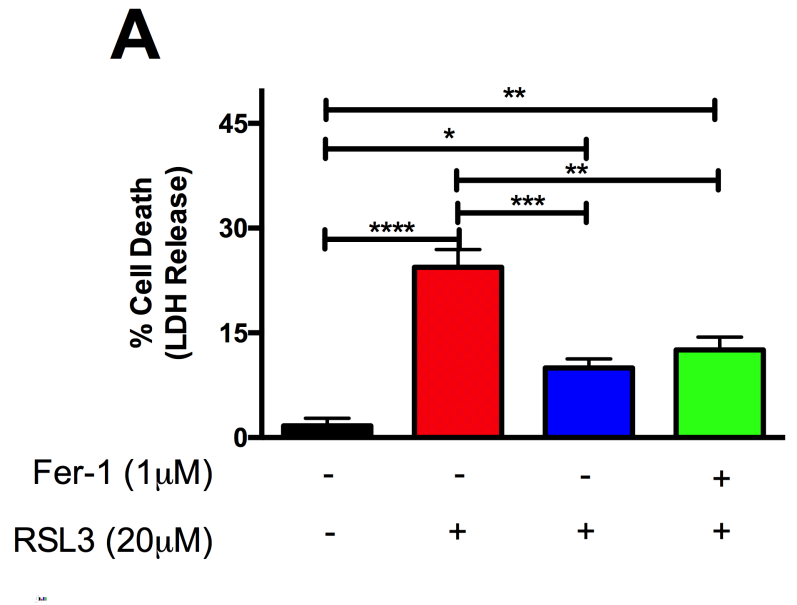


Figure 5.3. Fer-1 pre-treatment maintains sGSIS in human islets treated with erastin.

Human islets were pre-treated with or without 1 μM Fer-1 for 24 hours and subsequently challenged with or without 50 μM erastin for an additional 24 hours and assessed for sGSIS. Cell-free supernatants were assessed for insulin secretion via ELISA and expressed as stimulation index. Data represented as mean ± SEM, triplicate samples from three human pancreata. *P<0.05 (one-way ANOVA, followed by Tukey's multiple comparison test).

5.4.6 – Human islets pre-treated with erastin do not exhibit impaired engraftment.

Transplant recipients of human islets pre-treated for 24 hours with or without 1 μM Fer-1 and additional 24 hour challenge with or without 50 μM erastin revealed no significant difference in restoration of euglycemia between all transplant groups (**Figure 5.6A**). Non-fasting, mean blood glucose profiles of all four transplant groups revealed similar glycemic trends (**Figure 5.6B**). Recipients in all groups reverted back to hyperglycemia upon graft recovery nephrectomy, thus confirming graft-dependent euglycemia. To evaluate *in vivo* graft function, intraperitoneal glucose tolerance testing (IPGTT) was performed on transplant recipients 40 days post-transplant. Islet transplant recipients from all groups revealed similar blood glucose profiles post-dextrose infusion and exhibited a restoration of normoglycemia within 120 minutes (**Figure 5.6C**). No discernable difference in blood glucose mean area under the curve (AUC) was observed between groups (**Figure 5.6D**). These data suggest that erastin does not exacerbate the amount of ferroptosis that spontaneously occurs in this scenario.


A
% Cell Death (LDH Release)
45
30
15
0
**
*
**
***
****
Fer-1 (1μM) - - - +
RSL3 (20μM) - + + +

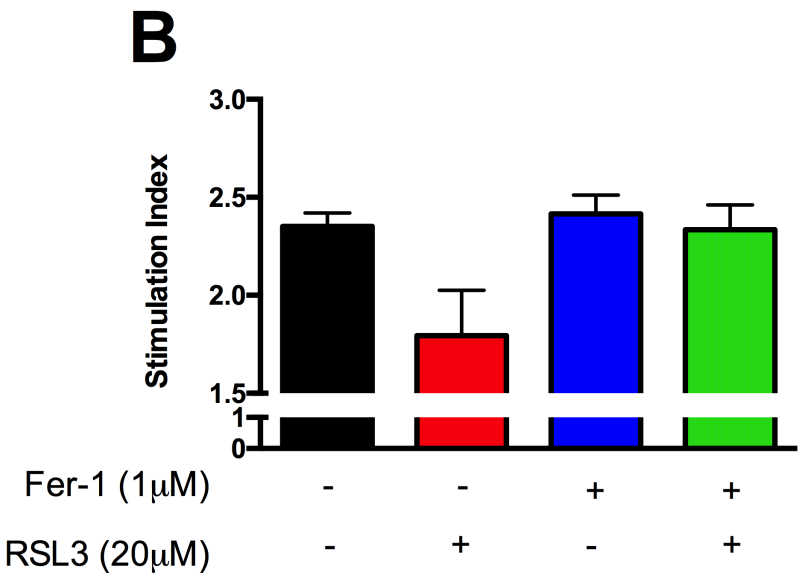
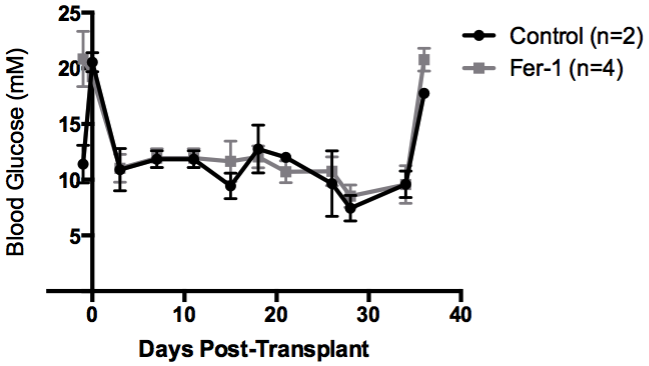

B
Stimulation Index
3.0
2.5
2.0
1.5
1
0
Fer-1 (1μM) - - + +
RSL3 (20μM) - + - +

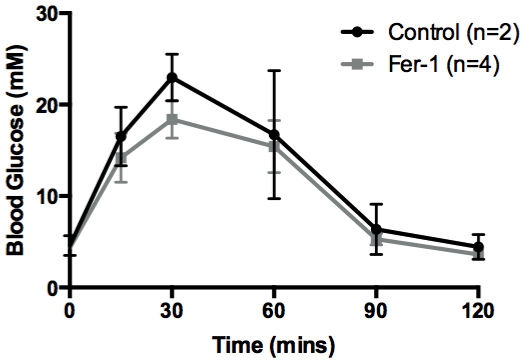
Figure 5.4. Evaluation of *in vitro* human islet viability and function in the presence of the ferroptosis-inducing agent, RSL3.

Human islets were pre-treated with or without 1 μM Fer-1 for 24 hours and subsequently challenged with or without 20 μM RSL3 for an additional 24 hours. (**A**) Human islets exhibit significantly increased cell death as assessed by LDH in the presence of 20 μM RSL3. Pre-treatment with 1 μM Fer-1 preserves islet viability in the presence of RSL3. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001 (one-way ANOVA). (**B**) sGSIS evaluation of the insulin-secreting capacity of human islets cultured alone, pre-conditioned with 1 μM Fer-1 or in the presence of 20 μM RSL3. Cell-free supernatants were assessed for insulin secretion via ELISA and expressed as stimulation index. Data represented as mean ± SEM, triplicate samples from three human pancreata.

A


Blood Glucose (mM)
25
20
15
10
5
0
10
20
30
40
Days Post-Transplant
Control (n=2)
Fer-1 (n=4)

B


Blood Glucose (mM)
30
20
10
0
0
30
60
90
120
Time (mins)
Control (n=2)
Fer-1 (n=4)

C

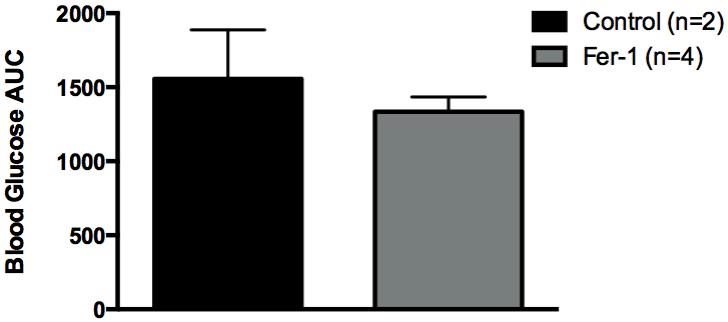

Blood Glucose AUC
2000
1500
1000
500
0
Control (n=2)
Fer-1 (n=4)

Figure 5.5. Efficacy of marginal dose human islet transplants under the renal capsule of C57BL/6 RAG$^{-/-}$ recipients.

(**A**) Non-fasting blood glucose measurements of euglycemic recipients post-transplant. Recipients of control and 1 μM Fer-1 marginal (500 IEQ) human islets exhibited robust glycemic control until graft retrieval (arrow). (**B**) Blood glucose prolife post-dextrose bolus of control (black, n=2) and 1 μM Fer-1 (grey, n=4). (**C**) Blood glucose area under the curve (AUC) analysis did not differ between control and 1 μM Fer-1-treated islet recipients. Mice were administered 3 mg/kg 25% dextrose i.p. Blood glucose measurements were monitored at t=0, 15, 30, 60, 90 and 120 minutes.

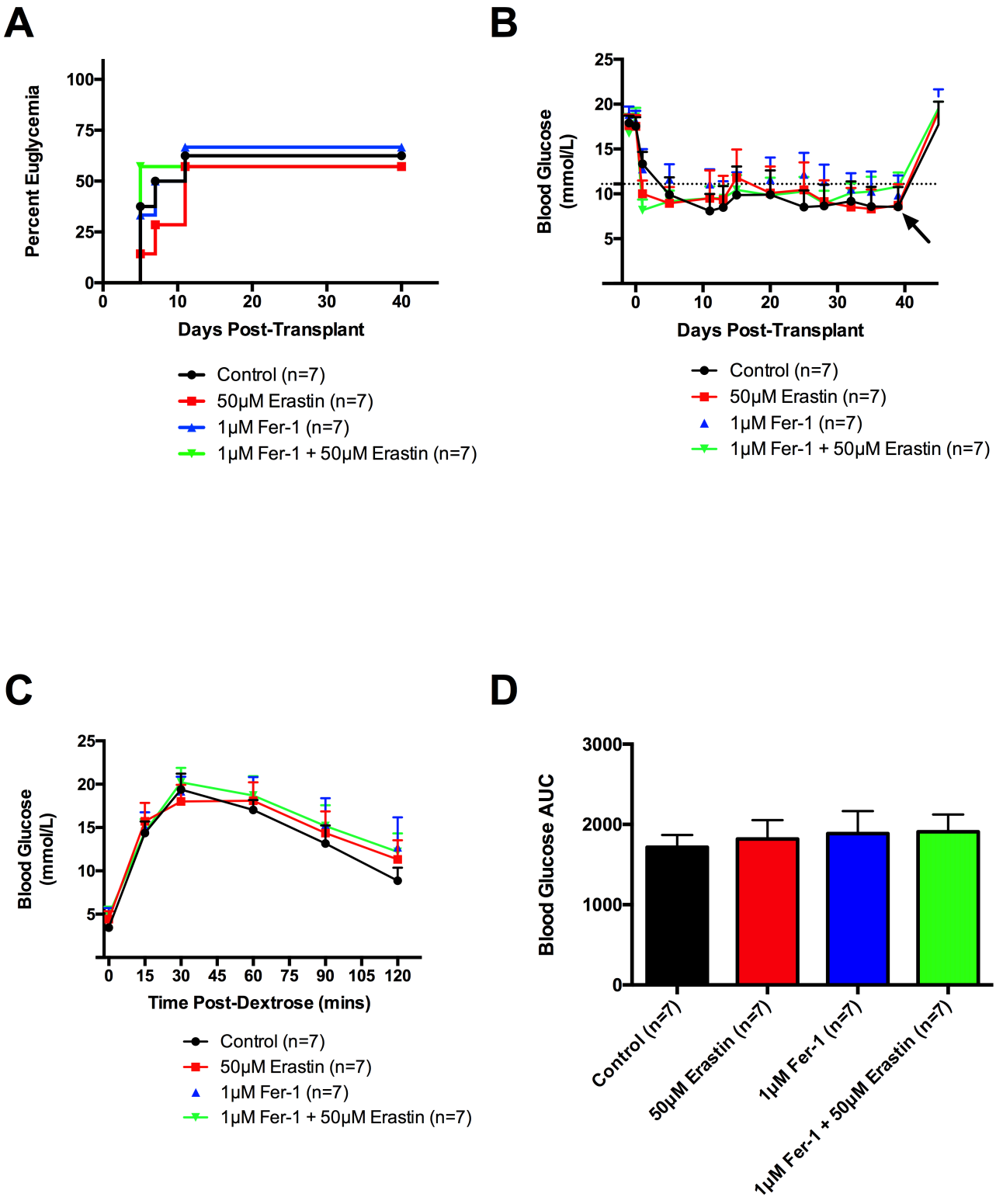

A
Percent Euglycemia
100
75
50
25
0
0
10
20
30
40
Days Post-Transplant
Control (n=7)
50μM Erastin (n=7)
1μM Fer-1 (n=7)
1μM Fer-1 + 50μM Erastin (n=7)
B
Blood Glucose (mmol/L)
25
20
15
10
5
0
10
20
30
40
Days Post-Transplant
Control (n=7)
50μM Erastin (n=7)
1μM Fer-1 (n=7)
1μM Fer-1 + 50μM Erastin (n=7)
C
Blood Glucose (mmol/L)
25
20
15
10
5
0
15
30
45
60
75
90
105
120
Time Post-Dextrose (mins)
Control (n=7)
50μM Erastin (n=7)
1μM Fer-1 (n=7)
1μM Fer-1 + 50μM Erastin (n=7)
D
Blood Glucose AUC
3000
2000
1000
0
Control (n=7)
50μM Erastin (n=7)
1μM Fer-1 (n=7)
1μM Fer-1 + 50μM Erastin (n=7)

Figure 5.6. Efficacy of full dose (1500 IE) human islet transplants under the renal capsule of C57BL/6 RAG$^{-/-}$ recipients.

(**A**) Percent euglycemia of full-dose (1500 IE) human islet transplant recipients receiving islets cultured alone (Control, n=7), or challenged with 50 μM erastin (red, n=7), 1 μM Fer-1 alone (blue, n=7) or 1 μM Fer-1 ± 50 μM erastin (green, n=7). (**B**) Non-fasting blood glucose measurements of human islet recipients post-transplant. Euglycemic recipients maintained glycemic control throughout the duration of engraftment until graft retrieval (arrow). Dotted line exhibits maximum threshold of normoglycemia (≤11.1 mmol/L). Data represented as mean ± SEM per group. (**C**) Blood glucose profile post-dextrose bolus of control (black, n=7), 20 μM erastin (red, n=7), 1 μM Fer-1 alone (blue, n=7) or 1 μM Fer-1 ± 50 μM erastin (green, n=7). (**D**) Mean blood glucose area under the curve (AUC). Mice were administered 3mg/kg 25% dextrose i.p. Blood glucose measurements were monitored at t=0, 15, 30, 60, 90 and 120 minutes. Data represented as mean ± SEM per group.

5.5 – DISCUSSION

Strategies to ameliorate early islet loss in culture and in the acute transplant period may contribute positively to long-term engraftment outcomes. During organ procurement, islet isolation and transplantation, islets experience considerable oxidative stress that contributes to islet injury and loss. GSH has been identified as an important intracellular antioxidant capable of mitigating the accumulation of ROS. Erastin has demonstrated the ability to induce ferroptosis by minimizing cystine uptake, reducing intracellular GSH levels, thus contributing to ferroptosis-induced cell death in various disease models.[17] Moreover, the GPX4 inhibitor, RSL3 has also been identified as an additional inducing agent of ferroptosis. Given the importance of GSH and GPX4 in islet viability and function, the role of ferroptosis has yet to be elucidated in islet transplantation.

The inability to resolve accumulating ROS results in oxidative stress. It has previously been demonstrated that considerable ROS is generated during isolation and transplantation.[26, 27] Prolonged oxidative stress has been associated with compromised islet viability and function.[28] As such, host antioxidant systems play an integral role in reducing excessive ROS thus minimizing cellular damage and impairment. Islets exhibit reduced intrinsic antioxidant enzyme expression and activity relative to other host tissues and in an effort to preserve islet function, the administration of exogenous antioxidants has provided substantial cytoprotective benefit.[23, 24, 29] GSH, a tri-peptide synthesized from glutamate, cysteine and glycine has been established as an important intracellular antioxidant.[30] The exogenous administration of glutamine, which contributes to glutamate synthesis, a GSH precursor, have exhibited improved islet

function.[31, 32] Intra-ductal administration of glutathione precursors, such as L-glutamine, have previously demonstrated the ability to augment intracellular glutathione pools, and reduce oxidative injury during human pancreatic islet isolation.[32] Disruption of the cell's glutamine-synthesizing capacity may alternatively contribute to the cell's demise.

In the present study, we evaluated whether human islet viability and function could be compromised in the presence of FIAs, erastin and RSL3. Our results revealed that erastin and RSL3 exacerbated LDH release, indicative of islet cell ferroptosis. Erastin exhibited the ability to impair the insulin secreting capacity of islets when assessed via sGSIS, and for islets pre-conditioned with Fer-1, the effect was abolished. These findings are novel, as these studies represent first-in-human testing of Fer-1 pathways in human tissues, and specifically in human islets. These observations may be attributed to erastin's ability to inhibit cystine uptake and contribute to subsequent GSH depletion.[17] Prior studies have implicated GSH as a crucial antioxidant alleviating oxidative stress in islets. Glutamine, a precursor to GSH has previously been shown to enhance insulin secretion in response to glucose as well as reduce lipid peroxidation levels.[32, 33, 34] Miwa and colleagues demonstrated that islets treated with various lipid peroxidation products inhibited glucose-induced insulin secretion.[27] Ample evidence suggests that when elevated in β-cells, fatty acids impair insulin gene expression, glucose-stimulated insulin secretion, and increase cell death.[35] It remains to be determined in more detail if these fatty acids also contain polyunsaturated fatty acids (PUFAs) that were recently demonstrated to contribute to ferroptosis, dependent on the molecule PEBP1. However, little is known about ferroptosis in human tissues.

In our study, human islets pre-treated with Fer-1 and subsequently challenged with erastin exhibited improved viability and function than islets treated with erastin alone. Fer-1's ability to mitigate the deleterious events associated with ferroptosis has previously been demonstrated in various cellular models, including Huntington's disease,[25] and likely accounts for improved islet viability. It is possible that impaired insulin secretion observed in our study was a result of downstream events associated with engagement of the Xc^- antiporter. GPX4 is capable of reducing intracellular reactive oxygen species and requires GSH as an essential enzymatic cofactor.[19] Erastin treatment has previously demonstrated the ability to indirectly inhibits GPX4 activity by depleting GSH levels.[20] Koulajian *et al.* demonstrated improved *in vitro* and *in vivo* β-cell function in islets over-expressing GPX4 in the presence of lipid peroxidation products,[28] further substantiating the necessity of GPX4 in preserving islet viability. On this premise, we sought to evaluate whether *in vitro* viability and function could indeed be compromised in the presence of the GPx4-specific inhibitor and FIA, RSL3. Given the importance of GPX4 in maintaining islet function and viability, increased levels of LDH indicate impaired viability. Islets challenged with RSL3 indeed revealed reduced viability, which was rescued in the presence of the ferroptosis inhibitor, Fer-1. However, the insulin-secreting capacity of islets exposed to 20 μM RSL3 exhibited only a modest insignificant decrease in sGSIS relative to non-treated islets and islets pre-conditioned with Fer-1. While this dose was capable of sufficiently inducing cell death, a higher dose of RSL3 may be required to confer impairment in insulin secreting capacity, or regulated necrosis pathways other than ferroptosis (e.g. necroptosis or pyroptosis) may be involved.

Though treatment of human islets with both FIAs utilized in this study revealed compromised *in vitro* viability, pre-treatment of islets with erastin failed to compromise subsequent engraftment when transplanted under the renal capsule of immune-deficient recipients with a transplant dose (1500 IEQ). Early observations in the *in vivo* marginal study revealed both control islet recipients became euglycemic in parallel to all Fer-1-treated islet recipients. Control and Fer-1-treated islets exhibited similar LDH levels *in vitro*, suggesting minimal cytotoxicity in culture without erastin treatment. An important explanation for differences between the positive impact of Fer-1 *in vitro*, but not seen *in vivo,* is that Fer-1 has a short half-life, and therefore may have had limited efficacy if ferroptosis was ongoing *in vivo.*

It is important to emphasize in the current study that human islets only became available for research study 24 to 72 hours post-isolation and subsequently assessed an additional 48 hours in our experimental conditions. It is likely that any major negative contribution of ferroptosis would have already occurred within the unstable multi-organ donor, during prolonged cold ischemia, and the preceding islet isolation and culture periods. The impact we observed *in vitro* therefore reflects a secondary wave of inducible injury from the FIAs. This, coupled with the fact that we recounted and compensated for any numeric loss of human islets sustained during erastin or RSL3 secondary culture when we transplanted the human islets across groups, may explain why the dominant negative impact of FIAs was seen only *in vitro* but masked *in vivo*. The negative impact of both FIAs *in vitro* was relatively modest (approximately 20% inducible cell death only, even at maximal doses). This further suggests that the FIAs are acting through non-dominant pathways, and that the majority of islets remain

preserved. The present study therefore does not completely define the full potential of ferroptosis-inhibition in islet transplantation, especially if this could be applied early in the multi-organ donor, and across all steps of transport, islet isolation and culture, which was not addressed herein.

Utilizing Fer-1 during islet isolation and acutely post-culture may confer benefit to human islets and would be worth investigating in the experimental setting to determine its prospective clinical utility. It may also be necessary to evaluate the potential cytoprotective effect of Fer-1 using a more clinically relevant transplant site, like the hepatic portal vein. Our group previously revealed that the cytoprotective effects of the apoptosis inhibitor, F573, conferred varying engraftment outcomes dependent on the site of transplantation.[36] Moreover, this study also revealed the necessity to administer the drug to the organ donors and/or recipients could potentially confer improved engraftment outcomes. In this regard, administration of Fer-1 or other more potent ferroptosis inhibitors to donors or recipients may be necessary to confer maximal benefit to islet engraftment. More stable ferrostatins are currently being designed.

We, and others, have suggested that Fer-1 exhibits lower *in vivo* stability, and have developed a third-generation ferroptosis inhibitor that exhibits improved plasma and metabolic stability.[17] Expanding the utility of new generation inhibitors of ferroptosis during islet isolation to prevent accumulated damage may be an attractive avenue to explore. The administration of ferrostatins as a co-therapy to islet transplant recipients may also yield improved clinical outcomes. Deterring early islet loss during isolation and subsequent clinical transplant is critical for long-term graft function. Utilizing novel cell death inhibitors to diminish islet damage *in vitro* and in the acute

transplant period may be an attractive combination therapy to preserve islet mass and improve engraftment.

5.6 – REFERENCES

1. Bruni A, Gala-Lopez B, Pepper AR, Abualhassan NS, Shapiro AJ. Islet cell transplantation for the treatment of type 1 diabetes: recent advances and future challenges. *Diabetes Metab Syndr Obes* 2014, **7:** 211-223.
2. 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**(4)**:** 230-238.
3. 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**(7)**:** 2060-2069.
4. McCall MD, Maciver AM, Kin T, Emamaullee J, Pawlick R, Edgar R*, et al.* Caspase inhibitor IDN6556 facilitates marginal mass islet engraftment in a porcine islet autotransplant model. *Transplantation* 2012, **94**(1)**:** 30-35.
5. Davalli AM, Scaglia L, Zangen DH, Hollister J, Bonner-Weir S, Weir GC. Vulnerability of islets in the immediate posttransplantation period. Dynamic changes in structure and function. *Diabetes* 1996, **45**(9)**:** 1161-1167.
6. 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**(1)**:** 66-72.
7. Emamaullee JA, Stanton L, Schur C, Shapiro AM. Caspase inhibitor therapy enhances marginal mass islet graft survival and preserves long-term function in islet transplantation. *Diabetes* 2007, **56**(5)**:** 1289-1298.

8. Linkermann A, Stockwell BR, Krautwald S, Anders HJ. Regulated cell death and inflammation: an auto-amplification loop causes organ failure. *Nat Rev Immunol* 2014, **14**(11)**:** 759-767.

9. Linkermann A. Nonapoptotic cell death in acute kidney injury and transplantation. *Kidney Int* 2016, **89**(1)**:** 46-57.

10. Pasparakis M, Vandenabeele P. Necroptosis and its role in inflammation. *Nature* 2015, **517**(7534)**:** 311-320.

11. Wallach D, Kang TB, Dillon CP, Green DR. Programmed necrosis in inflammation: Toward identification of the effector molecules. *Science* 2016, **352**(6281)**:** aaf2154.

12. Schwarznau A, Hanson MS, Sperger JM, Schram BR, Danobeitia JS, Greenwood KK*, et al.* IL-1beta receptor blockade protects islets against pro-inflammatory cytokine induced necrosis and apoptosis. *J Cell Physiol* 2009, **220**(2)**:** 341-347.

13. SoRelle JA, Itoh T, Peng H, Kanak MA, Sugimoto K, Matsumoto S*, et al.* Withaferin A inhibits pro-inflammatory cytokine-induced damage to islets in culture and following transplantation. *Diabetologia* 2013, **56**(4)**:** 814-824.

14. Emamaullee JA, Davis J, Pawlick R, Toso C, Merani S, Cai SX*, et al.* The caspase selective inhibitor EP1013 augments human islet graft function and longevity in marginal mass islet transplantation in mice. *Diabetes* 2008, **57**(6)**:** 1556-1566.

15. Batinic-Haberle I, Tovmasyan A, Roberts ER, Vujaskovic Z, Leong KW, Spasojevic I. SOD therapeutics: latest insights into their structure-activity relationships and impact on the cellular redox-based signaling pathways. *Antioxid Redox Signal* 2014, **20**(15)**:** 2372-2415.

16. McCall M, Toso C, Emamaullee J, Pawlick R, Edgar R, Davis J*, et al.* The caspase inhibitor IDN-6556 (PF3491390) improves marginal mass engraftment after islet transplantation in mice. *Surgery* 2011, **150**(1)**:** 48-55.

17. Linkermann A, Skouta R, Himmerkus N, Mulay SR, Dewitz C, De Zen F*, et al.* Synchronized renal tubular cell death involves ferroptosis. *Proc Natl Acad Sci U S A* 2014, **111**(47)**:** 16836-16841.

18. Dixon SJ, Lemberg KM, Lamprecht MR, Skouta R, Zaitsev EM, Gleason CE*, et al.* Ferroptosis: an iron-dependent form of nonapoptotic cell death. *Cell* 2012, **149**(5)**:** 1060-1072.

19. Stockwell BR, Friedmann Angeli JP, Bayir H, Bush AI, Conrad M, Dixon SJ*, et al.* Ferroptosis: A Regulated Cell Death Nexus Linking Metabolism, Redox Biology, and Disease. *Cell* 2017, **171**(2)**:** 273-285.

20. Yang WS, Stockwell BR. Ferroptosis: Death by Lipid Peroxidation. *Trends Cell Biol* 2016, **26**(3)**:** 165-176.

21. Dixon SJ, Stockwell BR. The role of iron and reactive oxygen species in cell death. *Nat Chem Biol* 2014, **10**(1)**:** 9-17.

22. Reed JC, Pellecchia M. Ironing out cell death mechanisms. *Cell* 2012, **149**(5)**:** 963-965.

23. Bottino R, Balamurugan AN, Bertera S, Pietropaolo M, Trucco M, Piganelli JD. Preservation of human islet cell functional mass by anti-oxidative action of a novel SOD mimic compound. *Diabetes* 2002, **51**(8)**:** 2561-2567.

24. Bottino R, Balamurugan AN, Tse H, Thirunavukkarasu C, Ge X, Profozich J*, et al.* Response of human islets to isolation stress and the effect of antioxidant treatment. *Diabetes* 2004, **53**(10)**:** 2559-2568.

25. Skouta R, Dixon SJ, Wang J, Dunn DE, Orman M, Shimada K*, et al.* Ferrostatins inhibit oxidative lipid damage and cell death in diverse disease models. *J Am Chem Soc* 2014, **136**(12)**:** 4551-4556.

26. Hennige AM, Lembert N, Wahl MA, Ammon HP. Oxidative stress increases potassium efflux from pancreatic islets by depletion of intracellular calcium stores. *Free Radic Res* 2000, **33**(5)**:** 507-516.

27. Miwa I, Ichimura N, Sugiura M, Hamada Y, Taniguchi S. Inhibition of glucose-induced insulin secretion by 4-hydroxy-2-nonenal and other lipid peroxidation products. *Endocrinology* 2000, **141**(8)**:** 2767-2772.

28. Koulajian K, Ivovic A, Ye K, Desai T, Shah A, Fantus IG*, et al.* Overexpression of glutathione peroxidase 4 prevents beta-cell dysfunction induced by prolonged elevation of lipids in vivo. *Am J Physiol Endocrinol Metab* 2013, **305**(2)**:** E254-262.

29. Finkel T. Oxygen radicals and signaling. *Curr Opin Cell Biol* 1998, **10**(2)**:** 248-253.

30. 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**(2)**:** e55288.

31. Jang HJ, Kwak JH, Cho EY, We YM, Lee YH, Kim SC*, et al.* Glutamine induces heat-shock protein-70 and glutathione expression and attenuates ischemic damage in rat islets. *Transplant Proc* 2008, **40**(8)**:** 2581-2584.

32. Avila J, Barbaro B, Gangemi A, Romagnoli T, Kuechle J, Hansen M*, et al.* Intra-ductal glutamine administration reduces oxidative injury during human pancreatic islet isolation. *Am J Transplant* 2005, **5**(12)**:** 2830-2837.

33. Carobbio S, Ishihara H, Fernandez-Pascual S, Bartley C, Martin-Del-Rio R, Maechler P. Insulin secretion profiles are modified by overexpression of glutamate dehydrogenase in pancreatic islets. *Diabetologia* 2004, **47**(2)**:** 266-276.

34. Li C, Buettger C, Kwagh J, Matter A, Daikhin Y, Nissim IB*, et al.* A signaling role of glutamine in insulin secretion. *J Biol Chem* 2004, **279**(14)**:** 13393-13401.

35. Robertson RP, Harmon J, Tran PO, Poitout V. Beta-cell glucose toxicity, lipotoxicity, and chronic oxidative stress in type 2 diabetes. *Diabetes* 2004, **53 Suppl 1:** S119-124.

36. Pepper AR, Bruni A, Pawlick R, Wink J, Rafiei Y, Gala-Lopez B*, et al.* Engraftment Site and Effectiveness of the Pan-Caspase Inhibitor F573 to Improve Engraftment in Mouse and Human Islet Transplantation in Mice. *Transplantation* 2017, **101**(10)**:** 2321-2329.

37. 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**(11)**:** 1029-1035.

CHAPTER 6.

CONCLUSIONS AND FUTURE DIRECTIONS

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6.1 – ISLET TRANSPLANTATION: WHERE WE WERE, WHERE WE ARE AND WHERE WE WILL GO

Diabetes affects more than 200 million people worldwide, with projections that this scourge will affect 5% of the world population by 2025, and therefore represents a huge burden to global healthcare systems.[1] Of these, it is estimated that approximately 10% are diagnosed with T1DM. As the most severe form of this disease, T1DM is characterized as a chronic disorder resulting in the autoimmune destruction of insulin producing β-cells within the pancreatic islets of Langerhans. The discovery of insulin by Banting, Best, Collip and McLeod has indeed transitioned T1DM from a death sentence to a chronically manageable condition.[2,3] Despite optimal insulin therapy, many still suffer from progressive long-term complications including nephropathy, neuropathy, retinopathy, peripheral vascular disease and coronary artery disease. Such chronic micro- and macrovascular complications cause substantial morbidity and mortality. The Diabetes Control and Complications Trials (DCCT) investigated the outcomes of intensive insulin therapy[4-6] and elucidated that this therapy was able to partially mitigate cardiovascular disease, retinopathy and nephropathy, though a substantial increase in the number of adverse hypoglycemic events were evident.[5]

Insulin pumps, dynamic continuous glucose monitoring and closed loop systems have been developed as a means to tighten glycemic control and have contributed to improved glycemia, reduced hypoglycemic risk, and moderate improved protection from secondary diabetic complications. While these technological advances offer patients improved benefit, they continue to fall short as a definitive, robust cure for diabetes. Concerted efforts to ameliorate the symptoms and complications of diabetes

have spanned beyond administration of exogenous insulin to the restoration of β-cell mass through whole vascularized pancreas transplantation or with islet transplantation.

First attempted in 1966, whole pancreas transplantation was associated with dismal clinical outcomes.[7] Today, considerable advances in surgical technique, immunosuppressive strategies and management have made improvements to the safety and efficacy of this approach, though this procedure requires major intra-abdominal surgery with risk of complications and occasional mortality. Conversely, the transplantation of the islets of Langerhans offers a more attractive alternative as this procedure poses far less risk for patients as it is much less invasive.

Pivotal work by Paul E. Lacy, a pathologist by training, in the early 1970's established experimental islet transplantation as a potential means to correct hyperglycemia in diabetes. By isolating hypertrophic rodent islets enzymatically from obese rats, then later refining techniques to isolate hundreds of metabolically active, intact islets from normal rat pancreases[8], Lacy and Kostianovsky set the stage. Extracting high yields of islets from larger animals and humans proved far more elusive, however, as these pancreas organs are more dense and fibrous. The human pancreas is estimated to contain between 1-2 million islets.[9] While Lacy's work established the liver as an ideal site for islet transplantation[10], important work by John Najarian and David Sutherland demonstrated high early success rates with injection of unpurified human islet digests back into patients' own livers after total pancreatectomy for chronic pancreatitis. These autotransplants did not require immunosuppression, and were not subject to recurrent autoimmunity explaining their success. The infusion of unpurified islet digests into the portal vein occasionally led to fatal risk of complete portal vein

thrombosis however. The success rates of the first attempts at islet allotransplantation in patients with T1DM immunosuppressed with azathioprine and corticosteroids was exceedingly low by contrast.[11] Inroads in human islet isolation and purification came as a result of the development of the Ricordi chamber, developed by Camillo Ricordi in 1988, while working in Paul E. Lacy's laboratory in St. Louis. He introduced a semi-automated process that was instrumental in isolating and purifying large islet quantities, the principles of which constitute the basis of modern human islet isolation in routine practice today.[12,13] In spite of these milestones, <10% of the 267 islet transplant recipients completed up to 1999 were insulin independent for >1 year.[14]

The establishment of the Edmonton Protocol by Shapiro and colleagues at the University of Alberta significantly contrasted previous clinical islet transplantation protocols in two distinct manners: (*i*) by utilizing an immunosuppressive protocol void of corticosteroids and use of potent immunosuppression with combined sirolimus, tacrolimus and anti-CD25 antibody to protect against rejection and recurrent autoimmunity; and (*ii*) increasing the initial islet mass (>13,000 islet equivalents (IE)/kg recipient body weight) derived from two fresh islet preparations.[15] As a consequence of this refined approach, seven consecutive patients receiving an islet transplant achieved insulin independence up to one year post-transplant, a feat unprecedented at the time. Early follow-up reports revealed that full insulin independence was not durable, with approximately 11% of patients maintaining sustained insulin independence by 5 years posttransplant, although approximately 70% of grafts maintained more than sufficient function at 5 years to protect against severe hypoglycemic events (SHEs), to correct HbA1C ≤6.5% with detectable human C-peptide production of transplant cell origin.[16]

Certainly, modifications in islet transplantation over the decade have revealed improvements in short- and long-term outcomes. As per the 2014 CITR report, 864 islet allotransplants have been completed, with insulin independence achieved in 80% of patients after initial or subsequent infusions. Five year follow-up rates have also improved as a result of T-cell depletion induction and TNF anti-inflammatory treatment, with approximately 50% of patients remaining insulin independent post-transplant.[16-18]

The Edmonton group has reported that the majority of patients (approximately 70%) exhibit maintained C-peptide secretion post-islet transplantation, with stabilized glycemic control and the elimination of hypoglycemic unawareness. At the University of Alberta, insulin independence is maintained in approximately 25% of patients at five years post-transplant. Perhaps more importantly, these rates double to approximately 50% in those receiving newer T-depletional induction, immunosuppression and combination anti-inflammatory induction protocols. The precise cause of islet graft deterioration is rarely if ever defined on an individual case-by-case, but multiple culprits likely contribute to graft deterioration over time. To achieve insulin independence, most patients require two islet transplant procedures, and in some cases, three infusions. The achievement of insulin independence after single-donor islet infusion has occurred in few transplant centers globally, with the most success reported from the University of Minnesota.[19,20] In these series, patient selection bias toward low body weight and low baseline insulin requirement contribute to higher rates of single donor islet engraftment success. While the islet infusion risk has improved considerably over time, and risk of portal vein thrombosis or life-threatening bleeding is exceedingly low in larger centres, the perceived risks associated with long-term immunosuppression, post-transplant

lymphoma, life-threatening sepsis and malignancies continue to limit broader application of islet transplantation in those with less severe forms of diabetes including children.[21]

The Clinical Islet Transplant (CIT) Consortium recently reported results from their license-enabling, multicenter phase 3 clinical trial (CIT-07; NCT00434811).[22] Designed with a primary endpoint evaluating safety and efficacy in islet transplant recipients, participants included patients with T1DM for at least 5 years duration and persistent impaired awareness of hypoglycemia and SHEs despite expert physician management for 1 year prior to enrollment. The primary end-point was the achievement of HbA1c <7.0% at day 365 and freedom from SHEs from day 28 to day 365 after the first islet transplant. The positive trial findings support a Biological Licensure Application through the Food and Drug Administration (FDA) as the primary endpoints were successfully achieved, with 87.5% of subjects at 1 year and by 71% at 2 years post-transplant achieving a median HbA1c level of 5.6% at both 1 and 2 years. Moreover, dramatic improvements in Clarke and HYPO scores were achieved, thus confirming a restoration of hypoglycemia awareness. Nevertheless, these results are critical in establishing islet transplantation as a real, safe clinical therapy in the United States, as it is currently supported through scarce research funding at limited institutions nationally.

From its early inception, to its establishment as a safe and efficacious therapy, improvements in clinical islet transplant outcomes have been observed over the last three decades. However, as noted, considerable refinements are still required to

overcome the obstacles impeding long-term outcomes, as well as single-donor transplant success.

Herein, I provide areas of focus from the body of work presented that contribute to some of the shortfalls observed in islet transplantation and present interventional strategies to overcome these obstacles. The chapter also shares new areas of interest that will expand the availability of cellular sources for β-cell replacement therapy, mechanisms to induce tolerance in the recipient to avoid the need for chronic, systemic immunosuppression, as well as genetic approaches to immunologically reset T1DM disease onset. The chapter concludes with final remarks.

6.2 – IMPROVING ISLET ENGRAFTMENT - CONSIDERATIONS FOR THERAPEUTIC INTERVENTION

6.2.1 – Donor criteria

To achievable durable rates of insulin independence, it is widely accepted that multiple islet infusions are required to ameliorate the consequences of brittle T1DM in most islet transplantation centers globally. A considerable burden is placed on the availability of quality donor pancreases suitable for islet transplantation, and as such donor selection is an important consideration to improve engraftment outcomes. Retrospective studies at single centers have identified several donor-related variables that may contribute to islet isolation and transplantation outcomes such as donor age, cause of death, and body mass index (BMI), among others.[20,23-29] O'Gorman et al developed a scoring system based on donor characteristics that can predict islet isolation

outcomes.[30] This tool has proven useful in determining whether an organ should proceed to islet isolation, given that the isolation process is involved and costly.[31] It should be noted, however, that this donor scoring system is not a predictive tool for transplantation outcomes.

A prospective means to broaden the donor pool for islet transplantation is the utility of donation after cardiac death (DCD) organs. To date, the success of DCD islets in the context of clinical islet transplantation has been evaluated at few single centers worldwide, with limited long-term successful outcomes[32-34] Japan, at one point, held the most extensive experience using DCD donors for organ transplantation, contributing to optimizing organ retrieval techniques, as well as establishing the Kyoto preservation solution and an oxygen-carrier two-layer preservation method.[35] In 2014, Anazawa and colleagues from the Japan Islet Transplant Registry evaluated long-term clinical islet transplantation outcomes using pancreases from uncontrolled DCD donors, whereby 18 subjects with T1DM collectively received 34 islet transplants from sixty-four isolations, with a mean follow-up of 76 months. The results of this study showed relatively low rates of C-peptide survival over time (30% by 5 years), with low rates of insulin independence achieved in only three recipients.[33]

The DCD experience here at the University of Alberta's Clinical Islet Transplant Program has eclipsed that of the Japan Islet Transplant Registry, and has now become the site with the most experience with DCD pancreases. To date, the CIT Program has performed 47 islet isolations, 20 of which have resulted in an islet transplant (data provided by Dr. T. Kin). In 2016, Andres and colleagues shared a report highlighting the DCD experience at the University of Alberta.[36] Fifteen human DCD pancreases that

experienced a maximal warm ischemia limit of 30 minutes exhibited no discernable difference in islet yield in comparison to standard neurological determination of death (NDD) pancreata. Examination of insulin requirement one month post-transplant in recipients of islets from NDD or DCD pancreases revealed no significant difference between groups.[36] While the earlier long-term results from the Japanese experience may be discouraging, available long-term data from Alberta suggests that DCD outcomes are equivalent to NDD islet transplants. Until then, pre-clinical models may serve as useful tools to evaluate the utility of DCD and extended criteria pancreases in islet transplantation.

In Chapter 3, we established a murine DCD model and sought to evaluate whether administration of BMX-001 during organ procurement could augment islet yield, *in vitro* function after a brief culture period, as well as improve engraftment in a syngeneic, marginal transplant model. Our study revealed that the translation of rodent DCD models to the clinical setting is quite limited, as a 15-minute warm ischemic (WI) period significantly impaired islet yield relative to standard murine islet donors that did not experience WI. In a 30-minute WI rat islet isolation model, Avila and colleauges also observed a significant reduction in islet yield immediately post-isolation.[37]

This contrast in islet yield post-isolation in the pre-clinical setting relative to clinical findings noted above may be attributed to the metabolic differences between rodents and large mammals, like humans. Giraud *et al* revealed that 30 minutes of WI in humans is equivalent to 3.5 minutes of WI in mice.[38] It is far more likely however that the intraductal administration of collagenase did not percolate through the entire pancreas at the time of distension, thus affecting islet yield, as the pancreatic capsule in

mice is gossamer thin, and does not tolerate short periods of ischemia. Moreover, the digestion time utilized in the protocol was optimized for standard murine isolation procedures. It may be possible that the time of digestion was insufficient in our DCD model and required further optimization to permit an increased islet yield per pancreas. As such, pre-clinical evaluation of DCD islet isolation and transplantation certainly does not accurately translate to the clinical setting. To more accurately represent a clinical DCD model in the pre-clinical setting, large animal studies utilizing pigs may be more translational, albeit carrying far greater costs and technical expertise. We currently therefore do not have any reliable small animal model of DCD donation that satisfactorily corresponds to the clinical scenario.

The utility of DCD and extended criteria donors for islet transplantation have a potentially greater capacity to be utilized in the clinic as a result of commercially available cold pumped or normothermic machine perfusion systems. These systems, which were initially utilized to preserve kidneys from marginal donors, have gained considerable acceptance in all other solid organs.[39] They offer considerable advantages to typical cold storage conditions, as machine perfusion can mimic physiologic, normothermic conditions, as well as permit continuous perfusion of preservation solutions at homeostatic pressures and flow rates. Perfusion also permits the measurement of biomarkers from the circulating perfusate, as means to assess organ quality prior to transplantation. Perhaps among the numerous attractions of *ex vivo* perfusion is the permissibility of delivering therapeutic agents to the solid organ during perfusion as a means to preserve organ viability prior to transplantation.[40-42] Utilizing *ex vivo* organ perfusion machines as a means to preserve organ survival prior to islet

isolation and transplantation is certainly an attractive option. Further work in the pre-clinical setting will prove instrumental in determining its benefit in the clinical realm, and may indeed offer the added benefit of preserving the quality of marginal organs thus expanding the availability of organs for islet transplantation.

6.2.2 – Islet isolation & culture

The pancreas itself is a unique organ in that it is composed of multiple cell types of exocrine and endocrine origin. As a result of the multi-dimensional composition of the pancreas, enzymatic digestion and mechanical agitation of the pancreas is required to dissociate the islets from the exocrine pancreas. Successful islet transplantation is initially contingent on the isolation of high islet yields, ensuring that this process inflicts significantly minimal damage. Subsequent to isolation, islets are then purified by density gradient centrifugation.[43] Poor islet isolation outcomes, including reduced yield (number of islets isolated from the pancreas), decreased purity (excessive exocrine tissue) and prolonged enzyme exposure can lead to loss of islet viability and integrity.[44] The post-isolation culture period is critical for recovery from isolation-induced damage. As such, nutrient supply and sufficient oxygen are paramount during this culture period as a means to circumvent significant islet mass loss. Indeed, culture conditions have been shown to vary between islet isolation centers and despite considerable research efforts aimed to delineate optimal culture conditions, protocols have yet to be standardized.[45]

As noted in **Chapter 2 and Chapter 3**, the use of antioxidants during islet isolation and culture is a rational approach given that islets are susceptible, in part, to

oxidative injury which can be generated during these periods. Islets are also especially sensitive since they exhibit reduced endogenous antioxidant levels relative to other tissues.[46] The overwhelming imbalance between free radical production relative to endogenous antioxidant expression results in oxidative stress, thus driving the translation of pro-inflammatory cytokines (TNF- a, IL-1β, IL-6, and IFN-γ), as well as chemokine synthesis.[47,48] Utilizing antioxidants to bolster host defenses, including metabolites, vitamins, trace elements, herbal products and enzymatic antioxidants as a means to ameliorate the aforementioned deleterious events has been approached in islet isolation and transplantation. To this end, our group previously published findings whereby glutathione-ethyl-ester administration during murine pancreas procurement and culture could increase viability, protect from apoptosis and promote engraftment.[49] The delivery of cytoprotective agents prior to islet isolation is an attractive option to mitigate the deleterious events associated with isolation-associated oxidative injury. Current clinical trials here at the University of Alberta aim to deliver cytoprotective agents, such as BMX-010, directly to the islet microvasculature via the pancreas, using the splenic or superior mesenteric artery shortly after donor aortic cross-clamp and preservation solution flush. Again, on arrival of the pancreas at the GMP facility, the vasculature is again flushed with cytoprotective agent to maximize delivery to the islet microvasculature. It is anticipated that uptake of a drug will confer greater protection than delivery during the culture period alone or subsequent to transplantation when delivered to the recipient.

These observations provided the rationale for utilizing the catalytic, SOD-mimetic, BMX-001, in islet isolation and transplantation. Our results demonstrated that

when administering a physiologic dose of 34μM improved *in vitro* islet function, reduced cell death and augmented engraftment in a syngeneic, marginal transplant model. These observations are likely attributed to reduced ROS production, as established in **Chapter 3**, thus preserving pre-transplant islet potency in the acute transplant period. BMX-010, an earlier generation manganese SOD-mimetic has been previously evaluated in the clinic, and exhibited a limited ability to enhance islet isolation yield or engraftment outcomes relative to standard isolation.[50] Our human islet data comparing BMX-010 and BMX-001 revealed greater cytoprotection *in vitro* with BMX-001. Therefore, its application in the clinical setting could enhance isolation and transplant outcomes. It is conceivable that the administration of cytoprotective agents, such as metalloporphyrins, during islet isolation and culture can decrease the production of free radicals and aberrant downstream inflammatory cascades, reducing the incidence of primary non-function and prospectively increase the incidence of insulin independence from single-donor islet infusions. A main difference in our pre-clinical islet isolation model is that we relied on ductal delivery of BMX-001 to murine pancreases, as venous delivery is less feasible in mice due to their size and would present additional technical challenges.

6.2.3 – The subcutaneous space as an alternative to intraportal islet infusion

Despite being the only utilized efficacious transplant site clinically, infusion of islets into the hepatic portal vein may compromise long-term islet engraftment. Survival and function of islets post-infusion is reliant on numerous factors to maximize survive post-transplantation. Islets must adequately engraft by developing new capillaries and

vessels with the native islet vasculature. The engraftment process initiates 1 to 3 days post-transplant, with initial neovascularization occurring at days 10 - 14 post-transplant, whereby the new network develops from the recipient's blood vessels in concert with the remnants of the donor islet endothelium.[51] Further microvascular remodeling occurs over several months post-transplant. During this time, islets are subjected to considerable environmental stresses within the first days of transplant that can contribute to a reduction in the original islet mass by 50 – 70%.[44,51,52]

The instant blood-mediated inflammatory reaction (IBMIR) is the primary culprit that accounts for robust islet loss in the acute transplant period. IBMIR negatively influences islet engraftment through a cascade initiated by the expression of tissue factor, resulting in platelet adherence, activation, clot formation and lymphocyte recruitment.[53,54] Considerable work by Korsgren *et al* have elucidated that IBMIR is initiated upon infusion of islets into the portal vasculature.[55] These results suggest that despite being the current clinically utilized site for islet transplantation, the portal vein presents itself as a less than ideal environment for transplantation.

Considerable work in the Shapiro laboratory, and others, are exploring the subcutaneous space through control of the foreign body response to prevascularize this site. However, it should be clearly noted that the hepatic portal vein is the only site that to date has reliably led to high rates of early insulin independence, and this has been very difficult to achieve in every other site tested. Recently, one promising alternative to the portal vein was explored by the Miami group, whereby a degradable biological scaffold was created using the greater omentum and human plasma/thrombin matrix.[56] Initial reports in their first subject appeared promising, with insulin independence

achieved at 17 days post-transplant, and marked improvements in various efficacy measures including mixed meal tests and BETA-2 scores up to 6 months post-transplant. However, a marked functional decrease was observed at 12 months post-transplant, suggesting that further work is required to optimize this site as a surrogate to portal vein infusion. The clinical utility of any alternative transplant site will need to meet or exceed the efficacy attained with the current gold standard of intraportal islet infusion if it is to be widely accepted.

As noted, intraportal islet infusion currently serves as the clinical site of transplantation, and routinely regulates glycemic control in subjects with brittle diabetes.[57] While the procedure itself is minimally invasive, it is not void of procedural risks and complications, including bleeding to portal vein thrombosis.[58] Moreover, the initiation of inflammatory cascades through platelet activation and coagulation can confer significant islet loss. These events have propagated considerable experimental and clinical interest in identifying an alternative anatomical site for clinical islet transplantation. It is proposed that the ideal site for islet transplantation and subsequent engraftment should be in close proximity to vascular networks that supply physiologically sufficient oxygen and nutrients, as well as provide ample space to accommodate the necessary transplant volume. Furthermore, a site that can be easily monitored and is retrievable is also desirable, particularly if insulin-producing stem cells are to be transferred into clinical practice.[59]

The translation of alternative transplant sites from experimental models to clinical practice has been met with considerable limitations. For example, the kidney capsule has demonstrated the ability accommodate a smaller islet mass capable of

restoring euglycemia in rodent models, however when translated to humans, this site has failed to achieve success. Clinical experience using the kidney capsule has been limited to one trial whereby Groth *et al* transplanted fetal porcine islet cell clusters transplanted under the renal capsule of a T1DM patient. While circulating porcine C-peptide was present, neither insulin independence nor the restoration of euglycemia were achieved. Moreover, from an anatomic perspective, the durability of the kidney capsule in mice is not as pronounced in humans, and as such, does not separate from the kidney to feasibly permit transplantation of human islets in this site. Numerous additional transplant sites have been explored experimentally, including the spleen, muscle and immune privileged sites, however, these sites have not conferred significant benefit. Within recent years, the subcutaneous space has garnered significant attention as an ideal site for islet transplantation for multiple reasons, including the ability to accommodate large transplant volumes, is easily accessible, and retrievable, if required.[60] A primary limitation to the subcutaneous space is that it presents with poor blood supply, relative to the portal site, of which could compromise islet viability in the acute transplant period. As such, the utility of subcutaneous devices to promote neovascularization prior to islet infusion have been explored, and in some cases have been met with limited clinical success.[61] Our laboratory has established an effective means to exploit the foreign-body response to create a vascularized network in the subcutaneous space by the temporary transplantation of an angiocatheter up to four weeks post-implantation.[60] Removal of the angiocatheter extinguishes the foreign body response, and the resulting void space is capable of accommodating the survival of human islets as well insulin-producing stem cells.[60,62] This modified device-less (DL) approach eliminates the need for long-term

implantation of a subcutaneous device, while sufficiently correcting hyperglycemia in rodent models. Strategies aimed to utilize the subcutaneous space as an alternative site for islet engraftment holds significant promise for current and future clinical applications. Indeed, while less invasive, the clinical efficacy of the DL site as an alternative to intraportal islet transplantation remains unknown and warrants investigation. As a retrievable site, the DL approach holds potential promise for insulin-producing stem cell transplantation, as concerns of teratoma formation exist, and retrievability of such transplants may be an early pre-requisite for clinical testing.[60,62] Stem cell delivery via the intraportal route might require major liver resection should teratoma or other uncontrolled function or growth occur, which may be difficult to secure approval by ethics boards and regulatory bodies for early pilot trials, at least until more safety data is accrued.

In Chapter 4, we evaluated the utility of the pan-caspase inhibitor, F573, in murine and human islet transplants in syngeneic and immunocompromised murines recipients, respectively. We evaluated the efficacy of this drug in promoting engraftment in various transplant sites, including the kidney capsule, portal vein and DL site. The administration of F573 *in vitro* and to the recipient was required to permit sufficient cytoprotection to augment islet engraftment outcomes. Of particular importance, the administration of F573 improved syngeneic engraftment in the DL site. These results suggest that the subcutaneous site, while attractive for numerous reasons, is not void of compromising engraftment despite modification prior to transplant and that therapeutic intervention may be required in this setting to further enhance islet survivability (or other cellular sources) in the subcutaneous space.

6.2.4 Regulated Cell Death – Implications in Islet Transplantation

Therapeutic strategies to ameliorate islet cell death in the acute and peri-transplant period provide an attractive approach to preserve early islet mass, potentially improving long-term engraftment outcomes. As previously noted, apoptosis has been the most extensively described form of regulated cell death in numerous pathologies and diseases, and within the context of islet transplantation, apoptosis has been identified as a contributor to cellular demise. Therapeutic strategies that subvert the initiation and consequences of apoptotic cell death have shown promise in pre-clinical models, with particular emphasis from the Shapiro laboratory evaluating a plethora of anti-apoptotic agents, including results presented in **Chapter 4**.[63,64,65,66,67] However, the recent identification of novel, alternative regulated cell death pathways that are morphologically and biochemically distinct from apoptosis has opened new avenues of therapeutic intervention in other organ models, including renal ischemia reperfusion injury.[68-70] Given that some of the key mediators that contribute to these regulated cell death pathways have also been implicated in islet dysfunction and demise, it is likely that these pathways may also have substantial relevance in islet transplantation, despite not being explicitly identified to date.

In **Chapter 5**, we examined whether human islets were susceptible to ferroptosis, as this regulated cell death pathway had yet to be clearly defined in context to islet dysfunction and loss. Ferroptosis, an iron-dependent form of non-apoptotic cell death, was first described in parallel to the identification ferrostatin-1 (Fer-1), an inhibitor of this cell death pathway that functioned to prevent erastin-induced cell death.[68-71] The small potent molecule, erastin, exhibited the selective ability to inhibit the X_c^-

cystine/glutamate antiporter required for glutathione (GSH) biosynthesis, thus initiating this cell death cascade. [69,71] The subsequent depletion of GSH, compromises the function of glutathione peroxidase 4 (GPX4), the GSH-dependent, lipid repair enzyme resulting in aberrant downstream accumulation of reactive oxygen species (ROS).[69,71-74] As previously noted, relative to other tissues, islets exhibit reduced antioxidant defences, and as a result are susceptible to the dysregulation of free radical production and subsequent oxidative stress.[75,76] Prior pre-clinical studies have revealed the importance of GSH in preserving islet viability and function, as GSH has been implicated as a crucial antioxidant capable of alleviating oxidative stress in islets.[49] Moreover, lipid peroxidation byproducts have exhibited the ability to impair glucose-induced insulin secretion[77], and when elevated, fatty acids impair insulin gene expression, glucose-stimulated insulin secretion, and increase cell death.[78] In contrast, the administration of GSH precursors have demonstrated improved insulin secretion in response to glucose, as well as reduce lipid peroxidation levels.[79-81] These observations were instrumental in forming the rationale for exploring whether ferroptosis can be initiated in human islets. Results from our study revealed that the ferroptosis-inducing agents, erastin and RSL3, could indeed induce islet cell death, as evidenced by increased LDH release. Further, islet function was impaired when assessed by sGSIS in the presence of these agents. Pre-treatment with Fer-1, the ferroptosis-specific inhibitor resulted in rescued islet function and viability, further suggesting that ferroptosis can contribute to islet dysfunction and demise.

Indeed, the results from our study suggest that alternative cell death pathways, aside from apoptosis, may contribute to cellular dysfunction and substantially

compromise engraftment outcomes. Given that various extracellular stimuli have demonstrated the ability to induce multiple cell death modalities, it is more than plausible that considerable crosstalk exists among these pathways. Examples in other transplant modalities reveal that mediators of some regulated cell death pathways may have implications in other programmed cell death mechanisms. For example, RIPK3 a key mediator of necroptosis has been implicated in the processing of pro-IL-1β as a result of promoting the NLRP3 inflammasome, independent of necroptotic cell death.[82] Moreover, the death receptors of the TNF superfamily, including TNF receptor-1 and CD95, also called Fas, have been implicated in the activation of the initiator, caspase-8, which in turn cleaves and activates the executioner caspases, thereby driving apoptosis.[83] However, the binding of TNF to TNFR1 in concomitant inhibition of caspase-8 has also demonstrated the ability to induce necroptosis.[84] Given the shared upstream elements of apoptosis and necroptosis, both pathways are tightly regulated and it is now believed that the most important role of caspase-8 is the prevention of necroptotic cell death.[4,85,86] The introduction of therapeutic agents aimed to ameliorate broad caspase activation should be approached with caution, as the inhibition of caspase-8 may mitigate the onset of apoptosis, but consequently initiate necroptosis. Further evidence has also linked the necroptosis and ferroptosis pathways. The once perceived necroptosis-specific inhibitor, Nec-1, has also demonstrated the capacity to inhibit ferroptosis, prospectively suggesting implications in off-target, to be determined mechanisms.[87] It is also unknown exactly how important the signaling from injured and ischemic transplanted islet cells may be in recruiting neovascularization to secure long-term functional survival. Within the context of islet transplantation, cross talk of the

various regulated cell death pathways has yet to be fully elucidated, but certainly warrants further investigation, as potential key mediators linking these pathways could be used as interventional targets. Considerable pre-clinical and clinical evidence suggests that inflammation plays a critical role leading to β-cell dysfunction and death.[88,89] The inflammatory cytokines, TNF-α and IL-1β, among others, have demonstrated the capacity to induce considerable islet dysfunction, leading to islet cell death and diminished engraftment outcomes.

Therapeutic strategies aimed to subvert the deleterious consequences of such inflammatory cytokines have been met with considerable success within pre-clinical and clinical investigation. In a murine, syngeneic islet transplant model, Farney and colleagues demonstrated improved engraftment outcomes as a result of TNF-α blockade.[90] Concomitant Anakinra and Etanercept administration in rodent islet transplantation models from our laboratory also potentiated engraftment outcomes, suggesting that inhibiting the actions of these cytokines in the acute transplant period likely mitigates downstream signaling cascades. In a single-donor clinical transplant protocol utilizing Etanercept, Hering *et al.* achieved insulin independence in all 8 patients transplanted.[91] While encouraging, caveats exist in that the study was underpowered, subjects received substantial transplant mass of 7271 ± 1035 IE, and patients weighing >70 kg (150 pounds) were excluded. To put this into context, patients enrolled here in the Clinical Islet Transplant Program, are excluded if their body mass index exceeds 35 kg/m^2 at the time of screening, which roughly translates to 112 kg (260 pounds) for a 183 cm (6 feet) tall individual. Therefore, the translation of these findings may be limited. Bellin *et al.* also revealed that islet transplant recipients

receiving an induction therapy T-cell depleting antibodies with TNF-α-inhibition (TNF-α-i) exhibited significantly improved insulin-independence rates up to 5 years post-transplant than recipients who did not receive TNF-α-i, regardless of maintenance immunosuppression.[17] These results clearly suggest that inflammatory cascades in the acute transplant period impact engraftment outcomes, and ameliorating the consequences associated with these events is an imperative strategy to support single-donor success. Indeed, as regulated cell death pathways become more defined in the context of islet transplantation, targets for therapeutic intervention can be identified to promote islet viability and deter deleterious events that may contribute to islet dysfunction thus compromising engraftment outcomes.

6.3 – ALTERNATIVE β-CELL REPLACEMENT THERAPIES

As previously noted, the major limitation to broader application of islet transplantation is both a need for chronic high-dose immunosuppression and a serious shortage of donor pancreas organs. The organ donor supply cannot possibly match the global burden of diabetes. According to the World Health Organization, in 2014 it was estimated that 422 million people suffered from diabetes globally.[92] For this same year, 27,396 deceased organ donors were registered in the Global Observatory on Donation and Transplantation.[93] If one were to assume that all pancreases from these donors were fit for islet transplantation, a meager 0.0065% of the global diabetic population (suffering from either T1DM or T2DM) could receive an islet transplant derived from a single donor. Expressed in an alternative manner, if the incidence of diabetes, as well as the number of organ donors remained fixed, it would take 15,403 years to transplant the

current global diabetic population. A major priority remains securing a less limited, alternative source of insulin-producing cells if patients with diabetes are to be more broadly treated with cellular therapies.

Throughout this thesis, I have sought to identify mechanisms that contribute to islet cell dysfunction and death, as well as identify prospective therapeutic treatments that can augment islet engraftment outcomes. While most of these efforts have occurred in pre-clinical rodent models, the relevance of these therapeutic agents in clinical islet transplantation remains to be investigated. Given that multiple islet infusions are consistently required to confer clinical benefit, and islet transplantation is limited, in part, by the availability of available donor pancreases, it is evident that if ***all*** patients with T1DM are to be offered cellular replacement, development of alternative, more ubiquitous insulin-producing cellular source is imperative.

6.3.1 – Xenotransplantation

Since numerous islet infusions are often required to correct the consequences associated with T1DM, identification of a xenogeneic supply of islets may be an attractive option to circumvent need for human donor pancreases. This approach could provide an unlimited source. One such xenogeneic option is the pig, a widely available source capable of producing insulin that is functional in humans. In 1994, Groth *et al* reported a first-in-human series where fetal porcine islet cell clusters were transplanted beneath the renal capsule of patients undergoing kidney transplantation. Clusters were capable of surviving in the human body as evidenced by detectible levels of porcine C-

peptide in the urine, but no improvement in euglycemia or insulin requirement was observed.[94]

Despite limited clinical success within the last decade, the testing of porcine islets in non-human primate models has generated considerable interest. Notably, improvements in aggressive immunosuppressive protocols combined with genetic manipulation of the pig genome has improved success.[95-99] Notably, in immunosuppressed diabetic non-human primate studies where wild-type or genetically-engineered porcine islets have been transplanted, achievement of normoglycemia has been observed for >1 year post-transplant.[100,101] This is an impressive accomplishment when one considers that xenografts would only last minutes to hours a decade or two ago. Indeed, considerable efforts are currently being undertaken to establish highly monitored clean pig colonies to eliminate the potential for the transmission of zoonotic infections or endogenous retroviruses.[99,102,103]

As a means to subvert the xenogeneic immune response, efforts to transplant encapsulated neonatal porcine islets in non-immunosuppressed diabetic patients is currently underway in New Zealand. The results from this clinical trial revealed modest if any detectible clinical function, as only a marginal reduction in hypoglycemic unawareness was reported.[104] Results from this initial trial resulted in a second nationally regulated clinical trial in Argentina which claimed improvement in HbA1c and reduced hypoglycemic unawareness events up to 2 years post transplant, though only a marginal reduction in insulin dose was observed.[105] The universal lack of detectible porcine C-peptide in these studies to date raises major concern that the porcine cells may be providing little or no useful function in patients. Until this issue is

resolved, outcome data must be interpreted with great caution. While transmission of porcine endogenous retroviruses has been raised as a potential concern, this at least does not seen to have been an issue in any of the clinical trials to date.[100,105,106] Further refinements in porcine islet xenotransplantation are necessary if these pilot trials are to advance to larger-scale clinical trials.[99] To date, results do not match whatsoever with those routinely obtained by clinical islet transplantation using allogeneic donor cells.

While efforts to encapsulate porcine islets are being pursued, genetic modification of porcine islets is an important alternative, strategy that may subvert the xenogeneic response. Avoidance of IBMIR and accelerated xenogenic destruction by elimination of galactose-α-1,3-galactose (α-Gal) epitopes was introduced by David White *et al* as a means to overcome a major target for primate anti-porcine antibodies. Elimination of α-Gal, and generation of α-1,3-galactosyltransferase gene knockout (GTKO) proved useful in subverting chronic rejection.[107] Human glycoprotein decay accelerating factor (DAF, CD55) is responsible for the regulation of complement activation, and was identified by White *et al* as a major target in xenotransplantation. Expression of CD55 may inhibit complement-mediated damage of transplanted porcine islets, though results reported by Mandel and colleagues in a renal subcapsular monkey study did not reveal any detectible benefit.[108] In 2007, Rood *et al* were the first to report the transplantation of islets from GTKO pigs into the portal vein of cynomolgus monkeys. Results indicated that GTKO islets exhibited similar *in vitro* and *in vivo* functionality compared to wild-type porcine counterparts.[109] Further work by Thompson *et al* transplanted GTKO neonatal porcine islets transplanted into the portal vein of Rhesus monkeys and 4 of 5 recipients became euglycemic, ranging 50 – 249 days post-

transplant. These data suggest that GTKO islets are more resistant to the deleterious effects associated with IBMIR.[110] CD46 is an additional human protein target that could potentially mitigate complement-mediated porcine islet destruction in primates. Van der Windt *et al* transplanted porcine islets expressing humanized CD46 (hCD46) into diabetic Cynomolgus recipients and reported long-term islet function up to 346 days, the longest survival of xenoislets a non-human primates to date. While hCD46 was unable to preserve initial islet mass loss from IBMIR, histological assessment liver biopsies revealed more viable islets than controls.[98] The above studies suggest that expression of humanized proteins on porcine islets may indeed augment engraftment outcomes in xenotransplantation.

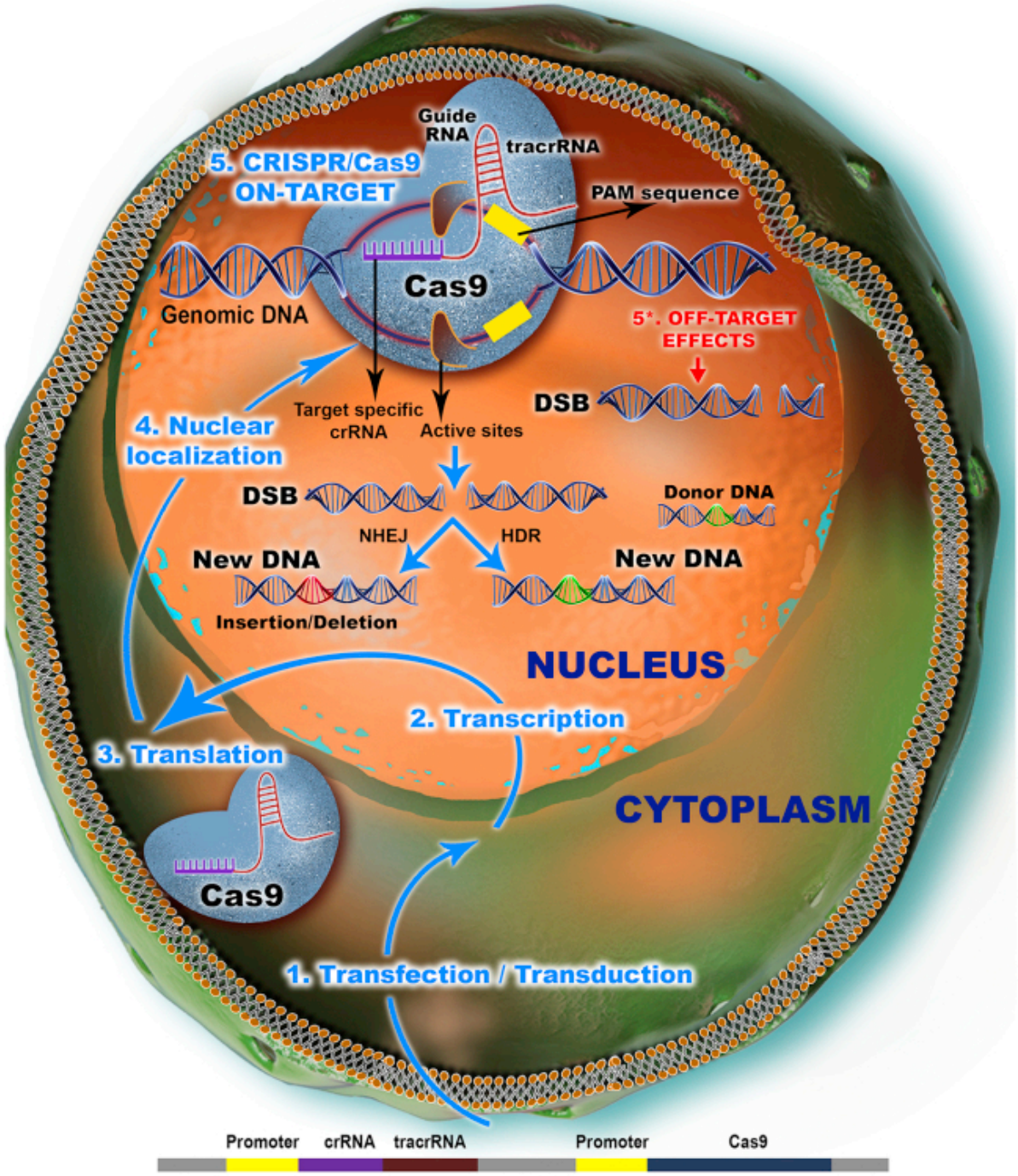
In 1987, Ishino *et al* observed DNA repeats with unknown function in the genome of *Escherichia coli*.[111] Mojica *et al* termed these repeats Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR).[112] While initially their function was unknown, these CRISPR sequences were later identified as an adaptive, anti-viral immune response by the bacterial host, whereby viral DNA could be excised from the nucleus through Cas nucleases.[113] Such gene editing processes involve generation of a double-stranded break within a targeted DNA sequence. The double-stranded break subsequently triggers homology-directed repair, with homologous recombination of a donor DNA sequence with precise insertion of specific sequences in the targeted locus (i.e. insertion of a DNA sequence encoding a desired trait) (**Figure 6.1)**. An attractive feature of CRISPR/Cas9 system is that it is site-specific, provides the opportunity to delete multiple genes simultaneously and is relatively inexpensive compared with other

gene modification systems. Moreover, the utility of CRISPR/Cas9 has remarkable potential application in xenotransplantation.

Early application of CRISPR technologies have already proven useful in xenotransplantation, and Niu *et al* demonstrated inactivation of porcine endogenous retrovirus (PERV) in pigs with this approach.[114] The authors eliminated PERV from the FFF3 cells by mutating the *pol* gene, and successfully inactivated 25 copies of this gene. The *pol*-mutated FFF3 cells were used subsequently for generation of embryos by somatic cell nuclear transfer, with 20 – 40% efficiency, typical of porcine cells. Embryos were implanted into surrogate sows. Despite a slightly reduced pregnancy rate, the number of piglets born per embryo implanted was similar between PERV-inactivated and wild-type cells. Moreover, from 17 surrogate sows, 37 PERV-inactivated piglets were born, 15 of which remained alive. At the time of this report, piglets were 4 months old and remained PERV-free. The results from this groundbreaking experience could potentially accelerate utility of xeno-derived islets in the clinic, eliminating one of the remaining impediments. One important factor to consider, however, is whether these PERV-inactivated animals are completely protected from PERV infection. A conceivable next step would be to shelter PERV-inactivated and wild-type pigs together to confirm transmission of PERV is completely abrogated.

The emergence of CRSPR/Cas9 technology will likely generate interventional strategies in xenotransplantation that will deliver more compatible, humanized pigs for transplantation for both solid organ and cellular transplantation, including islets. However, as with the establishment of any technology that permits genetic manipulation of a living organism, ethical issues warrant careful consideration. CRISPR/Cas9 genome

editing has the potential to generate chimeric organisms, defined as an organism whose composition consists of two or more cellular entities from the same or different species.[115] If the surrogate host contains humanized elements, it may be difficult to differentiate how "human" the surrogate host is. As such, this raises ethical concerns relating to handling, use and disposal of the surrogate, as animal or as human. Genome editing of human embryos also raises concerns with respect to unintended off-target mutations. Off-target mutations can occur when CRISPR/Cas9 cleaves homologous DNA sequences to that of the intended DNA target. It has been demonstrated that off-target mutations can cause transformation, or even cell death.[116] These ethical considerations require open dialogue with the public, ethicists, government entities and the scientific community if guidelines and policies are to be established that are mutually acceptable, and applied broadly, but certainly this technology holds considerable promise in accelerated introduction of xenotransplantation in the treatment of human disease.


Guide RNA
tracrRNA
5. CRISPR/Cas9 ON-TARGET
PAM sequence
Cas9
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5*. OFF-TARGET EFFECTS
DSB
Target specific crRNA
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DSB
Donor DNA
NHEJ
HDR
New DNA
New DNA
Insertion/Deletion
NUCLEUS
2. Transcription
3. Translation
CYTOPLASM
Cas9
1. Transfection / Transduction
Promoter
crRNA
tracrRNA
Promoter
Cas9

Figure 6.1 CRISPR/Cas9 Mechanism of Action

The original bacterial CRISPR/Cas9 design has been translated into an engineered instrument for genome editing purposes and is capable of introducing specific modifications in the target cell. In this regard, the vector comprising the crRNA and tracrRNA that together constitute the RNA molecule for Cas9 guidance (gRNA) is introduced in the desired cell, where it passes the cytoplasmic milieu toward the nucleus. After delivery to the nucleus, the Cas9 gene encoded by the experimental vector is transcribed and exported into the cytoplasm for translation of Cas9 nuclease. After synthesis of the active protein, the gRNA, transcribed by its own promoter, interacts with the Cas9 nuclease, resulting in the ribonucleic-protein effector complex that is internalized back into the nucleus. The cleavage of the double-stranded genomic DNA takes place in a guided manner, where the crRNA sequence of gRNA directs Cas9 toward the specific locus, based on sequence complementarity, which is positioned adjacent to the PAM. When cleaved, the continuity of the host DNA can be restored through NHEJ, where the hanging ends join together, creating small indels, or through HDR in the presence of a donor DNA. (Reproduced with permission from Chira *et al., Mol Ther Nucleic Acids*. 2017 Jun 16; 7: 211–222).

6.3.2 – Stem Cell Transplantation

Inroads in developmental biology and regenerative medicine have contributed in a major way to research efforts currently underway to identify suitable islet precursor cells. Given that donor criteria, pancreas digestion and islet isolation can all contribute to compromised islet engraftment outcomes and that the identification precursors with the potential to differentiate into an unlimited source of insulin-producing β-cells is an attractive approach to negate the need for pancreas donors. History has proven, however, that such a feat has been challenging to achieve; the ability to produce such cells capable of secreting insulin in a physiologically responsive manner whilst proliferating in a controlled manner.[117,118]

Diverse efforts have been undertaken to establish a renewable source of insulin-producing cells. For example, some approaches have been undertaken to exploit the pancreas's native ability to re-establish its β-cell population in response to injury by utilizing various transcription factors.[119,120] Others have sought to utilize cells derived from the hematopoietic lineage, including bone marrow-derived cells, as well as umbilical cord blood, as precursors to insulin-producing cells. Umbilical cord blood as a precursor is advantageous in that it can be easily obtained and would avoid some of the ethical implications associated with the use of stem cells. However, some animal studies utilizing hematopoietic lineages as a means to augment endogenous β-cell levels were inconclusive.[121,122] In a clinical study conducted by Haller and colleagues, newly diagnosed patients receiving autologous umbilical cord blood revealed lower $HbA1_c$ and reduced insulin requirements.[123]

The self-renewing capacity and pluripotent properties of embryonic stem cells (ESCs) have drawn considerable attention in recent years. Strategies to differentiate ESCs into insulin-producing cells have been underway since the early 2000s, and while initially promising, were met by impediments including low numbers of insulin-positive cells and a lack of glucose sensitivity.[124-127] Aside from scientific impediments, in August 2001 the U.S. Government under the Bush Administration stifled progress further through a federal ban on research funding supporting newly created human ES cells for scientific research. Studies utilizing ES lines created prior to this ban were still eligible for grant funding sponsored by the National Institutes of Health, however, only 21 lines proved to be of any use to investigators.[128] Moreover, collaborative efforts between U.S. and International centres were impeded, as U.S. scientists were limited in their ability to contribute substantially as a result of this ban. The fact that the 21 eligible cell lines were limited in genetic and/or ethnic diversity left uncertainty with respect to cellular processes in underrepresented ethnic groups.[129] Given the above limitations, one must question how this ban impeded scientific progress, not just in T1DM, but in other disease states such as Parkinson's disease too.

In 2006, pivotal work by D'Amour *et al* set precedence in establishing pancreatic hormone-expressing cells derived from embryonic stem cells.[118] Utilizing a five-stage, *ex vivo* differentiation process, the authors reported the ability to mimic *in vivo* organogenesis, with an end result of differentiated endocrine cells capable of synthesizing islet-specific hormones insulin, glucagon, somatostatin, ghrelin, and pancreatic polypeptide. This 11 – 18+ day protocol varied in conditions from medium, growth factors and duration for each stage and transitioned from definitive endoderm

(Stage 1) to hormone expressing endocrine cells (Stage 5). This work was fundamental to the subsequent differentiation protocols established by Kroon *et al*, whereby glucose-responsive pancreatic endoderm cells were derived from ES cells using a 12-day, 4-stage protocol.[130] Notably, at day 12, these cellular aggregates exhibit low expression of insulin or glucagon, though these hormone positive cells would express insulin and glucagon or insulin and somatostatin. Perhaps most importantly, transplanted into the epdidymal fat pad of SCID-beige mice, sufficient levels of human C-peptide could be measured 3 months post-transplant, confirming *in vivo* differentation from an immature to a more mature phenotype.

Subsequent improvements resulted in development of glucose-sensitive cells capable of restoring euglycemia in diabetic rodent models.[130] Novel approaches have also been undertaken to differentiate human ESCs into pancreatic progenitor cells *in vitro* with final stages of differentiation into glucose-responsive, insulin-producing *β* cells *in vivo*.[130-135] Strategies to consistently produce PEC in sufficient quantities for clinical trials have also been developed [136] with the capacity to restore normoglycemia.[137,138]

Many of these seminal studies were paramount in the establishment of a US Food and Drug Administration- and Health Canada-approved, first-in-human pilot phase 1/2 clinical trial to be conducted by ViaCyte Inc., a commercial leader in regenerative medicine technologies. Approved in 2014, this trial sought to test the VC-01 combination product, PEC-Encap™, which combines CyT49 hESC-derived PEC contained within an immune-protective, macroencapsulated device transplanted subcutaneously in a small cohort of patients with T1DM (NCT02239354). These trials

have been especially interesting to us in Edmonton as the Shapiro group has been a major contributor to patient enrolment. The results from this ongoing pilot study have thus far confirmed that the device is safe and offers at least a degree of immune protection, with evidence of vascularization, engraftment and differentiation of PEC-01 cells *in vivo* (unpublished data). However, ViaCyte also reports that refinements to the Encaptra device are required to control a destructive foreign-body giant cell type response, which must be overcome if cell survival is to be further optimized.

The observations of this VC-01 pilot study lead to further approval of a safety and tolerability study of Viacyte's PEC-Direct VC-02 combination product in patients with T1DM (NCT03162926). In contrast to the VC-01 combination product, the PEC-Direct system permits vascularization to the PEC-01 cells situated within the device, therefore the unit is not immune-protected, and as such patients participating in this trial require immunosuppression therapy for the duration of the clinical trial. By utilizing sentinel devices for various extended implantation periods, immunohistological examination of explanted devices will assist with determining vascularization, engraftment, and differentiation of PEC-01 cells *in situ.* Indeed these findings will be critical in moving this technology forward as a more effective source to treat T1DM. These pilot studies will help to confirm the safety and tolerability of these devices. The number of PEC-01 cells administered to each patient, including the number of devices that will be required to feasibly correct hyperglycemia has yet to be determined. It is unlikely that results from murine studies could be interpolated to determine the appropriate dose to restore euglycemia. While large animal pre-clinical porcine model would normally be warranted in parallel to safety studies conducted in the clinic, they

would constitute xenotransplants rather than allotransplants in these models as the origin cells are human ESCs. The macroencapsulation devices are insufficient to protect from xenoimmunity, making pilot trials in patients far more relevant than would otherwise be the case.

Alternative protocols are being established whereby pluripotent stem cells undergo longer *in vitro* differentiation resulting in generation of insulin-producing cells. In 2014, Rezania *et al* from Tim Kieffer's group in Vancouver, together with J&J, reported a 7-Stage differentiation protocol whereby *in vitro* assessment of these cells exhibited functional characteristics similar to human β-cells, as determined by insulin secretion and calcium imaging. The authors assert, however, that these differentiated cells cannot be considered mature β-cells, as only 5 – 10% of the Stage 7 population exhibited physiological characteristics resembling β-cells.[138] When transplanted into diabetic murine recipients under the renal capsule, Stage 7 cells were capable of restoring euglycemia within 40 days, four-fold faster than their pancreatic progenitor (Stage 4) counterparts. Moreover, the work by Doug Melton's laboratory has also demonstrated the ability to develop functional Stage 7 insulin-producing cells utilizing an *in vitro* 27 – 34 day differentiation protocol.[137] These cells also exhibited glucose responsiveness *in vitro*, as well as the ability to restore euglycemia in diabetic murine recipients, within 18 days post-transplant.

Whether it is indeed important to transplant Stage 4 or more mature Stage 7 cell remains to be seen. A patient that has had longstanding T1DM can surely afford to wait for a few months for their cells to differentiate and become fully functional. Further studies utilizing pancreatic progenitor cells transplanted into extrahepatic, subcutaneous

devices will undoubtedly require further optimization and refinements to improve engraftment, oxygen delivery and metabolic exchange. The Shapiro laboratory have compared placement of PEC-01 cells placed into the modified subcutaneous DL space, and shown that PEC-01 cells can mature and be fully functional in this site. Importantly, before transplantation, the Stage-4 PEC-01 cells exhibited reduced metabolic capacity, as assessed by oxygen consumption rate (OCR).[62] The immature phenotype of these cells, and especially their metabolic quiescent state, may be an added advantage when cells are initially implanted to a hypoxic, subcutaneous environment. While these cells required approximately 100 days to differentiate into fully functional, glucose-responsive cells, one could surmise that this is a considerable advantage in contrast to a more differentiated β-cell lineage whose metabolic demands would be greater and would require sufficient oxygen to survive, engraft and function. Furthermore, graft-bearing immunohistological analysis also revealed robust presence of glucose-regulatory cells contained within a vascularized collagen scaffold. These results are especially encouraging, namely because the DL site may serve as a functional surrogate to subcutaneous devices that routinely fail long-term.

A considerable limitation in most pre-clinical models that use insulin-producing stem cells is that efficacy is typically evaluated in immune-deficient mice. These models have limitations, and the absence of an allogeneic or even an innate immune response could be underestimated, and immunosuppression is not required. The Shapiro lab is currently investigating what potential harmful effects immunosuppressive medications may have upon the differentiation process of these cells. Since PEC-01 cells require 2-3 months to differentiate into functioning, glucose-responsive cells, concerns persist in

terms of how harmful diabetogenic drugs such as tacrolimus may have on the development process. Results from the ongoing VC-02 trials will provide important foundations as ESC-derived islet alternatives move forward to the clinic.

The prospect of establishing a limitless supply of glucose-responsive, insulin-producing cells as means for β-cell replacement therapy is undeniably attractive. Concerns pertaining to the risk of teratogenicity, as well as ethical and religious considerations over the use of ESCs warrant notable caution to proceed.[139-141] However, if such a product can be ethically derived from a donated, discarded human embryonic blastocyst from an *in vitro* fertilization clinic, then insulin-producing ESCs may transition as a therapy for a select population of patients to a β-cell replacement therapy for all individuals suffering from T1DM.

6.4 IMMUNOMODULATORY THERAPIES IN NEW ONSET T1DM

Recurrent autoimmunity remains an almost insurmountable barrier in cellular replacement therapy in T1DM. [142] Recurrent autoimmunity almost certainly is detrimental to islet survival in patients.[143] Since similar observations occur in whole pancreas and islet transplantation, it is highly likely that stem cells differentiated into insulin-producing cellular sources that mimic β-cells will also be target of recurrent autoimmunity.

Studies that mitigate the autoimmune destruction of β-cells in T1DM and delay disease onset, have increasing relevance therefore in cell transplant trials. Chatenoud *et al* found that administration of potent anti-CD3 antibody in NOD mice for 5 days at the

time of disease onset was capable of inducing remission in 80% of recipients. Moreover, despite discontinuation of treatment, mice in remission did not become diabetic over time.[144] Further pre-clinical work exploring the utility of anti-CD3 monoclonal antibody in mice by Herold *et al* revealed the capacity of this therapy to prevent T1DM onset and tolerance induction.[145] These studies suggested that a similar therapy might confer benefit in the clinic; however, anti-CD3 (OKT3) exhibited considerable cytokine-release syndrome in otherwise healthy patients when used as an induction anti-rejection therapy.[146,147] As a means to eliminate such risks, Bluestone *et al* developed a humanized anti-CD3 monoclonal antibody, hOKT3γ1 (Ala-Ala), and examined its ability to delay new onset in patients with T1DM. Two year follow up of these patients revealed markedly improved C-peptide with prolongation of the 'honeymoon' period.[148] Moreover, the Immune Tolerance Network recently reported the findings of the Autoimmunity-Blocking Antibody for Tolerance in Recently Diagnosed Type 1 Diabetes (AbATE) trial. In a 4th trial utilizing the monoclonal antibody teplizumab (anti-CD3), patients who positively responded to treatment exhibited preserved β-cell function, and had preserved C-peptide function up to two years from diagnosis T1DM. The AbATE trial has demonstrated that in responders the mean preservation of C-peptide continues at baseline levels for 2 years.[149] As Skyler highlights, such positive experiences with anti-CD3 warrant full-scale phase 3 trials whereby an adequate treatment dose is paramount, along with an appropriate primary outcome (preservation of C-peptide) to confirm efficacy of this treatment in newly diagnosed type 1 diabetic patients.[150]

6.4.1 – Immunologic Reset

While islet transplantation has offered extreme benefit as a treatment modality for select patients with T1DM, it still falls short as a "curative" therapy. The notion to cure T1DM through prevention or through the reversal of new disease onset has translated poorly to the clinic, despite considerable promise in experimental settings. Almost 500 treatment strategies have demonstrated ability to prevent or reverse autoimmune diabetes in non-obese diabetic mice,[151-153] leading to large-scale clinical trials that have been met with failed clinical outcomes,[154] suggesting the NOD mouse model is a poor surrogate for human T1DM. A limited few interventional strategies have mitigated disease onset or sustained endogenous C-peptide in preliminary clinical pilot trials. MMF together with daclizumab,[155] B-cell depletion with rituximab,[156] and administration of vitamin D3 are three approaches that stand out.[157] Despite encouraging promise in pilot trials, results have been less than favourable in all approaches when expanded to randomized, powered trials.[158]

Perhaps the most promising approach explored within the last decade is "immunological reset" whereby auto-reactive T and B cells are non-specifically eliminated, followed by reconstitution of a tolerant immune system.[159] Most importantly with this approach is shifting the host response from autoimmunity to a state of tolerance. Much success with this approach has been experienced in Brazil, where, in 2007, Voltarelli *et al* reportedly eliminated autoreactive lymphocyte clones using thymoglobulin- and cyclophosphamide-based depletional therapy, and subsequently restored self-tolerance through autologous bone marrow transplant that had been mobilized *ex vivo* with granulocyte colony stimulating factor (G-CSF) in patients with

T1DM. In 20 of 23 children and adolescents with new-onset T1DM, insulin independence was achieved for periods ranging 6 to 35 months, which is a remarkable finding. Further follow up revealed a maintained state of insulin independence for a mean period of 31 months was achieved in just over 50% of these patients.[160-162]

Results from these experiences have garnered considerable attention, and as such have propagated the initiation of a clinical trial by Shapiro *et al* at the University of Alberta. In contrast to the Voltarelli protocol, cyclophosphamide has been eliminated and clonal T-cell depletion is being achieved with a single-dose alemtuzumab as opposed to thymoglobulin. Moreover, autologous bone marrow cells are being mobilized by a drug-based approach using plerixafor, rather than G-CSF. Long-acting glucagon-like peptide-1 (GLP-1) analogue will also be employed based on its trophic and metabolic protective effects.[163-165] Anti-inflammatory agents, Etanercept and Anakinra will also be employed post-infusion as a means to prolong cellular survival by dampening the initiating of deleterious inflammatory cascades. Given the successful experience with alemtuzumab, Etanercept and Anakinra within the islet patients, as well as prior successes observed with the Voltarelli protocol, one might anticipate that these modifications will have positive impact. It is likely that minimizing pro-inflammatory cascades in the acute infusion period will augment cellular survival. In tandem with robust T-cell depletion to mitigate recurrence of the autoimmune response, this much-anticipated trial may set the precedent in eliminating T1DM disease onset, thus alleviating the prospective global burden of this disease.

6.4.2 – Regulatory T-cells in Islet Transplantation

Allotransplantation, regardless of the organ, presents with the challenge of chronic immunosuppression management. While necessary to subvert the allogeneic response from the host to preserve graft viability, long-term immunosuppression also carries considerable morbidity and potential mortality risks to the transplant recipient. Moreover, chronic systemic immunosuppression can present with increased susceptibility to infection and post-transplant cancer as a result of subdued host immune surveillance.[166,167] Other morbidities, including but not limited to, nephrotoxicity, diabetes, hypertension and cardiovascular diseases, are also associated with off-target effects of long-term, global immunosuppression administration.[168-170] As a means to deter the consequences of these associated morbidities, a reduction or potential withdrawal of immunosuppression may be required but will ultimately contribute to graft failure. An attractive approach to minimize or eliminate the necessity of chronic systemic immunosuppression is to induce tolerance, a state of non-responsiveness to self- or foreign tissue.[171] One such approach is through adoptive transfer of regulatory T-cells (Treg), a population of $CD4^+CD25^+$ cells that rely on the transcription factor, FOXP3.[172]

Tregs are an attractive approach relative to chronic immunosuppression, primarily due to their ability to distinguish different antigens with extreme specificity, owing to their receptors. Tregs also possess the inherent ability to seek their targets throughout the body and elicit function at a localized site.[171,172] Using islet antigen-specific Tregs, Meagher and colleagues demonstrated the ability to protect against islet destruction in a mouse model of autoimmune diabetes.[173] The ability to harness antigen

specificity, as well as control responses of numerous immune cells, including but not limited to $CD4^+$ and $CD8^+$ T cells, natural killer cells, and B cells, identifies Tregs as a prime candidate to confer protection to the islet graft in the transplant setting. To effectively induce an adequate protective effect in the transplant setting, a sufficient population of Tregs must be transplanted as a means to outbalance the effector response.[88,171,172] To accommodate such an approach, *ex vivo* expansion of Tregs is required and has been successfully demonstrated from peripheral blood, as well as from discarded pediatric thymuses and holds great promise for clinical application.[174,175]

Given the promising ability to expand Tregs into sufficient numbers *ex vivo*, the stage has been set to explore the utility of this tolerance-inducing strategy in clinical islet transplantation. A first-in-human trial conducted jointly with Shapiro's group at the University of Alberta and Bluestone and Tang at the University of California San Francisco will hopefully shed light on the utility of this approach in the clinical setting. The clinical application of a tolerance-inducing strategy in islet transplantation will eliminate the need for chronic immunosuppression, thus broadening the T1DM patient population eligible for an islet transplant.

6.5 - CONCLUDING REMARKS

From its initial inception as a prospective tool to treat patients with T1DM, islet transplantation has transitioned into the 21st century as a real therapeutic approach. The pivotal pre-clinical and clinical experiences that ultimately contributed to the foundation of the monumentally successful Edmonton Protocol has indeed been critical in establishing islet transplantation as an efficacious therapy. Marked improvements in

islet transplant outcomes over the past two decades has revealed that islet transplantation may impede the evolution of several long-term secondary complications associated with T1DM. To achieve this, multiple infusions and a cumulative islet implant mass ≥10,000 islet equivalents per kilogram recipient weight (IE/kg) are often times required. Nevertheless, 5-year insulin independence rates for islet transplantation are now comparable to that of whole pancreas transplant outcomes.

The body of work undertaken in the last 4 years that contributed to this thesis sought to introduce therapeutic agents aimed to preserve islet cell mass by directly or indirectly impede the progression of cell death *in vitro* as well as in the acute transplant period. Intraductal administration of the MnSOD, BMX-001, during organ procurement and 24-hour culture demonstrated the ability to preserve murine islet function through sGSIS, as well as exhibit reduced death through TUNEL staining. Marginal islet transplants exhibited improved engraftment outcomes nearly two-fold in 34μM BMX-001 islet recipients than control islet recipients. The mechanism by which BMX-001 affords improved islet function remains to be elucidated, however, further work in the murine DCD model revealed that BMX-001 reduced the production of ROS. It is highly likely that this was also the case in our standard murine marginal, syngeneic model. Given that MnSOD is a critical mitochondrial antioxidant, it is likely that administration of BMX-001 augmented islet mitochondrial viability. Direct measurement of oxygen consumption rate could be further utilized to support this notion in future studies. Future clinical work administering BMX-001 at the time of cross-clamp and during isolation may reveal improved benefit post-transplant. Large animal pre-clinical studies will more accurately mimic clinical procurement practices and as such will be of greater

translational utility. Administration of BMX-001 to islet recipients pre-clinically has yet to be explored, but may also be an attractive adjuvant therapy given its known anti-inflammatory effects. Notably, we have extensively reviewed that the generation of pro-inflammatory cytokines in the acute transplant period can significantly impair engraftment outcomes. As such, it is conceivable that BMX-001 can deter the downstream consequences of such cytokines and preserve islet mass and subsequent engraftment outcomes.

Our work exploring the utility of the pan-caspase inhibitor, F573, corroborated prior work from the Shapiro laboratory elucidating the benefit of apoptotic inhibition in islet transplantation. The more important aspect of this study was in the comparison of anti-apoptotic agents to mitigate cell death in the modified subcutaneous DL site. In this model, we were able to routinely reverse diabetes with a marginal islet transplant mass. The administration of F573 to enhance DL-islet engraftment reveals that modifications to the DL site may be required to fully exploit its engraftment. While neovascularization is a pre-requisite for successful DL islet engraftment, the site remains hypoxic, and may still have deleterious consequences for metabolically active islets or *in vitro* differentiated insulin-producing cells derived from pancreatic endoderm. Given the direct access of the subcutaneous space, it would be interesting for future studies to directly measure oxygen tension in the DL space to dertermine if this site could be further optimized to accommodate a cellular graft. Future use of biodegradable materials that temporarily elute growth factors to promote neovascularization before transplantation could be an attractive approach that further warrants preclinical investigation.

Given the therapeutic benefit of Tregs and their potential in inducing tolerance in transplant recipients, ongoing studies by other graduate students in the Shapiro and Korbutt laboratories are investigating the potential role of co-transplanting Tregs with islets, or other immune-modifying cells into the DL space. Co-transplantation of such cells may further augment an immune-protected environment. Such a strategy could broaden the therapeutic reach of β-cell replacement therapy from patients with brittle diabetes, to patients suffering from insulin-dependent T2DM, as well as children.

The chemical induction of ferroptosis, and its inhibition in human islets, is the first study to our knowledge to address the incidence of this cell death modality in human tissue. The identification of this novel regulated cell death pathway should indeed lead to the exploration and potential identification of novel mediators that contribute to impaired islet function and cell death. As these mediators are identified in islets and other organ systems, future studies should aim to exploit these targets and drive the development of novel therapeutic agents. As previously noted, given the fact that TNF-α inhibition confers benefit on islet viability and engraftment, and TNF-α is a key mediator of necroptosis, the utility of necroptosis-specific inhibitors may be beneficial in islet isolation and transplantation.

Furthermore, the utility of the CRISPR/Cas9 system in islet transplantation may hold promise as a therapeutic tool to directly promote islet survival by selective insertion of one or multiple cytoprotective genes. In contrast to chemical delivery of protective agents directly to the pancreas, or by brief culture of islets post-isolation as a means to confer adequate uptake and subsequent therapeutic action, the CRISPR/Cas9 system could genetically modify islets with precision, mitigating the need for

therapeutic drugs. The identification of existing, as well as novel mediators that contribute to novel cell death pathways may serve as therapeutic targets that can be exploited utilizing this methodology. Such an approach would reduce the need for systemic administration of therapeutic agents that may have off-target or unwanted side effects. Exploring genetically modified islets in the pre-clinical arena may open avenues for this technology's utility in the clinical setting. However, as noted above, safety and ethical issues surrounding this technology should be addressed before genetically modified cells become commonplace in the clinic.

Efforts aimed at augmenting islet survival and function offer promise to circumvent a need for multiple donor pancreases. There are multiple areas for future investigation; from pancreas procurement, islet isolation and culture, as well as immediately post-transplant. The identification of novel, biochemically distinct regulated cell death pathways in other disease states and transplant modalities has opened a new area of investigation in islet transplantation, warrants further exploration. With the potential identification of novel targets, the development of novel adjuvant therapies may be used in a multi-therapeutic approach to prolong islet survival and engraftment.

As a means to alleviate the burden upon available donor pancreases, the utility of alternative cellular sources, including xenogeneic porcine islets, as well as insulin-producing "islet-like" cells derived from pancreatic endoderm, continue to offer promise as a means to treat patients with T1DM. As these cellular sources transcend from pre-clinical investigation to clinical translation, safety and efficacy are imperative milestones. Chronic, systemic immunosuppression is still required to mitigate recurrent

auto- and alloimmune responses in the transplant recipient. Therapies that facilitate immunological tolerance through co-transplantation of islets with Tregs are exciting avenues now entering clinical trials. There is an abundance of approaches that collectively will ultimately alleviate the burden of T1DM and offer hope that this disease can once and for all be eliminated.

6.6 - REFERENCES

1. King H, Aubert RE, Herman WH. Global burden of diabetes, 1995-2025: prevalence, numerical estimates, and projections. *Diabetes Care.* 1998;21(9):1414-1431.
2. Polonsky KS. The past 200 years in diabetes. *The New England journal of medicine.* 2012;367(14):1332-1340.
3. Banting FG, Best CH, Collip JB, Campbell WR, Fletcher AA. Pancreatic Extracts in the Treatment of Diabetes Mellitus. *Canadian Medical Association journal.* 1922;12(3):141-146.
4. 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. *The New England journal of medicine.* 1993;329(14):977-986.
5. Keen H. The Diabetes Control and Complications Trial (DCCT). *Health Trends.* 1994;26(2):41-43.
6. Nathan DM, Lachin J, Cleary P, et al. Intensive diabetes therapy and carotid intima-media thickness in type 1 diabetes mellitus. *The New England journal of medicine.* 2003;348(23):2294-2303.
7. Kelly WD, Lillehei RC, Merkel FK, Idezuki Y, Goetz FC. Allotransplantation of the pancreas and duodenum along with the kidney in diabetic nephropathy. *Surgery.* 1967;61(6):827-837.
8. Lacy PE, Kostianovsky M. Method for the isolation of intact islets of Langerhans from the rat pancreas. *Diabetes.* 1967;16(1):35-39.

9. Misler S. The isolated pancreatic islet as a micro-organ and its transplantation to cure diabetes: celebrating the legacy of Paul Lacy. *Islets.* 2010;2(4):210-224.

10. Scharp DW, Kemp CB, Knight MJ, Ballinger WF, Lacy PE. The use of ficoll in the preparation of viable islets of langerhans from the rat pancreas. *Transplantation.* 1973;16(6):686-689.

11. Najarian JS, Sutherland DE, Matas AJ, Steffes MW, Simmons RL, Goetz FC. Human islet transplantation: a preliminary report. *Transplantation proceedings.* 1977;9(1):233-236.

12. Shapiro AM. A historical perspective on experimental and clinical islet transplantation. *Informa Health Care.* 2007:1.

13. Ricordi C, Lacy PE, Scharp DW. Automated islet isolation from human pancreas. *Diabetes.* 1989;38 Suppl 1:140-142.

14. Brendel M HB, Shulz A, Bretzel R. International Islet Transplant Registry Report. 1999.

15. Shapiro AM, Lakey JR, Ryan EA, 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(4):230-238.

16. Ryan EA, Paty BW, Senior PA, et al. Five-year follow-up after clinical islet transplantation. *Diabetes.* 2005;54(7):2060-2069.

17. Bellin MD, Barton FB, Heitman A, et al. Potent induction immunotherapy promotes long-term insulin independence after islet transplantation in type 1 diabetes. *Am J Transplant.* 2012;12(6):1576-1583.

18. 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(2):343-347.

19. Hering BJ, Kandaswamy R, Ansite JD, et al. Single-donor, marginal-dose islet transplantation in patients with type 1 diabetes. *Jama.* 2005;293(7):830-835.

20. Hering BJ, Kandaswamy R, Harmon JV, et al. Transplantation of cultured islets from two-layer preserved pancreases in type 1 diabetes with anti-CD3 antibody. *Am J Transplant.* 2004;4(3):390-401.

21. Collaborative Islet Transplant Registry. 2015; http://www.citregistry.org.

22. Hering BJ, Clarke WR, Bridges ND, et al. Phase 3 Trial of Transplantation of Human Islets in Type 1 Diabetes Complicated by Severe Hypoglycemia. *Diabetes Care.* 2016;39(7):1230-1240.

23. Benhamou PY, Watt PC, Mullen Y, et al. Human islet isolation in 104 consecutive cases. Factors affecting isolation success. *Transplantation.* 1994;57(12):1804-1810.

24. Goto M, Eich TM, Felldin M, 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(9):1367-1375.

25. Ihm SH, Matsumoto I, Sawada T, et al. Effect of donor age on function of isolated human islets. *Diabetes.* 2006;55(5):1361-1368.

26. Lakey JR, Rajotte RV, Warnock GL, Kneteman NM. Human pancreas preservation prior to islet isolation. Cold ischemic tolerance. *Transplantation.* 1995;59(5):689-694.

27. Lakey JR, Warnock GL, Rajotte RV, et al. Variables in organ donors that affect the recovery of human islets of Langerhans. *Transplantation.* 1996;61(7):1047-1053.

28. Nano R, Clissi B, Melzi R, et al. Islet isolation for allotransplantation: variables associated with successful islet yield and graft function. *Diabetologia.* 2005;48(5):906-912.

29. Zeng Y, Torre MA, Karrison T, Thistlethwaite JR. The correlation between donor characteristics and the success of human islet isolation. *Transplantation.* 1994;57(6):954-958.

30. O'Gorman D, Kin T, Murdoch T, et al. The standardization of pancreatic donors for islet isolations. *Transplantation.* 2005;80(6):801-806.

31. Witkowski P, Liu Z, Cernea S, et al. Validation of the scoring system for standardization of the pancreatic donor for islet isolation as used in a new islet isolation center. *Transplantation proceedings.* 2006;38(9):3039-3040.

32. Saito T, Gotoh M, Satomi S, et al. Islet transplantation using donors after cardiac death: report of the Japan Islet Transplantation Registry. *Transplantation.* 2010;90(7):740-747.

33. Anazawa T, Saito T, Goto M, et al. Long-term outcomes of clinical transplantation of pancreatic islets with uncontrolled donors after cardiac death: a multicenter experience in Japan. *Transplantation proceedings.* 2014;46(6):1980-1984.

34. Markmann JF, Deng S, Desai NM, et al. The use of non-heart-beating donors for isolated pancreatic islet transplantation. *Transplantation.* 2003;75(9):1423-1429.

35. Noguchi H. Pancreas procurement and preservation for islet transplantation: personal considerations. *J Transplant.* 2011;2011:783168.

36. Andres A, Kin T, O'Gorman D, et al. Clinical islet isolation and transplantation outcomes with deceased cardiac death donors are similar to neurological determination of death donors. *Transpl Int.* 2016;29(1):34-40.

37. Avila JG, Tsujimura T, Oberholzer J, et al. Improvement of pancreatic islet isolation outcomes using glutamine perfusion during isolation procedure. *Cell Transplant.* 2003;12(8):877-881.

38. Giraud S, Hauet T, Eugene M, Mauco G, Barrou B. A new preservation solution (SCOT 15) Improves the islet isolation process from pancreata of non-heart-beating donors: a Murine model. *Transplantation proceedings.* 2009;41(8):3293-3295.

39. Sung RS, Christensen LL, Leichtman AB, et al. Determinants of discard of expanded criteria donor kidneys: impact of biopsy and machine perfusion. *Am J Transplant.* 2008;8(4):783-792.

40. Ploeg RJ, Friend PJ. New strategies in organ preservation: current and future role of machine perfusion in organ transplantation. *Transpl Int.* 2015;28(6):633.

41. Bruns H, Schemmer P. Machine perfusion in solid organ transplantation: where is the benefit? *Langenbecks Arch Surg.* 2014;399(4):421-427.

42. Van Raemdonck D, Neyrinck A, Rega F, Devos T, Pirenne J. Machine perfusion in organ transplantation: a tool for ex-vivo graft conditioning with mesenchymal stem cells? *Curr Opin Organ Transplant.* 2013;18(1):24-33.

43. Wolters GH, Vos-Scheperkeuter GH, van Deijnen JH, van Schilfgaarde R. An analysis of the role of collagenase and protease in the enzymatic dissociation of the rat pancreas for islet isolation. *Diabetologia.* 1992;35(8):735-742.

44. Wang X, Meloche M, Verchere CB, Ou D, Mui A, Warnock GL. Improving islet engraftment by gene therapy. *J Transplant.* 2011;2011:594851.

45. 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

.

46. Tiedge M, Lortz S, Drinkgern J, Lenzen S. Relation between antioxidant enzyme gene expression and antioxidative defense status of insulin-producing cells. *Diabetes.* 1997;46(11):1733-1742.

47. Tse HM, Milton MJ, Piganelli JD. Mechanistic analysis of the immunomodulatory effects of a catalytic antioxidant on antigen-presenting cells: implication for their use in targeting oxidation-reduction reactions in innate immunity. *Free Radic Biol Med.* 2004;36(2):233-247.

48. Adler V, Yin Z, Tew KD, Ronai Z. Role of redox potential and reactive oxygen species in stress signaling. *Oncogene.* 1999;18(45):6104-6111.

49. do Amaral AS, Pawlick RL, Rodrigues E, 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(2):e55288.

50. Gala-Lopez B, Kin T, O'Gorman D, et al. The metalloporphyrin BMX-010 in human islet isolation and clinical transplantation *CellR4.* 2016;4(3):8.

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

52. Merani S, Toso C, Emamaullee J, Shapiro AM. Optimal implantation site for pancreatic islet transplantation. *The British journal of surgery.* 2008;95(12):1449-1461.

53. Plesner A, Verchere CB. Advances and challenges in islet transplantation: islet procurement rates and lessons learned from suboptimal islet transplantation. *J Transplant.* 2011;2011:979527.

54. Emamaullee JA, Shapiro AM. Interventional strategies to prevent beta-cell apoptosis in islet transplantation. *Diabetes.* 2006;55(7):1907-1914.

55. Ozmen L, Ekdahl KN, Elgue G, Larsson R, Korsgren O, Nilsson B. Inhibition of thrombin abrogates the instant blood-mediated inflammatory reaction triggered by isolated human islets: possible application of the thrombin inhibitor melagatran in clinical islet transplantation. *Diabetes.* 2002;51(6):1779-1784.

56. Baidal DA, Ricordi C, Berman DM, et al. Bioengineering of an Intraabdominal Endocrine Pancreas. *The New England journal of medicine.* 2017;376(19):1887-1889.

57. Rajab A. Islet transplantation: alternative sites. *Curr Diab Rep.* 2010;10(5):332-337.

58. Barshes NR, Lee TC, Goodpastor SE, et al. Transaminitis after pancreatic islet transplantation. *J Am Coll Surg.* 2005;200(3):353-361.

59. Veriter S, Gianello P, Dufrane D. Bioengineered Sites for Islet Cell Transplantation. *Curr Diab Rep.* 2013.

60. 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(5):518-523.

61. 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 (5).

62. Pepper AR, Pawlick R, Bruni A, et al. Transplantation of Human Pancreatic Endoderm Cells Reverses Diabetes Post Transplantation in a Prevascularized Subcutaneous Site. *Stem Cell Reports.* 2017;8(6):1689-1700.

63. Emamaullee JA, Stanton L, Schur C, Shapiro AM. Caspase inhibitor therapy enhances marginal mass islet graft survival and preserves long-term function in islet transplantation. *Diabetes.* 2007;56(5):1289-1298.

64. Emamaullee JA, Davis J, Pawlick R, et al. The caspase selective inhibitor EP1013 augments human islet graft function and longevity in marginal mass islet transplantation in mice. *Diabetes.* 2008;57(6):1556-1566.

65. McCall M, Toso C, Emamaullee J, et al. The caspase inhibitor IDN-6556 (PF3491390) improves marginal mass engraftment after islet transplantation in mice. *Surgery.* 2011;150(1):48-55.

66. McCall MD, Maciver AM, Kin T, et al. Caspase inhibitor IDN6556 facilitates marginal mass islet engraftment in a porcine islet autotransplant model. *Transplantation.* 2012;94(1):30-35.

67. Pepper AR, Bruni A, Pawlick R, et al. Engraftment Site and Effectiveness of the Pan-Caspase Inhibitor F573 to Improve Engraftment in Mouse and Human Islet Transplantation in Mice. *Transplantation.* 2017;101(10):2321-2329.

68. Linkermann A. Nonapoptotic cell death in acute kidney injury and transplantation. *Kidney Int.* 2016;89(1):46-57.

69. Linkermann A, Skouta R, Himmerkus N, et al. Synchronized renal tubular cell death involves ferroptosis. *Proc Natl Acad Sci U S A.* 2014;111(47):16836-16841.

70. Linkermann A, Stockwell BR, Krautwald S, Anders HJ. Regulated cell death and inflammation: an auto-amplification loop causes organ failure. *Nat Rev Immunol.* 2014;14(11):759-767.

71. Dixon SJ, Lemberg KM, Lamprecht MR, et al. Ferroptosis: an iron-dependent form of nonapoptotic cell death. *Cell.* 2012;149(5):1060-1072.

72. Yang WS, Stockwell BR. Ferroptosis: Death by Lipid Peroxidation. *Trends Cell Biol.* 2016;26(3):165-176.

73. Dixon SJ, Stockwell BR. The role of iron and reactive oxygen species in cell death. *Nat Chem Biol.* 2014;10(1):9-17.

74. Reed JC, Pellecchia M. Ironing out cell death mechanisms. *Cell.* 2012;149(5):963-965.

75. Bottino R, Balamurugan AN, Bertera S, Pietropaolo M, Trucco M, Piganelli JD. Preservation of human islet cell functional mass by anti-oxidative action of a novel SOD mimic compound. *Diabetes.* 2002;51(8):2561-2567.

76. Bottino R, Balamurugan AN, Tse H, et al. Response of human islets to isolation stress and the effect of antioxidant treatment. *Diabetes.* 2004;53(10):2559-2568.

77. Miwa I, Ichimura N, Sugiura M, Hamada Y, Taniguchi S. Inhibition of glucose-induced insulin secretion by 4-hydroxy-2-nonenal and other lipid peroxidation products. *Endocrinology.* 2000;141(8):2767-2772.

78. Robertson RP, Harmon J, Tran PO, Poitout V. Beta-cell glucose toxicity, lipotoxicity, and chronic oxidative stress in type 2 diabetes. *Diabetes.* 2004;53 Suppl 1:S119-124.

79. Carobbio S, Ishihara H, Fernandez-Pascual S, Bartley C, Martin-Del-Rio R, Maechler P. Insulin secretion profiles are modified by overexpression of glutamate dehydrogenase in pancreatic islets. *Diabetologia.* 2004;47(2):266-276.

80. Li C, Buettger C, Kwagh J, et al. A signaling role of glutamine in insulin secretion. *J Biol Chem.* 2004;279(14):13393-13401.

81. Avila J, Barbaro B, Gangemi A, et al. Intra-ductal glutamine administration reduces oxidative injury during human pancreatic islet isolation. *Am J Transplant.* 2005;5(12):2830-2837.

82. Anders HJ, Muruve DA. The inflammasomes in kidney disease. *J Am Soc Nephrol.* 2011;22(6):1007-1018.

83. McIlwain DR, Berger T, Mak TW. Caspase functions in cell death and disease. *Cold Spring Harb Perspect Biol.* 2013;5(4):a008656.

84. Vercammen D, Beyaert R, Denecker G, et al. Inhibition of caspases increases the sensitivity of L929 cells to necrosis mediated by tumor necrosis factor. *J Exp Med.* 1998;187(9):1477-1485.

85. Kaiser WJ, Upton JW, Long AB, et al. RIP3 mediates the embryonic lethality of caspase-8-deficient mice. *Nature.* 2011;471(7338):368-372.

86. Oberst A, Dillon CP, Weinlich R, et al. Catalytic activity of the caspase-8-FLIP(L) complex inhibits RIPK3-dependent necrosis. *Nature.* 2011;471(7338):363-367.

87. Friedmann Angeli JP, Schneider M, Proneth B, et al. Inactivation of the ferroptosis regulator Gpx4 triggers acute renal failure in mice. *Nat Cell Biol.* 2014;16(12):1180-1191.

88. Bluestone JA, Herold K, Eisenbarth G. Genetics, pathogenesis and clinical interventions in type 1 diabetes. *Nature.* 2010;464(7293):1293-1300.

89. Butler AE, Janson J, Bonner-Weir S, Ritzel R, Rizza RA, Butler PC. Beta-cell deficit and increased beta-cell apoptosis in humans with type 2 diabetes. *Diabetes.* 2003;52(1):102-110.

90. Farney AC, Xenos E, Sutherland DE, et al. Inhibition of pancreatic islet beta cell function by tumor necrosis factor is blocked by a soluble tumor necrosis factor receptor. *Transplantation proceedings.* 1993;25(1 Pt 2):865-866.

91. Hering BJ. Achieving and maintaining insulin independence in human islet transplant recipients. *Transplantation.* 2005;79(10):1296-1297.

92. http://www.who.int/mediacentre/factsheets/fs312/en/.

93. http://www.transplant-observatory.org/data-charts-and-tables/.

94. Groth CG, Korsgren O, Tibell A, et al. Transplantation of porcine fetal pancreas to diabetic patients. *Lancet.* 1994;344(8934):1402-1404.

95. Cardona K, Korbutt GS, Milas Z, et al. Long-term survival of neonatal porcine islets in nonhuman primates by targeting costimulation pathways. *Nat Med.* 2006;12(3):304-306.

96. Hering BJ, Walawalkar N. Pig-to-nonhuman primate islet xenotransplantation. *Transpl Immunol.* 2009;21(2):81-86.

97. Hering BJ, Wijkstrom M, Graham ML, et al. Prolonged diabetes reversal after intraportal xenotransplantation of wild-type porcine islets in immunosuppressed nonhuman primates. *Nat Med.* 2006;12(3):301-303.

98. van der Windt DJ, Bottino R, Casu A, et al. Long-term controlled normoglycemia in diabetic non-human primates after transplantation with hCD46 transgenic porcine islets. *Am J Transplant.* 2009;9(12):2716-2726.

99. van der Windt DJ, Bottino R, Kumar G, et al. Clinical islet xenotransplantation: how close are we? *Diabetes.* 2012;61(12):3046-3055.

100. Ekser B, Bottino R, Cooper DK. Clinical Islet Xenotransplantation: A Step Forward. *EBioMedicine.* 2016;12:22-23.

101. Park CG, Bottino R, Hawthorne WJ. Current status of islet xenotransplantation. *Int J Surg.* 2015;23(Pt B):261-266.

102. Bottino R, Trucco M. Use of genetically-engineered pig donors in islet transplantation. *World J Transplant.* 2015;5(4):243-250.

103. Fishman JA, Patience C. Xenotransplantation: infectious risk revisited. *Am J Transplant.* 2004;4(9):1383-1390.

104. Matsumoto S, Tan P, Baker J, et al. Clinical porcine islet xenotransplantation under comprehensive regulation. *Transplantation proceedings.* 2014;46(6):1992-1995.

105. Matsumoto S, Abalovich A, Wechsler C, Wynyard S, Elliott RB. Clinical Benefit of Islet Xenotransplantation for the Treatment of Type 1 Diabetes. *EBioMedicine.* 2016;12:255-262.

106. 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.

107. Phelps CJ, Koike C, Vaught TD, et al. Production of alpha 1,3-galactosyltransferase-deficient pigs. *Science.* 2003;299(5605):411-414.

108. Mandel TE, Koulmanda M, Cozzi E, et al. Transplantation of normal and DAF-transgenic fetal pig pancreas into cynomolgus monkeys. *Transplantation proceedings.* 1997;29(1-2 /01):940.

109. Rood PP, Bottino R, Balamurugan AN, et al. Reduction of early graft loss after intraportal porcine islet transplantation in monkeys. *Transplantation.* 2007;83(2):202-210.

110. Thompson P, Badell IR, Lowe M, et al. Islet xenotransplantation using gal-deficient neonatal donors improves engraftment and function. *Am J Transplant.* 2011;11(12):2593-2602.

111. Ishino Y, Shinagawa H, Makino K, Amemura M, Nakata A. Nucleotide sequence of the iap gene, responsible for alkaline phosphatase isozyme

conversion in Escherichia coli, and identification of the gene product. *J Bacteriol.* 1987;169(12):5429-5433.

112. Mojica FJ, Diez-Villasenor C, Soria E, Juez G. Biological significance of a family of regularly spaced repeats in the genomes of Archaea, Bacteria and mitochondria. *Mol Microbiol.* 2000;36(1):244-246.

113. Barrangou R, Fremaux C, Deveau H, et al. CRISPR provides acquired resistance against viruses in prokaryotes. *Science.* 2007;315(5819):1709-1712.

114. Niu D, Wei HJ, Lin L, et al. Inactivation of porcine endogenous retrovirus in pigs using CRISPR-Cas9. *Science.* 2017;357(6357):1303-1307.

115. Lensch MW, Schlaeger TM, Zon LI, Daley GQ. Teratoma formation assays with human embryonic stem cells: a rationale for one type of human-animal chimera. *Cell Stem Cell.* 2007;1(3):253-258.

116. Zhang F, Wen Y, Guo X. CRISPR/Cas9 for genome editing: progress, implications and challenges. *Hum Mol Genet.* 2014;23(R1):R40-46.

117. Bonner-Weir S, Weir GC. New sources of pancreatic beta-cells. *Nat Biotechnol.* 2005;23(7):857-861.

118. Otonkoski T, Gao R, Lundin K. Stem cells in the treatment of diabetes. *Ann Med.* 2005;37(7):513-520.

119. Bouwens L, Houbracken I, Mfopou JK. The use of stem cells for pancreatic regeneration in diabetes mellitus. *Nat Rev Endocrinol.* 2013;9(10):598-606.

120. Zhou Q, Brown J, Kanarek A, Rajagopal J, Melton DA. In vivo reprogramming of adult pancreatic exocrine cells to beta-cells. *Nature.* 2008;455(7213):627-632.

121. 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(6):843-850.

122. 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(5768):1778-1780.

123. 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(6):710-715.

124. Assady S, Maor G, Amit M, Itskovitz-Eldor J, Skorecki KL, Tzukerman M. Insulin production by human embryonic stem cells. *Diabetes.* 2001;50(8):1691-1697.

125. 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(25):16105-16110.

126. 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(5520):1389-1394.

127. Soria B, Roche E, Berna G, Leon-Quinto T, Reig JA, Martin F. Insulin-secreting cells derived from embryonic stem cells normalize glycemia in streptozotocin-induced diabetic mice. *Diabetes.* 2000;49(2):157-162.

128. Murugan V. Embryonic stem cell research: a decade of debate from Bush to Obama. *Yale J Biol Med.* 2009;82(3):101-103.

129. Daley GQ. Missed opportunities in embryonic stem-cell research. *The New England journal of medicine.* 2004;351(7):627-628.

130. Kroon E, Martinson LA, Kadoya K, et al. Pancreatic endoderm derived from human embryonic stem cells generates glucose-responsive insulin-secreting cells in vivo. *Nat Biotechnol.* 2008;26(4):443-452.

131. Bruin JE, Rezania A, Xu J, et al. Maturation and function of human embryonic stem cell-derived pancreatic progenitors in macroencapsulation devices following transplant into mice. *Diabetologia.* 2013;56(9):1987-1998.

132. D'Amour KA, Bang AG, Eliazer S, et al. Production of pancreatic hormone-expressing endocrine cells from human embryonic stem cells. *Nat Biotechnol.* 2006;24(11):1392-1401.

133. Kelly OG, Chan MY, Martinson LA, et al. Cell-surface markers for the isolation of pancreatic cell types derived from human embryonic stem cells. *Nat Biotechnol.* 2011;29(8):750-756.

134. Rezania A, Bruin JE, Riedel MJ, 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(8):2016-2029.

135. Schulz TC, Young HY, Agulnick AD, et al. A scalable system for production of functional pancreatic progenitors from human embryonic stem cells. *PLoS One.* 2012;7(5):e37004.

136. Schulz TC. Concise Review: Manufacturing of Pancreatic Endoderm Cells for Clinical Trials in Type 1 Diabetes. *Stem Cells Transl Med.* 2015;4(8):927-931.

137. Pagliuca FW, Millman JR, Gurtler M, et al. Generation of functional human pancreatic beta cells in vitro. *Cell.* 2014;159(2):428-439.

138. Rezania A, Bruin JE, Arora P, et al. Reversal of diabetes with insulin-producing cells derived in vitro from human pluripotent stem cells. *Nat Biotechnol.* 2014;32(11):1121-1133.

139. Liu X, Wang Y, Li Y, Pei X. Research status and prospect of stem cells in the treatment of diabetes mellitus. *Sci China Life Sci.* 2013;56(4):306-312.

140. Werbowetski-Ogilvie TE, Bosse M, Stewart M, et al. Characterization of human embryonic stem cells with features of neoplastic progression. *Nat Biotechnol.* 2009;27(1):91-97.

141. Vanikar AV, Trivedi HL, Thakkar UG. Stem cell therapy emerging as the key player in treating type 1 diabetes mellitus. *Cytotherapy.* 2016;18(9):1077-1086.

142. Pearson T, Markees TG, Serreze DV, et al. Islet cell autoimmunity and transplantation tolerance: two distinct mechanisms? *Ann N Y Acad Sci.* 2003;1005:148-156.

143. Bosi E, Braghi S, Maffi P, et al. Autoantibody response to islet transplantation in type 1 diabetes. *Diabetes.* 2001;50(11):2464-2471.

144. Chatenoud L, Thervet E, Primo J, Bach JF. Anti-CD3 antibody induces long-term remission of overt autoimmunity in nonobese diabetic mice. *Proc Natl Acad Sci U S A.* 1994;91(1):123-127.

145. Herold KC, Bluestone JA, Montag AG, et al. Prevention of autoimmune diabetes with nonactivating anti-CD3 monoclonal antibody. *Diabetes.* 1992;41(3):385-391.

146. Chatenoud L. Humoral immune response against OKT3. *Transplantation proceedings.* 1993;25(2 Suppl 1):68-73.

147. Chatenoud L. OKT3-induced cytokine-release syndrome: prevention effect of anti-tumor necrosis factor monoclonal antibody. *Transplantation proceedings.* 1993;25(2 Suppl 1):47-51.

148. Herold KC, Gitelman SE, Masharani U, et al. A single course of anti-CD3 monoclonal antibody hOKT3gamma1(Ala-Ala) results in improvement in C-peptide responses and clinical parameters for at least 2 years after onset of type 1 diabetes. *Diabetes.* 2005;54(6):1763-1769.

149. Herold KC, Gitelman SE, Ehlers MR, et al. Teplizumab (anti-CD3 mAb) treatment preserves C-peptide responses in patients with new-onset type 1 diabetes in a randomized controlled trial: metabolic and immunologic features at baseline identify a subgroup of responders. *Diabetes.* 2013;62(11):3766-3774.

150. Skyler JS. The compelling case for anti-CD3 in type 1 diabetes. *Diabetes.* 2013;62(11):3656-3657.

151. Shoda LK, Young DL, Ramanujan S, et al. A comprehensive review of interventions in the NOD mouse and implications for translation. *Immunity.* 2005;23(2):115-126.

152. Roep BO, Atkinson M. Animal models have little to teach us about type 1 diabetes: 1. In support of this proposal. *Diabetologia.* 2004;47(10):1650-1656.

153. Roep BO, Atkinson M, von Herrath M. Satisfaction (not) guaranteed: re-evaluating the use of animal models of type 1 diabetes. *Nat Rev Immunol.* 2004;4(12):989-997.

154. Herold KC, Bluestone JA. Type 1 diabetes immunotherapy: is the glass half empty or half full? *Sci Transl Med.* 2011;3(95):95fs91.

155. Gottlieb PA, Quinlan S, Krause-Steinrauf H, et al. Failure to preserve beta-cell function with mycophenolate mofetil and daclizumab combined therapy in patients with new- onset type 1 diabetes. *Diabetes Care.* 2010;33(4):826-832.

156. Herold KC, Pescovitz MD, McGee P, et al. Increased T cell proliferative responses to islet antigens identify clinical responders to anti-CD20 monoclonal antibody (rituximab) therapy in type 1 diabetes. *J Immunol.* 2011;187(4):1998-2005.

157. Walter M, Kaupper T, Adler K, Foersch J, Bonifacio E, Ziegler AG. No effect of the 1alpha,25-dihydroxyvitamin D3 on beta-cell residual function and insulin requirement in adults with new-onset type 1 diabetes. *Diabetes Care.* 2010;33(7):1443-1448.

158. Skyler JS, Type 1 Diabetes TrialNet Study G. Update on worldwide efforts to prevent type 1 diabetes. *Ann N Y Acad Sci.* 2008;1150:190-196.

159. Roep BO, Tree TI. Immune modulation in humans: implications for type 1 diabetes mellitus. *Nat Rev Endocrinol.* 2014;10(4):229-242.

160. Voltarelli JC, Couri CE, Stracieri AB, et al. Autologous nonmyeloablative hematopoietic stem cell transplantation in newly diagnosed type 1 diabetes mellitus. *JAMA.* 2007;297(14):1568-1576.

161. Voltarelli JC, Couri CE, Stracieri AB, et al. Autologous hematopoietic stem cell transplantation for type 1 diabetes. *Ann N Y Acad Sci.* 2008;1150:220-229.

162. Couri CE, Voltarelli JC. Autologous stem cell transplantation for early type 1 diabetes mellitus. *Autoimmunity.* 2008;41(8):666-672.

163. Merani S, Truong W, Emamaullee JA, Toso C, Knudsen LB, Shapiro AM. Liraglutide, a long-acting human glucagon-like peptide 1 analog, improves glucose homeostasis in marginal mass islet transplantation in mice. *Endocrinology.* 2008;149(9):4322-4328.

164. Toso C, McCall M, Emamaullee J, et al. Liraglutide, a long-acting human glucagon-like peptide 1 analogue, improves human islet survival in culture. *Transpl Int.* 2010;23(3):259-265.

165. Drucker DJ, Dritselis A, Kirkpatrick P. Liraglutide. *Nat Rev Drug Discov.* 2010;9(4):267-268.

166. Euvrard S, Kanitakis J, Claudy A. Skin cancers after organ transplantation. *The New England journal of medicine.* 2003;348(17):1681-1691.

167. Penn I. Cancers complicating organ transplantation. *The New England journal of medicine.* 1990;323(25):1767-1769.

168. Berenson GS, Wattigney WA, Tracy RE, et al. Atherosclerosis of the aorta and coronary arteries and cardiovascular risk factors in persons aged 6 to 30 years and studied at necropsy (The Bogalusa Heart Study). *Am J Cardiol.* 1992;70(9):851-858.

169. Textor SC, Taler SJ, Canzanello VJ, Schwartz L, Augustine JE. Posttransplantation hypertension related to calcineurin inhibitors. *Liver Transpl.* 2000;6(5):521-530.

170. Ojo AO, Held PJ, Port FK, et al. Chronic renal failure after transplantation of a nonrenal organ. *The New England journal of medicine.* 2003;349(10):931-940.

171. Bluestone JA, Tang Q. Immunotherapy: making the case for precision medicine. *Sci Transl Med.* 2015;7(280):280ed283.

172. Bluestone JA, Buckner JH, Fitch M, et al. Type 1 diabetes immunotherapy using polyclonal regulatory T cells. *Sci Transl Med.* 2015;7(315):315ra189.

173. Meagher C, Tang Q, Fife BT, et al. Spontaneous development of a pancreatic exocrine disease in CD28-deficient NOD mice. *J Immunol.* 2008;180(12):7793-7803.

174. Dijke IE, Hoeppli RE, Ellis T, et al. Discarded Human Thymus Is a Novel Source of Stable and Long-Lived Therapeutic Regulatory T Cells. *Am J Transplant.* 2016;16(1):58-71.

175. Putnam AL, Safinia N, Medvec A, et al. Clinical grade manufacturing of human alloantigen-reactive regulatory T cells for use in transplantation. *Am J Transplant.* 2013;13(11):3010-3020.

GENERAL BIBLIOGRAPHY

1. The CITR Coordinating Center and Investigators. The Collaborative Islet Transplant Registry (CITR) 2011 Seventh Annual Report
2. http://www.who.int/mediacentre/factsheets/fs312/en/.
3. 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. *The New England journal of medicine.* 1993;329(14):977-986.
4. Collaborative Islet Transplant Registry. 2015; http://www.citregistry.org.
5. The CITR Coordinating Center and Investigators. *The Collaborative Islet Transplant Registry (CITR)* 2016 Ninth Annual Report. US Department of Health and Human Services. Bethesda, MD, USA; 2016. Available from https://citregistry.org/system/files/9AR_Report.pdf. Accessed June 3, 2017.
6. Adler V, Yin Z, Tew KD, Ronai Z. Role of redox potential and reactive oxygen species in stress signaling. *Oncogene.* 1999;18(45):6104-6111.
7. Agarwal A, Brayman KL. Update on islet cell transplantation for type 1 diabetes. *Seminars in interventional radiology.* 2012;29(2):90-98.
8. Aglietti RA, Estevez A, Gupta A, et al. GsdmD p30 elicited by caspase-11 during pyroptosis forms pores in membranes. *Proceedings of the National Academy of Sciences of the United States of America.* 2016;113(28):7858-7863.
9. Anazawa T, Saito T, Goto M, et al. Long-term outcomes of clinical transplantation of pancreatic islets with uncontrolled donors after cardiac death:

a multicenter experience in Japan. *Transplantation proceedings.* 2014;46(6):1980-1984.

10. Anders HJ, Muruve DA. The inflammasomes in kidney disease. *J Am Soc Nephrol.* 2011;22(6):1007-1018.

11. Andersen HU, Jorgensen KH, Egeberg J, Mandrup-Poulsen T, Nerup J. Nicotinamide prevents interleukin-1 effects on accumulated insulin release and nitric oxide production in rat islets of Langerhans. *Diabetes.* 1994;43(6):770-777.

12. Andreone T, Meares GP, Hughes KJ, Hansen PA, Corbett JA. Cytokine-mediated beta-cell damage in PARP-1-deficient islets. *Am J Physiol Endocrinol Metab.* 2012;303(2):E172-179.

13. Andres A, Kin T, O'Gorman D, et al. Clinical islet isolation and transplantation outcomes with deceased cardiac death donors are similar to neurological determination of death donors. *Transpl Int.* 2016;29(1):34-40.

14. Armann B, Hanson MS, Hatch E, Steffen A, Fernandez LA. Quantification of basal and stimulated ROS levels as predictors of islet potency and function. *American journal of transplantation : official journal of the American Society of Transplantation and the American Society of Transplant Surgeons.* 2007;7(1):38-47.

15. Assady S, Maor G, Amit M, Itskovitz-Eldor J, Skorecki KL, Tzukerman M. Insulin production by human embryonic stem cells. *Diabetes.* 2001;50(8):1691-1697.

16. Avila J, Barbaro B, Gangemi A, et al. Intra-ductal glutamine administration reduces oxidative injury during human pancreatic islet isolation. *American*

journal of transplantation : official journal of the American Society of Transplantation and the American Society of Transplant Surgeons. 2005;5(12):2830-2837.

17. Avila JG, Tsujimura T, Oberholzer J, et al. Improvement of pancreatic islet isolation outcomes using glutamine perfusion during isolation procedure. *Cell transplantation.* 2003;12(8):877-881.

18. Baekkeskov S, Aanstoot HJ, Christgau S, et al. Identification of the 64K autoantigen in insulin-dependent diabetes as the GABA-synthesizing enzyme glutamic acid decarboxylase. *Nature.* 1990;347(6289):151-156.

19. Baidal DA, Ricordi C, Berman DM, et al. Bioengineering of an Intraabdominal Endocrine Pancreas. *The New England journal of medicine.* 2017;376(19):1887-1889.

20. Banting FG, Best CH, Collip JB, Campbell WR, Fletcher AA. Pancreatic Extracts in the Treatment of Diabetes Mellitus. *Canadian Medical Association journal.* 1922;12(3):141-146.

21. Bao J, Cai Y, Sun M, Wang G, Corke H. Anthocyanins, flavonols, and free radical scavenging activity of Chinese bayberry (Myrica rubra) extracts and their color properties and stability. *J Agric Food Chem.* 2005;53(6):2327-2332.

22. Barkai U, Weir GC, Colton CK, et al. Enhanced oxygen supply improves islet viability in a new bioartificial pancreas. *Cell transplantation.* 2013;22(8):1463-1476.

23. Barrangou R, Fremaux C, Deveau H, et al. CRISPR provides acquired resistance against viruses in prokaryotes. *Science.* 2007;315(5819):1709-1712.

24. Barshes NR, Lee TC, Goodpastor SE, et al. Transaminitis after pancreatic islet transplantation. *J Am Coll Surg.* 2005;200(3):353-361.

25. Barshes NR, Wyllie S, Goss JA. Inflammation-mediated dysfunction and apoptosis in pancreatic islet transplantation: implications for intrahepatic grafts. *Journal of leukocyte biology.* 2005;77(5):587-597.

26. Barton FB, Rickels MR, Alejandro R, et al. Improvement in outcomes of clinical islet transplantation: 1999-2010. *Diabetes care.* 2012;35(7):1436-1445.

27. Batinic-Haberle I, Tovmasyan A, Roberts ER, Vujaskovic Z, Leong KW, Spasojevic I. SOD therapeutics: latest insights into their structure-activity relationships and impact on the cellular redox-based signaling pathways. *Antioxid Redox Signal.* 2014;20(15):2372-2415.

28. Belghith M, Bluestone JA, Barriot S, Megret J, Bach JF, Chatenoud L. TGF-beta-dependent mechanisms mediate restoration of self-tolerance induced by antibodies to CD3 in overt autoimmune diabetes. *Nature medicine.* 2003;9(9):1202-1208.

29. Bellin MD, Barton FB, Heitman A, et al. Potent induction immunotherapy promotes long-term insulin independence after islet transplantation in type 1 diabetes. *American journal of transplantation : official journal of the American Society of Transplantation and the American Society of Transplant Surgeons.* 2012;12(6):1576-1583.

30. Benhamou PY, Watt PC, Mullen Y, et al. Human islet isolation in 104 consecutive cases. Factors affecting isolation success. *Transplantation.* 1994;57(12):1804-1810.

31. Berenson GS, Wattigney WA, Tracy RE, et al. Atherosclerosis of the aorta and coronary arteries and cardiovascular risk factors in persons aged 6 to 30 years and studied at necropsy (The Bogalusa Heart Study). *Am J Cardiol.* 1992;70(9):851-858.

32. Berney T, Johnson PR. Donor pancreata: evolving approaches to organ allocation for whole pancreas versus islet transplantation. *Transplantation.* 2010;90(3):238-243.

33. Bertera S, Crawford ML, Alexander AM, et al. Gene transfer of manganese superoxide dismutase extends islet graft function in a mouse model of autoimmune diabetes. *Diabetes.* 2003;52(2):387-393.

34. Bhatt S, Fung JJ, Lu L, Qian S. Tolerance-inducing strategies in islet transplantation. *International journal of endocrinology.* 2012;2012:396524.

35. 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(1):66-72.

36. Bloch K, Vennang J, Lazard D, Vardi P. Different susceptibility of rat pancreatic alpha and beta cells to hypoxia. *Histochemistry and cell biology.* 2012;137(6):801-810.

37. Bloom SR, Polak JM. Somatostatin. *British medical journal.* 1987;295(6593):288-290.

38. Bluestone JA, Buckner JH, Fitch M, et al. Type 1 diabetes immunotherapy using polyclonal regulatory T cells. *Sci Transl Med.* 2015;7(315):315ra189.

39. Bluestone JA, Herold K, Eisenbarth G. Genetics, pathogenesis and clinical interventions in type 1 diabetes. *Nature.* 2010;464(7293):1293-1300.

40. Bluestone JA, Tang Q. Immunotherapy: making the case for precision medicine. *Sci Transl Med.* 2015;7(280):280ed283.

41. Bonner-Weir S, Weir GC. New sources of pancreatic beta-cells. *Nature biotechnology.* 2005;23(7):857-861.

42. Bosi E, Braghi S, Maffi P, et al. Autoantibody response to islet transplantation in type 1 diabetes. *Diabetes.* 2001;50(11):2464-2471.

43. Bottino R, Balamurugan AN, Bertera S, Pietropaolo M, Trucco M, Piganelli JD. Preservation of human islet cell functional mass by anti-oxidative action of a novel SOD mimic compound. *Diabetes.* 2002;51(8):2561-2567.

44. Bottino R, Balamurugan AN, Tse H, et al. Response of human islets to isolation stress and the effect of antioxidant treatment. *Diabetes.* 2004;53(10):2559-2568.

45. Bottino R, Trucco M. Use of genetically-engineered pig donors in islet transplantation. *World J Transplant.* 2015;5(4):243-250.

46. Bouwens L, Houbracken I, Mfopou JK. The use of stem cells for pancreatic regeneration in diabetes mellitus. *Nat Rev Endocrinol.* 2013;9(10):598-606.

47. Bradley B, Prowse SJ, Bauling P, Lafferty KJ. Desferrioxamine treatment prevents chronic islet allograft damage. *Diabetes.* 1986;35(5):550-555.

48. Brandhorst D, Iken M, Bretzel RG, Brandhorst H. Pancreas storage in oxygenated perfluorodecalin does not restore post-transplant function of isolated pig islets pre-damaged by warm ischemia. *Xenotransplantation.* 2006;13(5):465-470.

49. Brandhorst H, Brandhorst D, Hering BJ, Federlin K, Bretzel RG. Body mass index of pancreatic donors: a decisive factor for human islet isolation. *Exp Clin Endocrinol Diabetes.* 1995;103 Suppl 2:23-26.

50. Brendel M HB, Shulz A, Bretzel R. International Islet Transplant Registry Report. 1999.

51. Bruin JE, Rezania A, Xu J, et al. Maturation and function of human embryonic stem cell-derived pancreatic progenitors in macroencapsulation devices following transplant into mice. *Diabetologia.* 2013;56(9):1987-1998.

52. Bruni A, Gala-Lopez B, Pepper AR, Abualhassan NS, Shapiro AJ. Islet cell transplantation for the treatment of type 1 diabetes: recent advances and future challenges. *Diabetes Metab Syndr Obes.* 2014;7:211-223.

53. Bruni A, Pepper AR, Gala-Lopez B, 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(4):e1190058.

54. Bruns H, Schemmer P. Machine perfusion in solid organ transplantation: where is the benefit? *Langenbecks Arch Surg.* 2014;399(4):421-427.

55. Butler AE, Janson J, Bonner-Weir S, Ritzel R, Rizza RA, Butler PC. Beta-cell deficit and increased beta-cell apoptosis in humans with type 2 diabetes. *Diabetes.* 2003;52(1):102-110.

56. Cabrera O, Jacques-Silva MC, Berman DM, et al. Automated, high-throughput assays for evaluation of human pancreatic islet function. *Cell transplantation.* 2008;16(10):1039-1048.

57. Cai H, Yang B, Xu Z, et al. Cyanidin-3-O-glucoside enhanced the function of syngeneic mouse islets transplanted under the kidney capsule or into the portal vein. *Transplantation.* 2015;99(3):508-514.

58. Cardona K, Korbutt GS, Milas Z, et al. Long-term survival of neonatal porcine islets in nonhuman primates by targeting costimulation pathways. *Nature medicine.* 2006;12(3):304-306.

59. Carobbio S, Ishihara H, Fernandez-Pascual S, Bartley C, Martin-Del-Rio R, Maechler P. Insulin secretion profiles are modified by overexpression of glutamate dehydrogenase in pancreatic islets. *Diabetologia.* 2004;47(2):266-276.

60. Chatenoud L. Humoral immune response against OKT3. *Transplantation proceedings.* 1993;25(2 Suppl 1):68-73.

61. Chatenoud L. OKT3-induced cytokine-release syndrome: prevention effect of anti-tumor necrosis factor monoclonal antibody. *Transplantation proceedings.* 1993;25(2 Suppl 1):47-51.

62. Chatenoud L, Primo J, Bach JF. CD3 antibody-induced dominant self tolerance in overtly diabetic NOD mice. *Journal of immunology.* 1997;158(6):2947-2954.

63. Chatenoud L, Thervet E, Primo J, Bach JF. Anti-CD3 antibody induces long-term remission of overt autoimmunity in nonobese diabetic mice. *Proceedings of the National Academy of Sciences of the United States of America.* 1994;91(1):123-127.

64. Conrad M, Angeli JP, Vandenabeele P, Stockwell BR. Regulated necrosis: disease relevance and therapeutic opportunities. *Nat Rev Drug Discov.* 2016;15(5):348-366.

65. Contreras JL, Eckstein C, Smyth CA, et al. Brain death significantly reduces isolated pancreatic islet yields and functionality in vitro and in vivo after transplantation in rats. *Diabetes.* 2003;52(12):2935-2942.

66. Cook JJ, Hudson I, Harrison LC, et al. Double-blind controlled trial of azathioprine in children with newly diagnosed type I diabetes. *Diabetes.* 1989;38(6):779-783.

67. Couri CE, Voltarelli JC. Autologous stem cell transplantation for early type 1 diabetes mellitus. *Autoimmunity.* 2008;41(8):666-672.

68. Curtin NJ, Szabo C. Therapeutic applications of PARP inhibitors: anticancer therapy and beyond. *Mol Aspects Med.* 2013;34(6):1217-1256.

69. D'Alessandro A M, Hoffmann RM, Knechtle SJ, et al. Liver transplantation from controlled non-heart-beating donors. *Surgery.* 2000;128(4):579-588.

70. D'Amour KA, Bang AG, Eliazer S, et al. Production of pancreatic hormone-expressing endocrine cells from human embryonic stem cells. *Nature biotechnology.* 2006;24(11):1392-1401.

71. Daley GQ. Missed opportunities in embryonic stem-cell research. *The New England journal of medicine.* 2004;351(7):627-628.

72. Daly KA, Liu S, Agrawal V, et al. Damage associated molecular patterns within xenogeneic biologic scaffolds and their effects on host remodeling. *Biomaterials.* 2012;33(1):91-101.

73. Davalli AM, Scaglia L, Zangen DH, Hollister J, Bonner-Weir S, Weir GC. Vulnerability of islets in the immediate posttransplantation period. Dynamic changes in structure and function. *Diabetes.* 1996;45(9):1161-1167.

74. Delmastro-Greenwood MM, Tse HM, Piganelli JD. Effects of metalloporphyrins on reducing inflammation and autoimmunity. *Antioxid Redox Signal.* 2014;20(15):2465-2477.

75. Dijke IE, Hoeppli RE, Ellis T, et al. Discarded Human Thymus Is a Novel Source of Stable and Long-Lived Therapeutic Regulatory T Cells. *American journal of transplantation : official journal of the American Society of Transplantation and the American Society of Transplant Surgeons.* 2016;16(1):58-71.

76. Dinarello CA, Simon A, van der Meer JW. Treating inflammation by blocking interleukin-1 in a broad spectrum of diseases. *Nat Rev Drug Discov.* 2012;11(8):633-652.

77. Dixon SJ, Lemberg KM, Lamprecht MR, et al. Ferroptosis: an iron-dependent form of nonapoptotic cell death. *Cell.* 2012;149(5):1060-1072.

78. Dixon SJ, Stockwell BR. The role of iron and reactive oxygen species in cell death. *Nat Chem Biol.* 2014;10(1):9-17.

79. do Amaral AS, Pawlick RL, Rodrigues E, 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(2):e55288.

80. Dolma S, Lessnick SL, Hahn WC, Stockwell BR. Identification of genotype-selective antitumor agents using synthetic lethal chemical screening in engineered human tumor cells. *Cancer Cell.* 2003;3(3):285-296.

81. Drognitz O, Obermaier R, Liu X, et al. Effects of organ preservation, ischemia time and caspase inhibition on apoptosis and microcirculation in rat pancreas transplantation. *American journal of transplantation : official journal of the American Society of Transplantation and the American Society of Transplant Surgeons.* 2004;4(7):1042-1050.

82. Drucker DJ, Dritselis A, Kirkpatrick P. Liraglutide. *Nat Rev Drug Discov.* 2010;9(4):267-268.

83. Dufrane D, Goebbels RM, Gianello P. Alginate macroencapsulation of pig islets allows correction of streptozotocin-induced diabetes in primates up to 6 months without immunosuppression. *Transplantation.* 2010;90(10):1054-1062.

84. Eich T, Eriksson O, Lundgren T, Nordic Network for Clinical Islet T. Visualization of early engraftment in clinical islet transplantation by positron-emission tomography. *The New England journal of medicine.* 2007;356(26):2754-2755.

85. Ekser B, Bottino R, Cooper DK. Clinical Islet Xenotransplantation: A Step Forward. *EBioMedicine.* 2016;12:22-23.

86. Elliott RB, Crossley JR, Berryman CC, James AG. Partial preservation of pancreatic beta-cell function in children with diabetes. *Lancet.* 1981;2(8247):631-632.

87. Emamaullee JA, Davis J, Pawlick R, et al. The caspase selective inhibitor EP1013 augments human islet graft function and longevity in marginal mass islet transplantation in mice. *Diabetes.* 2008;57(6):1556-1566.

88. Emamaullee JA, Davis J, Pawlick R, et al. Caspase inhibitor therapy synergizes with costimulation blockade to promote indefinite islet allograft survival. *Diabetes.* 2010;59(6):1469-1477.

89. Emamaullee JA, Shapiro AM. Interventional strategies to prevent beta-cell apoptosis in islet transplantation. *Diabetes.* 2006;55(7):1907-1914.

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

91. Emamaullee JA, Stanton L, Schur C, Shapiro AM. Caspase inhibitor therapy enhances marginal mass islet graft survival and preserves long-term function in islet transplantation. *Diabetes.* 2007;56(5):1289-1298.

92. England H, Summersgill HR, Edye ME, Rothwell NJ, Brough D. Release of interleukin-1alpha or interleukin-1beta depends on mechanism of cell death. *J Biol Chem.* 2014;289(23):15942-15950.

93. Espes D, Lau J, Quach M, Banerjee U, Palmer AF, Carlsson PO. Cotransplantation of Polymerized Hemoglobin Reduces beta-Cell Hypoxia and Improves beta-Cell Function in Intramuscular Islet Grafts. *Transplantation.* 2015;99(10):2077-2082.

94. Euvrard S, Kanitakis J, Claudy A. Skin cancers after organ transplantation. *The New England journal of medicine.* 2003;348(17):1681-1691.

95. Farney AC, Xenos E, Sutherland DE, et al. Inhibition of pancreatic islet beta cell function by tumor necrosis factor is blocked by a soluble tumor necrosis factor receptor. *Transplantation proceedings.* 1993;25(1 Pt 2):865-866.

96. Finkel T. Oxygen radicals and signaling. *Curr Opin Cell Biol.* 1998;10(2):248-253.

97. Fishman JA, Patience C. Xenotransplantation: infectious risk revisited. *American journal of transplantation : official journal of the American Society of Transplantation and the American Society of Transplant Surgeons.* 2004;4(9):1383-1390.

98. Frank A, Deng S, Huang X, et al. Transplantation for type I diabetes: comparison of vascularized whole-organ pancreas with isolated pancreatic islets. *Ann Surg.* 2004;240(4):631-640; discussion 640-633.

99. Friedmann Angeli JP, Schneider M, Proneth B, et al. Inactivation of the ferroptosis regulator Gpx4 triggers acute renal failure in mice. *Nat Cell Biol.* 2014;16(12):1180-1191.

100. Gabardi S, Martin ST, Roberts KL, Grafals M. Induction immunosuppressive therapies in renal transplantation. *American journal of health-system pharmacy : AJHP : official journal of the American Society of Health-System Pharmacists.* 2011;68(3):211-218.

101. Gala-Lopez B, Kin T, O'Gorman D, et al. The metalloporphyrin BMX-010 in human islet isolation and clinical transplantation *CellR4.* 2016;4(3):8.

102. Gala-Lopez B, Pepper AR, Shapiro AM. Biologic agents in islet transplantation. *Curr Diab Rep.* 2013;13(5):713-722.

103. 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 (5).

104. Gandy SE, Buse MG, Crouch RK. Protective role of superoxide dismutase against diabetogenic drugs. *J Clin Invest.* 1982;70(3):650-658.

105. Gatto C, Callegari M, Folin M, et al. Effects of cryopreservation and coculture with pancreatic ductal epithelial cells on insulin secretion from human pancreatic islets. *International journal of molecular medicine.* 2003;12(6):851-854.

106. Gibly RF, Graham JG, Luo X, Lowe WL, Jr., Hering BJ, Shea LD. Advancing islet transplantation: from engraftment to the immune response. *Diabetologia.* 2011;54(10):2494-2505.

107. Giovannoni L, Muller YD, Lacotte S, et al. Enhancement of islet engraftment and achievement of long-term islet allograft survival by Toll-like receptor 4 blockade. *Transplantation.* 2015;99(1):29-35.

108. Giraud S, Hauet T, Eugene M, Mauco G, Barrou B. A new preservation solution (SCOT 15) Improves the islet isolation process from pancreata of non-heart-beating donors: a Murine model. *Transplantation proceedings.* 2009;41(8):3293-3295.

109. Goto M, Eich TM, Felldin M, 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(9):1367-1375.

110. Goto M, Tjernberg J, Dufrane D, et al. Dissecting the instant blood-mediated inflammatory reaction in islet xenotransplantation. *Xenotransplantation.* 2008;15(4):225-234.

111. Gottlieb PA, Quinlan S, Krause-Steinrauf H, et al. Failure to preserve beta-cell function with mycophenolate mofetil and daclizumab combined therapy in patients with new- onset type 1 diabetes. *Diabetes care.* 2010;33(4):826-832.

112. Grankvist K, Marklund SL, Taljedal IB. CuZn-superoxide dismutase, Mn-superoxide dismutase, catalase and glutathione peroxidase in pancreatic islets and other tissues in the mouse. *Biochem J.* 1981;199(2):393-398.

113. Gray DW, Sutton R, McShane P, Peters M, Morris PJ. Exocrine contamination impairs implantation of pancreatic islets transplanted beneath the kidney capsule. *J Surg Res.* 1988;45(5):432-442.

114. Groth CG, Korsgren O, Tibell A, et al. Transplantation of porcine fetal pancreas to diabetic patients. *Lancet.* 1994;344(8934):1402-1404.

115. 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(6):710-715.

116. Hangauer MJ, Viswanathan VS, Ryan MJ, et al. Drug-tolerant persister cancer cells are vulnerable to GPX4 inhibition. *Nature.* 2017;551(7679):247-250.

117. Hanley SC, Paraskevas S, Rosenberg L. Donor and isolation variables predicting human islet isolation success. *Transplantation.* 2008;85(7):950-955.

118. Harrison LC, Colman PG, Dean B, Baxter R, Martin FI. Increase in remission rate in newly diagnosed type I diabetic subjects treated with azathioprine. *Diabetes.* 1985;34(12):1306-1308.

119. Heller B, Wang ZQ, Wagner EF, et al. Inactivation of the poly(ADP-ribose) polymerase gene affects oxygen radical and nitric oxide toxicity in islet cells. *J Biol Chem.* 1995;270(19):11176-11180.

120. Hengartner MO. The biochemistry of apoptosis. *Nature.* 2000;407(6805):770-776.

121. Hennige AM, Lembert N, Wahl MA, Ammon HP. Oxidative stress increases potassium efflux from pancreatic islets by depletion of intracellular calcium stores. *Free Radic Res.* 2000;33(5):507-516.

122. Hering BJ. Repurification: rescue rather than routine remedy. *American journal of transplantation : official journal of the American Society of Transplantation and the American Society of Transplant Surgeons.* 2005;5(1):1-2.

123. Hering BJ. Achieving and maintaining insulin independence in human islet transplant recipients. *Transplantation.* 2005;79(10):1296-1297.

124. Hering BJ, Clarke WR, Bridges ND, et al. Phase 3 Trial of Transplantation of Human Islets in Type 1 Diabetes Complicated by Severe Hypoglycemia. *Diabetes care.* 2016;39(7):1230-1240.

125. Hering BJ, Kandaswamy R, Ansite JD, et al. Single-donor, marginal-dose islet transplantation in patients with type 1 diabetes. *JAMA : the journal of the American Medical Association.* 2005;293(7):830-835.

126. Hering BJ, Kandaswamy R, Harmon JV, et al. Transplantation of cultured islets from two-layer preserved pancreases in type 1 diabetes with anti-CD3 antibody. *American journal of transplantation : official journal of the American Society of*

Transplantation and the American Society of Transplant Surgeons. 2004;4(3):390-401.

127. Hering BJ, Walawalkar N. Pig-to-nonhuman primate islet xenotransplantation. *Transpl Immunol.* 2009;21(2):81-86.

128. Hering BJ, Wijkstrom M, Graham ML, et al. Prolonged diabetes reversal after intraportal xenotransplantation of wild-type porcine islets in immunosuppressed nonhuman primates. *Nature medicine.* 2006;12(3):301-303.

129. Herold KC, Bluestone JA. Type 1 diabetes immunotherapy: is the glass half empty or half full? *Sci Transl Med.* 2011;3(95):95fs91.

130. Herold KC, Bluestone JA, Montag AG, et al. Prevention of autoimmune diabetes with nonactivating anti-CD3 monoclonal antibody. *Diabetes.* 1992;41(3):385-391.

131. Herold KC, Gitelman SE, Ehlers MR, et al. Teplizumab (anti-CD3 mAb) treatment preserves C-peptide responses in patients with new-onset type 1 diabetes in a randomized controlled trial: metabolic and immunologic features at baseline identify a subgroup of responders. *Diabetes.* 2013;62(11):3766-3774.

132. Herold KC, Gitelman SE, Masharani U, et al. A single course of anti-CD3 monoclonal antibody hOKT3gamma1(Ala-Ala) results in improvement in C-peptide responses and clinical parameters for at least 2 years after onset of type 1 diabetes. *Diabetes.* 2005;54(6):1763-1769.

133. Herold KC, Pescovitz MD, McGee P, et al. Increased T cell proliferative responses to islet antigens identify clinical responders to anti-CD20 monoclonal

antibody (rituximab) therapy in type 1 diabetes. *Journal of immunology.* 2011;187(4):1998-2005.

134. Hori Y, Rulifson IC, Tsai BC, Heit JJ, Cahoy JD, Kim SK. Growth inhibitors promote differentiation of insulin-producing tissue from embryonic stem cells. *Proceedings of the National Academy of Sciences of the United States of America.* 2002;99(25):16105-16110.

135. http://www.transplant-observatory.org/data-charts-and-tables/.

136. 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(6):843-850.

137. 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

138. Ihm SH, Matsumoto I, Sawada T, et al. Effect of donor age on function of isolated human islets. *Diabetes.* 2006;55(5):1361-1368.

139. Ishino Y, Shinagawa H, Makino K, Amemura M, Nakata A. Nucleotide sequence of the iap gene, responsible for alkaline phosphatase isozyme conversion in Escherichia coli, and identification of the gene product. *J Bacteriol.* 1987;169(12):5429-5433.

140. Itoh T, Iwahashi S, Kanak MA, et al. Elevation of high-mobility group box 1 after clinical autologous islet transplantation and its inverse correlation with outcomes. *Cell transplantation.* 2014;23(2):153-165.

141. Itoh T, Takita M, SoRelle JA, et al. Correlation of released HMGB1 levels with the degree of islet damage in mice and humans and with the outcomes of islet transplantation in mice. *Cell transplantation.* 2012;21(7):1371-1381.

142. Jang HJ, Kwak JH, Cho EY, et al. Glutamine induces heat-shock protein-70 and glutathione expression and attenuates ischemic damage in rat islets. *Transplantation proceedings.* 2008;40(8):2581-2584.

143. Jang KH, Do YJ, Son D, Son E, Choi JS, Kim E. AIF-independent parthanatos in the pathogenesis of dry age-related macular degeneration. *Cell Death Dis.* 2017;8(1):e2526.

144. Ji M, Yi S, Smith-Hurst H, et al. The importance of tissue factor expression by porcine NICC in triggering IBMIR in the xenograft setting. *Transplantation.* 2011;91(8):841-846.

145. Jindal RM, Sidner RA, McDaniel HB, Johnson MS, Fineberg SE. Intraportal vs kidney subcapsular site for human pancreatic islet transplantation. *Transplantation proceedings.* 1998;30(2):398-399.

146. Jorgensen I, Miao EA. Pyroptotic cell death defends against intracellular pathogens. *Immunological reviews.* 2015;265(1):130-142.

147. Jorgensen I, Zhang Y, Krantz BA, Miao EA. Pyroptosis triggers pore-induced intracellular traps (PITs) that capture bacteria and lead to their clearance by efferocytosis. *J Exp Med.* 2016;213(10):2113-2128.

148. Kaczmarek A, Vandenabeele P, Krysko DV. Necroptosis: the release of damage-associated molecular patterns and its physiological relevance. *Immunity.* 2013;38(2):209-223.

149. Kaiser WJ, Upton JW, Long AB, et al. RIP3 mediates the embryonic lethality of caspase-8-deficient mice. *Nature.* 2011;471(7338):368-372.

150. Kang TB, Yang SH, Toth B, Kovalenko A, Wallach D. Caspase-8 blocks kinase RIPK3-mediated activation of the NLRP3 inflammasome. *Immunity.* 2013;38(1):27-40.

151. Keen H. The Diabetes Control and Complications Trial (DCCT). *Health Trends.* 1994;26(2):41-43.

152. Kelly OG, Chan MY, Martinson LA, et al. Cell-surface markers for the isolation of pancreatic cell types derived from human embryonic stem cells. *Nature biotechnology.* 2011;29(8):750-756.

153. Kelly WD, Lillehei RC, Merkel FK, Idezuki Y, Goetz FC. Allotransplantation of the pancreas and duodenum along with the kidney in diabetic nephropathy. *Surgery.* 1967;61(6):827-837.

154. Keymeulen B, Walter M, Mathieu C, et al. Four-year metabolic outcome of a randomised controlled CD3-antibody trial in recent-onset type 1 diabetic patients depends on their age and baseline residual beta cell mass. *Diabetologia.* 2010;53(4):614-623.

155. Kim JS, Jang HJ, Kim SS, et al. Red Ginseng Administration Before Islet Isolation Attenuates Apoptosis and Improves Islet Function and Transplant Outcome in a Syngeneic Mouse Marginal Islet Mass Model. *Transplantation proceedings.* 2016;48(4):1258-1265.

156. Kin T. Islet isolation for clinical transplantation. *Adv Exp Med Biol.* 2010;654:683-710.

157. Kin T, Murdoch TB, Shapiro AM, Lakey JR. Estimation of pancreas weight from donor variables. *Cell Transplant.* 2006;15(2):181-185.

158. 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(11):1029-1035.

159. King H, Aubert RE, Herman WH. Global burden of diabetes, 1995-2025: prevalence, numerical estimates, and projections. *Diabetes care.* 1998;21(9):1414-1431.

160. Koh A, Senior P, Salam A, et al. Insulin-heparin infusions peritransplant substantially improve single-donor clinical islet transplant success. *Transplantation.* 2010;89(4):465-471.

161. Kono H, Rock KL. How dying cells alert the immune system to danger. *Nat Rev Immunol.* 2008;8(4):279-289.

162. Korsgren E, Korsgren O. Glucose Effectiveness: The Mouse Trap in the Development of Novel ss-Cell Replacement Therapies. *Transplantation.* 2016;100(1):111-115.

163. Korsgren O, Lundgren T, Felldin M, et al. Optimising islet engraftment is critical for successful clinical islet transplantation. *Diabetologia.* 2008;51(2):227-232.

164. Koulajian K, Ivovic A, Ye K, et al. Overexpression of glutathione peroxidase 4 prevents beta-cell dysfunction induced by prolonged elevation of lipids in vivo. *Am J Physiol Endocrinol Metab.* 2013;305(2):E254-262.

165. Kroon E, Martinson LA, Kadoya K, et al. Pancreatic endoderm derived from human embryonic stem cells generates glucose-responsive insulin-secreting cells in vivo. *Nature biotechnology.* 2008;26(4):443-452.

166. Lacy PE, Kostianovsky M. Method for the isolation of intact islets of Langerhans from the rat pancreas. *Diabetes.* 1967;16(1):35-39.

167. Lacy PE, Ricordi C, Finke EH. Effect of transplantation site and alpha L3T4 treatment on survival of rat, hamster, and rabbit islet xenografts in mice. *Transplantation.* 1989;47(5):761-766.

168. Lakey JR, Rajotte RV, Warnock GL, Kneteman NM. Human pancreas preservation prior to islet isolation. Cold ischemic tolerance. *Transplantation.* 1995;59(5):689-694.

169. Lakey JR, Warnock GL, Rajotte RV, et al. Variables in organ donors that affect the recovery of human islets of Langerhans. *Transplantation.* 1996;61(7):1047-1053.

170. Lakey JR, Warnock GL, Shapiro AM, et al. Intraductal collagenase delivery into the human pancreas using syringe loading or controlled perfusion. *Cell transplantation.* 1999;8(3):285-292.

171. Lakey JR, Woods EJ, Zieger MA, et al. Improved islet survival and in vitro function using solubilized small intestinal submucosa. *Cell and tissue banking.* 2001;2(4):217-224.

172. Land WG, Agostinis P, Gasser S, Garg AD, Linkermann A. DAMP-Induced Allograft and Tumor Rejection: The Circle Is Closing. *American journal of*

transplantation : official journal of the American Society of Transplantation and the American Society of Transplant Surgeons. 2016;16(12):3322-3337.

173. Land WG, Agostinis P, Gasser S, Garg AD, Linkermann A. Transplantation and Damage-Associated Molecular Patterns (DAMPs). *American journal of transplantation : official journal of the American Society of Transplantation and the American Society of Transplant Surgeons.* 2016;16(12):3338-3361.

174. Larsen CM, Faulenbach M, Vaag A, et al. [Interleukin-1 receptor antagonist-treatment of patients with type 2 diabetes]. *Ugeskr Laeger.* 2007;169(45):3868-3871.

175. Lau A, Wang S, Jiang J, et al. RIPK3-mediated necroptosis promotes donor kidney inflammatory injury and reduces allograft survival. *American journal of transplantation : official journal of the American Society of Transplantation and the American Society of Transplant Surgeons.* 2013;13(11):2805-2818.

176. Lawlor KE, Khan N, Mildenhall A, et al. RIPK3 promotes cell death and NLRP3 inflammasome activation in the absence of MLKL. *Nat Commun.* 2015;6:6282.

177. Lensch MW, Schlaeger TM, Zon LI, Daley GQ. Teratoma formation assays with human embryonic stem cells: a rationale for one type of human-animal chimera. *Cell Stem Cell.* 2007;1(3):253-258.

178. Lenzen S. The mechanisms of alloxan- and streptozotocin-induced diabetes. *Diabetologia.* 2008;51(2):216-226.

179. Lenzen S, Drinkgern J, Tiedge M. Low antioxidant enzyme gene expression in pancreatic islets compared with various other mouse tissues. *Free Radic Biol Med.* 1996;20(3):463-466.

180. Li C, Buettger C, Kwagh J, et al. A signaling role of glutamine in insulin secretion. *J Biol Chem.* 2004;279(14):13393-13401.

181. Linkermann A. Nonapoptotic cell death in acute kidney injury and transplantation. *Kidney Int.* 2016;89(1):46-57.

182. Linkermann A, Green DR. Necroptosis. *The New England journal of medicine.* 2014;370(5):455-465.

183. Linkermann A, Hackl MJ, Kunzendorf U, Walczak H, Krautwald S, Jevnikar AM. Necroptosis in immunity and ischemia-reperfusion injury. *American journal of transplantation : official journal of the American Society of Transplantation and the American Society of Transplant Surgeons.* 2013;13(11):2797-2804.

184. Linkermann A, Skouta R, Himmerkus N, et al. Synchronized renal tubular cell death involves ferroptosis. *Proceedings of the National Academy of Sciences of the United States of America.* 2014;111(47):16836-16841.

185. Linkermann A, Stockwell BR, Krautwald S, Anders HJ. Regulated cell death and inflammation: an auto-amplification loop causes organ failure. *Nat Rev Immunol.* 2014;14(11):759-767.

186. Liu X, Wang Y, Li Y, Pei X. Research status and prospect of stem cells in the treatment of diabetes mellitus. *Sci China Life Sci.* 2013;56(4):306-312.

187. Ludwig B, Reichel A, Steffen A, et al. Transplantation of human islets without immunosuppression. *Proceedings of the National Academy of Sciences of the United States of America.* 2013;110(47):19054-19058.

188. 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(5520):1389-1394.

189. Ly TT, Nicholas JA, Retterath A, Lim EM, Davis EA, Jones TW. Effect of sensor-augmented insulin pump therapy and automated insulin suspension vs standard insulin pump therapy on hypoglycemia in patients with type 1 diabetes: a randomized clinical trial. *JAMA : the journal of the American Medical Association.* 2013;310(12):1240-1247.

190. Ma X, Ye B, Gao F, et al. Tissue factor knockdown in porcine islets: an effective approach to suppressing the instant blood-mediated inflammatory reaction. *Cell transplantation.* 2012;21(1):61-71.

191. Mandel TE, Koulmanda M, Cozzi E, et al. Transplantation of normal and DAF-transgenic fetal pig pancreas into cynomolgus monkeys. *Transplantation proceedings.* 1997;29(1-2 /01):940.

192. Markmann JF, Deng S, Desai NM, et al. The use of non-heart-beating donors for isolated pancreatic islet transplantation. *Transplantation.* 2003;75(9):1423-1429.

193. Masters SL, Dunne A, Subramanian SL, et al. Activation of the NLRP3 inflammasome by islet amyloid polypeptide provides a mechanism for enhanced IL-1beta in type 2 diabetes. *Nat Immunol.* 2010;11(10):897-904.

194. Matsumoto I, Sawada T, Nakano M, et al. Improvement in islet yield from obese donors for human islet transplants. *Transplantation.* 2004;78(6):880-885.

195. Matsumoto S, Abalovich A, Wechsler C, Wynyard S, Elliott RB. Clinical Benefit of Islet Xenotransplantation for the Treatment of Type 1 Diabetes. *EBioMedicine.* 2016;12:255-262.

196. Matsumoto S, Takita M, Chaussabel D, et al. Improving Efficacy of Clinical Islet Transplantation with Iodixanol Based Islet Purification, Thymoglobulin Induction and Blockage of IL-1-beta and TNF-alpha. *Cell transplantation.* 2011.

197. Matsumoto S, Takita M, Chaussabel D, et al. Improving efficacy of clinical islet transplantation with iodixanol-based islet purification, thymoglobulin induction, and blockage of IL-1beta and TNF-alpha. *Cell transplantation.* 2011;20(10):1641-1647.

198. Matsumoto S, Tan P, Baker J, et al. Clinical porcine islet xenotransplantation under comprehensive regulation. *Transplantation proceedings.* 2014;46(6):1992-1995.

199. McCall M, Pawlick R, Kin T, Shapiro AM. Anakinra potentiates the protective effects of etanercept in transplantation of marginal mass human islets in immunodeficient mice. *American journal of transplantation : official journal of the American Society of Transplantation and the American Society of Transplant Surgeons.* 2012;12(2):322-329.

200. McCall M, Toso C, Emamaullee J, et al. The caspase inhibitor IDN-6556 (PF3491390) improves marginal mass engraftment after islet transplantation in mice. *Surgery.* 2011;150(1):48-55.

201. McCall MD, Maciver AM, Kin T, et al. Caspase inhibitor IDN6556 facilitates marginal mass islet engraftment in a porcine islet autotransplant model. *Transplantation.* 2012;94(1):30-35.

202. McIlwain DR, Berger T, Mak TW. Caspase functions in cell death and disease. *Cold Spring Harb Perspect Biol.* 2013;5(4):a008656.

203. Meagher C, Tang Q, Fife BT, et al. Spontaneous development of a pancreatic exocrine disease in CD28-deficient NOD mice. *Journal of immunology.* 2008;180(12):7793-7803.

204. Mellgren A, Schnell Landstrom AH, Petersson B, Andersson A. The renal subcapsular site offers better growth conditions for transplanted mouse pancreatic islet cells than the liver or spleen. *Diabetologia.* 1986;29(9):670-672.

205. Melzi R, Mercalli A, Sordi V, et al. Role of CCL2/MCP-1 in islet transplantation. *Cell transplantation.* 2010;19(8):1031-1046.

206. Merani S, Shapiro AM. Current status of pancreatic islet transplantation. *Clin Sci (Lond).* 2006;110(6):611-625.

207. Merani S, Toso C, Emamaullee J, Shapiro AM. Optimal implantation site for pancreatic islet transplantation. *The British journal of surgery.* 2008;95(12):1449-1461.

208. Merani S, Truong W, Emamaullee JA, Toso C, Knudsen LB, Shapiro AM. Liraglutide, a long-acting human glucagon-like peptide 1 analog, improves glucose homeostasis in marginal mass islet transplantation in mice. *Endocrinology.* 2008;149(9):4322-4328.

209. Misler S. The isolated pancreatic islet as a micro-organ and its transplantation to cure diabetes: celebrating the legacy of Paul Lacy. *Islets.* 2010;2(4):210-224.

210. Miwa I, Ichimura N, Sugiura M, Hamada Y, Taniguchi S. Inhibition of glucose-induced insulin secretion by 4-hydroxy-2-nonenal and other lipid peroxidation products. *Endocrinology.* 2000;141(8):2767-2772.

211. Mojica FJ, Diez-Villasenor C, Soria E, Juez G. Biological significance of a family of regularly spaced repeats in the genomes of Archaea, Bacteria and mitochondria. *Mol Microbiol.* 2000;36(1):244-246.

212. Montolio M, Biarnes M, Tellez N, Escoriza J, Soler J, Montanya E. Interleukin-1beta and inducible form of nitric oxide synthase expression in early syngeneic islet transplantation. *The Journal of endocrinology.* 2007;192(1):169-177.

213. 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.

214. Murugan V. Embryonic stem cell research: a decade of debate from Bush to Obama. *Yale J Biol Med.* 2009;82(3):101-103.

215. Nagata NA, Inoue K, Tabata Y. Co-culture of extracellular matrix suppresses the cell death of rat pancreatic islets. *Journal of biomaterials science. Polymer edition.* 2002;13(5):579-590.

216. Najarian JS, Sutherland DE, Matas AJ, Steffes MW, Simmons RL, Goetz FC. Human islet transplantation: a preliminary report. *Transplantation proceedings.* 1977;9(1):233-236.

217. Nano R, Clissi B, Melzi R, et al. Islet isolation for allotransplantation: variables associated with successful islet yield and graft function. *Diabetologia.* 2005;48(5):906-912.

218. Narang AS, Cheng K, Henry J, et al. Vascular endothelial growth factor gene delivery for revascularization in transplanted human islets. *Pharmaceutical research.* 2004;21(1):15-25.

219. Nathan DM, Lachin J, Cleary P, et al. Intensive diabetes therapy and carotid intima-media thickness in type 1 diabetes mellitus. *The New England journal of medicine.* 2003;348(23):2294-2303.

220. Nilsson B, Ekdahl KN, Korsgren O. Control of instant blood-mediated inflammatory reaction to improve islets of Langerhans engraftment. *Current opinion in organ transplantation.* 2011;16(6):620-626.

221. Niu D, Wei HJ, Lin L, et al. Inactivation of porcine endogenous retrovirus in pigs using CRISPR-Cas9. *Science.* 2017;357(6357):1303-1307.

222. Noguchi H. Pancreas procurement and preservation for islet transplantation: personal considerations. *Journal of transplantation.* 2011;2011:783168.

223. Nomikos IN, Prowse SJ, Carotenuto P, Lafferty KJ. Combined treatment with nicotinamide and desferrioxamine prevents islet allograft destruction in NOD mice. *Diabetes.* 1986;35(11):1302-1304.

224. O'Gorman D, Kin T, Murdoch T, et al. The standardization of pancreatic donors for islet isolation. *Transplant Proc.* 2005;37(2):1309-1310.

225. O'Gorman D, Kin T, Murdoch T, et al. The standardization of pancreatic donors for islet isolations. *Transplantation.* 2005;80(6):801-806.

226. Oberst A, Dillon CP, Weinlich R, et al. Catalytic activity of the caspase-8-FLIP(L) complex inhibits RIPK3-dependent necrosis. *Nature.* 2011;471(7338):363-367.

227. Oberst A, Green DR. It cuts both ways: reconciling the dual roles of caspase 8 in cell death and survival. *Nat Rev Mol Cell Biol.* 2011;12(11):757-763.

228. Ojo AO, Held PJ, Port FK, et al. Chronic renal failure after transplantation of a nonrenal organ. *The New England journal of medicine.* 2003;349(10):931-940.

229. Otonkoski T, Gao R, Lundin K. Stem cells in the treatment of diabetes. *Ann Med.* 2005;37(7):513-520.

230. Otterbein LE, Fan Z, Koulmanda M, Thronley T, Strom TB. Innate immunity for better or worse govern the allograft response. *Current opinion in organ transplantation.* 2015;20(1):8-12.

231. Ozmen L, Ekdahl KN, Elgue G, Larsson R, Korsgren O, Nilsson B. Inhibition of thrombin abrogates the instant blood-mediated inflammatory reaction triggered by isolated human islets: possible application of the thrombin inhibitor melagatran in clinical islet transplantation. *Diabetes.* 2002;51(6):1779-1784.

232. Pagliuca FW, Millman JR, Gurtler M, et al. Generation of functional human pancreatic beta cells in vitro. *Cell.* 2014;159(2):428-439.

233. Paraskevas S, Maysinger D, Wang R, Duguid TP, Rosenberg L. Cell loss in isolated human islets occurs by apoptosis. *Pancreas.* 2000;20(3):270-276.

234. Paredes-Juarez GA, Sahasrabudhe NM, Tjoelker RS, et al. DAMP production by human islets under low oxygen and nutrients in the presence or absence of an immunoisolating-capsule and necrostatin-1. *Sci Rep.* 2015;5:14623.

235. Park CG, Bottino R, Hawthorne WJ. Current status of islet xenotransplantation. *Int J Surg.* 2015;23(Pt B):261-266.

236. Pasparakis M, Vandenabeele P. Necroptosis and its role in inflammation. *Nature.* 2015;517(7534):311-320.

237. Pavlosky A, Lau A, Su Y, et al. RIPK3-mediated necroptosis regulates cardiac allograft rejection. *American journal of transplantation : official journal of the American Society of Transplantation and the American Society of Transplant Surgeons.* 2014;14(8):1778-1790.

238. Pearson T, Markees TG, Serreze DV, et al. Islet cell autoimmunity and transplantation tolerance: two distinct mechanisms? *Annals of the New York Academy of Sciences.* 2003;1005:148-156.

239. Penn I. Cancers complicating organ transplantation. *The New England journal of medicine.* 1990;323(25):1767-1769.

240. Pepper AR, Bruni A, Pawlick R, et al. Engraftment Site and Effectiveness of the Pan-Caspase Inhibitor F573 to Improve Engraftment in Mouse and Human Islet Transplantation in Mice. *Transplantation.* 2017.

241. Pepper AR, Bruni A, Pawlick R, et al. Engraftment Site and Effectiveness of the Pan-Caspase Inhibitor F573 to Improve Engraftment in Mouse and Human Islet Transplantation in Mice. *Transplantation.* 2017;101(10):2321-2329.

242. Pepper AR, Gala-Lopez B, Pawlick R, Merani S, Kin T, Shapiro AM. A prevascularized subcutaneous device-less site for islet and cellular transplantation. *Nature biotechnology.* 2015;33(5):518-523.

243. Pepper AR, Gala-Lopez B, Ziff O, Shapiro AJ. Current status of clinical islet transplantation. *World J Transplant.* 2013;3(4):48-53.

244. Pepper AR, Gala-Lopez B, Ziff O, Shapiro AM. Revascularization of transplanted pancreatic islets and role of the transplantation site. *Clinical & developmental immunology.* 2013;2013:352315.

245. Pepper AR, Pawlick R, Bruni A, et al. Harnessing the Foreign Body Reaction in Marginal Mass Device-less Subcutaneous Islet Transplantation in Mice. *Transplantation.* 2016;100(7):1474-1479.

246. Pepper AR, Pawlick R, Bruni A, et al. Transplantation of Human Pancreatic Endoderm Cells Reverses Diabetes Post Transplantation in a Prevascularized Subcutaneous Site. *Stem Cell Reports.* 2017;8(6):1689-1700.

247. Phelps CJ, Koike C, Vaught TD, et al. Production of alpha 1,3-galactosyltransferase-deficient pigs. *Science.* 2003;299(5605):411-414.

248. Piemonti L, Leone BE, Nano R, et al. Human pancreatic islets produce and secrete MCP-1/CCL2: relevance in human islet transplantation. *Diabetes.* 2002;51(1):55-65.

249. Piganelli JD, Flores SC, Cruz C, et al. A metalloporphyrin-based superoxide dismutase mimic inhibits adoptive transfer of autoimmune diabetes by a diabetogenic T-cell clone. *Diabetes.* 2002;51(2):347-355.

250. Pleau JM, Fernandez-Saravia F, Esling A, Homo-Delarche F, Dardenne M. Prevention of autoimmune diabetes in nonobese diabetic female mice by treatment with recombinant glutamic acid decarboxylase (GAD 65). *Clinical immunology and immunopathology.* 1995;76(1 Pt 1):90-95.

251. Plesner A, Verchere CB. Advances and challenges in islet transplantation: islet procurement rates and lessons learned from suboptimal islet transplantation. *Journal of transplantation.* 2011;2011:979527.

252. Ploeg RJ, Friend PJ. New strategies in organ preservation: current and future role of machine perfusion in organ transplantation. *Transpl Int.* 2015;28(6):633.

253. Polonsky KS. The past 200 years in diabetes. *The New England journal of medicine.* 2012;367(14):1332-1340.

254. Posselt AM, Szot GL, Frassetto LA, 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(12):1595-1601.

255. Potter KJ, Scrocchi LA, Warnock GL, et al. Amyloid inhibitors enhance survival of cultured human islets. *Biochim Biophys Acta.* 2009;1790(6):566-574.

256. Putnam AL, Safinia N, Medvec A, et al. Clinical grade manufacturing of human alloantigen-reactive regulatory T cells for use in transplantation. *American journal of transplantation : official journal of the American Society of Transplantation and the American Society of Transplant Surgeons.* 2013;13(11):3010-3020.

257. Rabinovitch A, Suarez-Pinzon WL, Strynadka K, et al. Human pancreatic islet beta-cell destruction by cytokines is independent of nitric oxide production. *J Clin Endocrinol Metab.* 1994;79(4):1058-1062.

258. Rafael E, Wu GS, Hultenby K, Tibell A, Wernerson A. Improved survival of macroencapsulated islets of Langerhans by preimplantation of the

immunoisolating device: a morphometric study. *Cell transplantation.* 2003;12(4):407-412.

259. Rajab A. Islet transplantation: alternative sites. *Curr Diab Rep.* 2010;10(5):332-337.

260. Reckard CR, Ziegler MM, Barker CF. Physiological and immunological consequences of transplanting isolated pancreatic islets. *Surgery.* 1973;74(1):91-99.

261. Reed JC, Pellecchia M. Ironing out cell death mechanisms. *Cell.* 2012;149(5):963-965.

262. Rezania A, Bruin JE, Arora P, et al. Reversal of diabetes with insulin-producing cells derived in vitro from human pluripotent stem cells. *Nature biotechnology.* 2014;32(11):1121-1133.

263. Rezania A, Bruin JE, Riedel MJ, 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(8):2016-2029.

264. Ricordi C, Goldstein JS, Balamurugan AN, et al. NIH-sponsored Clinical Islet Transplantation Consortium Phase 3 Trial: Manufacture of a Complex Cellular Product at Eight Processing Facilities. *Diabetes.* 2016.

265. Ricordi C, Lacy PE, Finke EH, Olack BJ, Scharp DW. Automated method for isolation of human pancreatic islets. *Diabetes.* 1988;37(4):413-420.

266. Ricordi C, Lacy PE, Scharp DW. Automated islet isolation from human pancreas. *Diabetes.* 1989;38 Suppl 1:140-142.

267. Robertson RP, Harmon J, Tran PO, Poitout V. Beta-cell glucose toxicity, lipotoxicity, and chronic oxidative stress in type 2 diabetes. *Diabetes.* 2004;53 Suppl 1:S119-124.

268. Roep BO, Atkinson M. Animal models have little to teach us about type 1 diabetes: 1. In support of this proposal. *Diabetologia.* 2004;47(10):1650-1656.

269. Roep BO, Atkinson M, von Herrath M. Satisfaction (not) guaranteed: re-evaluating the use of animal models of type 1 diabetes. *Nat Rev Immunol.* 2004;4(12):989-997.

270. Roep BO, Tree TI. Immune modulation in humans: implications for type 1 diabetes mellitus. *Nat Rev Endocrinol.* 2014;10(4):229-242.

271. Rood PP, Bottino R, Balamurugan AN, et al. Reduction of early graft loss after intraportal porcine islet transplantation in monkeys. *Transplantation.* 2007;83(2):202-210.

272. Rosenberg L, Wang R, Paraskevas S, Maysinger D. Structural and functional changes resulting from islet isolation lead to islet cell death. *Surgery.* 1999;126(2):393-398.

273. Ryan EA, Bigam D, Shapiro AM. Current indications for pancreas or islet transplant. *Diabetes, obesity & metabolism.* 2006;8(1):1-7.

274. Ryan EA, Paty BW, Senior PA, et al. Five-year follow-up after clinical islet transplantation. *Diabetes.* 2005;54(7):2060-2069.

275. 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(2):343-347.

276. Saito T, Gotoh M, Satomi S, et al. Islet transplantation using donors after cardiac death: report of the Japan Islet Transplantation Registry. *Transplantation.* 2010;90(7):740-747.

277. Saito T, Gotoh M, Satomi S, et al. Islet transplantation using donors after cardiac death: report of the Japan Islet Transplantation Registry. *Transplantation.* 2010;90(7):740-747.

278. Sandler S, Bendtzen K, Borg LA, Eizirik DL, Strandell E, Welsh N. Studies on the mechanisms causing inhibition of insulin secretion in rat pancreatic islets exposed to human interleukin-1 beta indicate a perturbation in the mitochondrial function. *Endocrinology.* 1989;124(3):1492-1501.

279. Sangiuliano B, Perez NM, Moreira DF, Belizario JE. Cell death-associated molecular-pattern molecules: inflammatory signaling and control. *Mediators Inflamm.* 2014;2014:821043.

280. Scharp DW, Kemp CB, Knight MJ, Ballinger WF, Lacy PE. The use of ficoll in the preparation of viable islets of langerhans from the rat pancreas. *Transplantation.* 1973;16(6):686-689.

281. Schulz TC. Concise Review: Manufacturing of Pancreatic Endoderm Cells for Clinical Trials in Type 1 Diabetes. *Stem Cells Transl Med.* 2015;4(8):927-931.

282. Schulz TC, Young HY, Agulnick AD, et al. A scalable system for production of functional pancreatic progenitors from human embryonic stem cells. *PLoS One.* 2012;7(5):e37004.

283. Schwarznau A, Hanson MS, Sperger JM, et al. IL-1beta receptor blockade protects islets against pro-inflammatory cytokine induced necrosis and apoptosis. *J Cell Physiol.* 2009;220(2):341-347.

284. Selck FW, Grossman EB, Ratner LE, Renz JF. Utilization, outcomes, and retransplantation of liver allografts from donation after cardiac death: implications for further expansion of the deceased-donor pool. *Ann Surg.* 2008;248(4):599-607.

285. Senior PA, Kin T, Shapiro AMJ, Koh A. Islet Transplantation at the University of Alberta: Status Update and Review of Progress over the Last Decade. *Canadian Journal of Diabetes.* 2012;36:32-37.

286. Senior PA KT, Shapiro AMJ, Koh A. Islet transplantation at the University of Alberta: Status update and review of progress over the last decade. *Can J Diabetes* 2012(36):32 - 37.

287. Shapiro AM. A historical perspective on experimental and clinical islet transplantation. *Informa Health Care.* 2007:1.

288. Shapiro AM. A historical perspective on experimental and clinical islet transplantation. In: Shapiro AM, Shaw J.A., ed. *Islet transplantation and beta cell replacement therapy*. New York, London: Informa Healthcare; 2007.

289. Shapiro AM. Strategies toward single-donor islets of Langerhans transplantation. *Current opinion in organ transplantation.* 2011;16(6):627-631.

290. Shapiro AM. Immune antibody monitoring predicts outcome in islet transplantation. *Diabetes.* 2013;62(5):1377-1378.

291. Shapiro AM, Lakey JR, Ryan EA, 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(4):230-238.

292. Shapiro AM, Nanji SA, Lakey JR. Clinical islet transplant: current and future directions towards tolerance. *Immunological reviews.* 2003;196:219-236.

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

294. Shapiro AM, Ricordi C. Unraveling the secrets of single donor success in islet transplantation. *American journal of transplantation : official journal of the American Society of Transplantation and the American Society of Transplant Surgeons.* 2004;4(3):295-298.

295. Shapiro AMJ, Ricordi, C. Islet Cell Transplantation Procedure and Surgical Technique. In: A. D. Kirk SJK, C. P. Larsen, J. C. Madsen, T. C. Pearson and S. A. Webber, ed. *Textbook of Organ Transplantation* Oxford, UK: John Wiley & Sons; 2014.

296. Shi J, Zhao Y, Wang K, et al. Cleavage of GSDMD by inflammatory caspases determines pyroptotic cell death. *Nature.* 2015;526(7575):660-665.

297. Shoda LK, Young DL, Ramanujan S, et al. A comprehensive review of interventions in the NOD mouse and implications for translation. *Immunity.* 2005;23(2):115-126.

298. Silverstein J, Maclaren N, Riley W, Spillar R, Radjenovic D, Johnson S. Immunosuppression with azathioprine and prednisone in recent-onset insulin-

dependent diabetes mellitus. *The New England journal of medicine.* 1988;319(10):599-604.

299. Siskind E, Maloney C, Akerman M, et al. An analysis of pancreas transplantation outcomes based on age groupings--an update of the UNOS database. *Clin Transplant.* 2014;28(9):990-994.

300. Sklavos MM, Bertera S, Tse HM, et al. Redox modulation protects islets from transplant-related injury. *Diabetes.* 2010;59(7):1731-1738.

301. Skouta R, Dixon SJ, Wang J, et al. Ferrostatins inhibit oxidative lipid damage and cell death in diverse disease models. *J Am Chem Soc.* 2014;136(12):4551-4556.

302. Skyler JS. The compelling case for anti-CD3 in type 1 diabetes. *Diabetes.* 2013;62(11):3656-3657.

303. Skyler JS, Ricordi C. Stopping type 1 diabetes: attempts to prevent or cure type 1 diabetes in man. *Diabetes.* 2011;60(1):1-8.

304. Skyler JS, Type 1 Diabetes TrialNet Study G. Update on worldwide efforts to prevent type 1 diabetes. *Annals of the New York Academy of Sciences.* 2008;1150:190-196.

305. Sloan-Lancaster J, Abu-Raddad E, Polzer J, et al. Double-blind, randomized study evaluating the glycemic and anti-inflammatory effects of subcutaneous LY2189102, a neutralizing IL-1beta antibody, in patients with type 2 diabetes. *Diabetes care.* 2013;36(8):2239-2246.

306. SoRelle JA, Itoh T, Peng H, et al. Withaferin A inhibits pro-inflammatory cytokine-induced damage to islets in culture and following transplantation. *Diabetologia.* 2013;56(4):814-824.

307. Sorenby AK, Kumagai-Braesch M, Sharma A, Hultenby KR, Wernerson AM, Tibell AB. Preimplantation of an immunoprotective device can lower the curative dose of islets to that of free islet transplantation: studies in a rodent model. *Transplantation.* 2008;86(2):364-366.

308. Soria B, Roche E, Berna G, Leon-Quinto T, Reig JA, Martin F. Insulin-secreting cells derived from embryonic stem cells normalize glycemia in streptozotocin-induced diabetic mice. *Diabetes.* 2000;49(2):157-162.

309. Stockwell BR, Friedmann Angeli JP, Bayir H, et al. Ferroptosis: A Regulated Cell Death Nexus Linking Metabolism, Redox Biology, and Disease. *Cell.* 2017;171(2):273-285.

310. Sung RS, Christensen LL, Leichtman AB, et al. Determinants of discard of expanded criteria donor kidneys: impact of biopsy and machine perfusion. *American journal of transplantation : official journal of the American Society of Transplantation and the American Society of Transplant Surgeons.* 2008;8(4):783-792.

311. 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(5768):1778-1780.

312. Suzanne M, Steller H. Shaping organisms with apoptosis. *Cell Death Differ.* 2013;20(5):669-675.

313. Tamura Y, Chiba Y, Tanioka T, et al. NO donor induces Nec-1-inhibitable, but RIP1-independent, necrotic cell death in pancreatic beta-cells. *FEBS Lett.* 2011;585(19):3058-3064.

314. Tanaka T, Fujita M, Bottino R, et al. Endoscopic biopsy of islet transplants in the gastric submucosal space provides evidence of islet graft rejection in diabetic pigs. *Islets.* 2016;8(1):1-12.

315. Taylor RC, Cullen SP, Martin SJ. Apoptosis: controlled demolition at the cellular level. *Nat Rev Mol Cell Biol.* 2008;9(3):231-241.

316. Textor SC, Taler SJ, Canzanello VJ, Schwartz L, Augustine JE. Posttransplantation hypertension related to calcineurin inhibitors. *Liver Transpl.* 2000;6(5):521-530.

317. Thompson DM, Meloche M, Ao Z, et al. Reduced progression of diabetic microvascular complications with islet cell transplantation compared with intensive medical therapy. *Transplantation.* 2011;91(3):373-378.

318. Thompson P, Badell IR, Lowe M, et al. Islet xenotransplantation using gal-deficient neonatal donors improves engraftment and function. *American journal of transplantation : official journal of the American Society of Transplantation and the American Society of Transplant Surgeons.* 2011;11(12):2593-2602.

319. Tian J, Clare-Salzler M, Herschenfeld A, et al. Modulating autoimmune responses to GAD inhibits disease progression and prolongs islet graft survival in diabetes-prone mice. *Nature medicine.* 1996;2(12):1348-1353.

320. Tiedge M, Lortz S, Drinkgern J, Lenzen S. Relation between antioxidant enzyme gene expression and antioxidative defense status of insulin-producing cells. *Diabetes.* 1997;46(11):1733-1742.

321. Toso C, McCall M, Emamaullee J, et al. Liraglutide, a long-acting human glucagon-like peptide 1 analogue, improves human islet survival in culture. *Transpl Int.* 2010;23(3):259-265.

322. Tse HM, Milton MJ, Piganelli JD. Mechanistic analysis of the immunomodulatory effects of a catalytic antioxidant on antigen-presenting cells: implication for their use in targeting oxidation-reduction reactions in innate immunity. *Free Radic Biol Med.* 2004;36(2):233-247.

323. Tse HM, Milton MJ, Schreiner S, Profozich JL, Trucco M, Piganelli JD. Disruption of innate-mediated proinflammatory cytokine and reactive oxygen species third signal leads to antigen-specific hyporesponsiveness. *Journal of immunology.* 2007;178(2):908-917.

324. Uy B, McGlashan SR, Shaikh SB. Measurement of reactive oxygen species in the culture media using Acridan Lumigen PS-3 assay. *J Biomol Tech.* 2011;22(3):95-107.

325. Vaithilingam V, Oberholzer J, Guillemin GJ, Tuch BE. Beneficial effects of desferrioxamine on encapsulated human islets--in vitro and in vivo study. *American journal of transplantation : official journal of the American Society of Transplantation and the American Society of Transplant Surgeons.* 2010;10(9):1961-1969.

326. van der Windt DJ, Bottino R, Casu A, et al. Long-term controlled normoglycemia in diabetic non-human primates after transplantation with hCD46 transgenic porcine islets. *American journal of transplantation : official journal of the American Society of Transplantation and the American Society of Transplant Surgeons.* 2009;9(12):2716-2726.

327. van der Windt DJ, Bottino R, Kumar G, et al. Clinical islet xenotransplantation: how close are we? *Diabetes.* 2012;61(12):3046-3055.

328. Van Raemdonck D, Neyrinck A, Rega F, Devos T, Pirenne J. Machine perfusion in organ transplantation: a tool for ex-vivo graft conditioning with mesenchymal stem cells? *Current opinion in organ transplantation.* 2013;18(1):24-33.

329. Vanden Berghe T, Linkermann A, Jouan-Lanhouet S, Walczak H, Vandenabeele P. Regulated necrosis: the expanding network of non-apoptotic cell death pathways. *Nat Rev Mol Cell Biol.* 2014;15(2):135-147.

330. Vanikar AV, Trivedi HL, Thakkar UG. Stem cell therapy emerging as the key player in treating type 1 diabetes mellitus. *Cytotherapy.* 2016;18(9):1077-1086.

331. Velmurugan K, Balamurugan AN, Loganathan G, Ahmad A, Hering BJ, Pugazhenthi S. Antiapoptotic actions of exendin-4 against hypoxia and cytokines are augmented by CREB. *Endocrinology.* 2012;153(3):1116-1128.

332. Vercammen D, Beyaert R, Denecker G, et al. Inhibition of caspases increases the sensitivity of L929 cells to necrosis mediated by tumor necrosis factor. *J Exp Med.* 1998;187(9):1477-1485.

333. Veriter S, Gianello P, Dufrane D. Bioengineered Sites for Islet Cell Transplantation. *Current diabetes reports.* 2013.

334. Vilaysane A, Chun J, Seamone ME, et al. The NLRP3 inflammasome promotes renal inflammation and contributes to CKD. *J Am Soc Nephrol.* 2010;21(10):1732-1744.

335. Virag L, Robaszkiewicz A, Rodriguez-Vargas JM, Oliver FJ. Poly(ADP-ribose) signaling in cell death. *Mol Aspects Med.* 2013;34(6):1153-1167.

336. Vivot K, Jeandidier N, Dollinger C, et al. Role of islet culture on angiogenic and inflammatory mechanisms. *Transplantation proceedings.* 2011;43(9):3201-3204.

337. Voltarelli JC, Couri CE, Stracieri AB, et al. Autologous hematopoietic stem cell transplantation for type 1 diabetes. *Annals of the New York Academy of Sciences.* 2008;1150:220-229.

338. Voltarelli JC, Couri CE, Stracieri AB, et al. Autologous nonmyeloablative hematopoietic stem cell transplantation in newly diagnosed type 1 diabetes mellitus. *JAMA : the journal of the American Medical Association.* 2007;297(14):1568-1576.

339. Wallach D, Kang TB, Dillon CP, Green DR. Programmed necrosis in inflammation: Toward identification of the effector molecules. *Science.* 2016;352(6281):aaf2154.

340. Walter M, Kaupper T, Adler K, Foersch J, Bonifacio E, Ziegler AG. No effect of the 1alpha,25-dihydroxyvitamin D3 on beta-cell residual function and insulin requirement in adults with new-onset type 1 diabetes. *Diabetes care.* 2010;33(7):1443-1448.

341. Wang RN, Rosenberg L. Maintenance of beta-cell function and survival following islet isolation requires re-establishment of the islet-matrix relationship. *The Journal of endocrinology.* 1999;163(2):181-190.

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

343. Werbowetski-Ogilvie TE, Bosse M, Stewart M, et al. Characterization of human embryonic stem cells with features of neoplastic progression. *Nature biotechnology.* 2009;27(1):91-97.

344. Westermark GT, Davalli AM, Secchi A, et al. Further evidence for amyloid deposition in clinical pancreatic islet grafts. *Transplantation.* 2012;93(2):219-223.

345. Westermark GT, Westermark P, Berne C, Korsgren O, Nordic Network for Clinical Islet T. Widespread amyloid deposition in transplanted human pancreatic islets. *The New England journal of medicine.* 2008;359(9):977-979.

346. Westermark GT, Westermark P, Nordin A, Tornelius E, Andersson A. Formation of amyloid in human pancreatic islets transplanted to the liver and spleen of nude mice. *Ups J Med Sci.* 2003;108(3):193-203.

347. Wherrett DK, Bundy B, Becker DJ, et al. Antigen-based therapy with glutamic acid decarboxylase (GAD) vaccine in patients with recent-onset type 1 diabetes: a randomised double-blind trial. *Lancet.* 2011;378(9788):319-327.

348. Wilson GL, Hartig PC, Patton NJ, LeDoux SP. Mechanisms of nitrosourea-induced beta-cell damage. Activation of poly (ADP-ribose) synthetase and cellular distribution. *Diabetes.* 1988;37(2):213-216.

349. Witkowski P, Liu Z, Cernea S, et al. Validation of the scoring system for standardization of the pancreatic donor for islet isolation as used in a new islet isolation center. *Transplant Proc.* 2006;38(9):3039-3040.

350. Wolters GH, Vos-Scheperkeuter GH, van Deijnen JH, van Schilfgaarde R. An analysis of the role of collagenase and protease in the enzymatic dissociation of the rat pancreas for islet isolation. *Diabetologia.* 1992;35(8):735-742.

351. Xenos ES, Farney AC, Widmer MB, et al. Effect of tumor necrosis factor alpha and of the soluble tumor necrosis factor receptor on insulin secretion of isolated islets of Langerhans. *Transplantation proceedings.* 1992;24(6):2863-2864.

352. Yamamoto H, Uchigata Y, Okamoto H. DNA strand breaks in pancreatic islets by in vivo administration of alloxan or streptozotocin. *Biochem Biophys Res Commun.* 1981;103(3):1014-1020.

353. Yamamoto H, Uchigata Y, Okamoto H. Streptozotocin and alloxan induce DNA strand breaks and poly(ADP-ribose) synthetase in pancreatic islets. *Nature.* 1981;294(5838):284-286.

354. Yang WS, Stockwell BR. Synthetic lethal screening identifies compounds activating iron-dependent, nonapoptotic cell death in oncogenic-RAS-harboring cancer cells. *Chem Biol.* 2008;15(3):234-245.

355. Yang WS, Stockwell BR. Ferroptosis: Death by Lipid Peroxidation. *Trends Cell Biol.* 2016;26(3):165-176.

356. Zeng Y, Torre MA, Karrison T, Thistlethwaite JR. The correlation between donor characteristics and the success of human islet isolation. *Transplantation.* 1994;57(6):954-958.

357. Zhang F, Wen Y, Guo X. CRISPR/Cas9 for genome editing: progress, implications and challenges. *Hum Mol Genet.* 2014;23(R1):R40-46.

358. Zhou Q, Brown J, Kanarek A, Rajagopal J, Melton DA. In vivo reprogramming of adult pancreatic exocrine cells to beta-cells. *Nature.* 2008;455(7213):627-632.