Advancing Engraftment and Cell Survival in Experimental and Clinical Islet Transplantation

by

BORIS LUIS GALA LOPEZ

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

Doctor of Philosophy

in

Experimental Surgery

Department of Surgery
University of Alberta

© BORIS LUIS GALA LOPEZ, 2016

ABSTRACT

Islet transplantation is today a well-established treatment modality for selected patients with type 1 Diabetes mellitus. The procedure has experienced notable refinements over the decades due the continuous efforts of clinicians and scientists to make islet isolation a reality, with a resulting final product to the highest standards, capable of safely treating patients against this autoimmune disease responsible for impaired insulin secretion and hypoglycemia unawareness. After the success of the University of Alberta group with a modified approach to the immune protection of islets, the international experience grew along with the numbers of transplants in highly specialized centers. Yet, long-term analysis of those initial results from the Edmonton group indicated that insulin-independence was not durable and most patients return to modest amounts of insulin around the fifth year, without recurrent hypoglycemia events. This thesis presents the results from multiple projects aimed to improve some of those limiting factors for prolonged islet survival. We provide sufficient background for the reader to learn about the historical perspective, along with the latest efforts to improve islet engraftment, immune protection and ultimately, long-term graft survival. We present our efforts to enhance beta cell viability and potency in vitro through added protection using the Mangano-metalloporphyrin BMX-010 during the isolation and culture process. This molecule has been reported to provide anti-inflammatory and antioxidant effects in pre-clinical transplant models. We here present an assessment of this metalloporphyrin in clinical islet transplantation. Another area of research is the avoidance of immunosuppression toxicity, which is one of the contributing factors for graft loss overtime. We specifically explored the potential cytoprotective effect of Anti-aging Glycopeptide (AAGP), a synthetic analogue of anti-freeze proteins, and demonstrated significant

impairment of islets treated with high dose tacrolimus and effective protective effect from culture supplementation with the AAGP. Clinical results of islet transplantation are discussed and new immunosuppressive strategies are presented, along with quality assurance elements for the human islet preparation. In particular, we explore the possibility of microbial contamination of the preparation. Shifting focus to alternative sources for islet transplantation, we present developmental experimental studies towards the implementation of the subcutaneous space for islet and insulin-producing stem cells, which is a promising avenue of research with the potential to provide an unlimited supply for transplantation and a personalized approach to transplant medicine. Various alternatives are tested for prevascularized Cell Transplant under the skin, in experimental and clinical setting to accommodate the future implementation of stem Cell Transplant in humans. Complementary information is provided in appendices with systematic reviews on the advances of immunosuppression in islet transplantation towards the improvement of engraftment and graft durability. Moreover, a special case reports provides an opportunity to debate the practice of islet autotransplantation after total pancreatectomy. In this case, a new indication is presented in a patient with metastatic renal cell carcinoma, prompting for a new view of indication expansion when conditions allow for it. Many phenomena have been identified as limiting factor for the islet engraftment and survival, and today all efforts are aimed to improve the quality of islets and their engrafting process, as well as more optimized immunosuppression to facilitate tolerance and ultimately, better long-term survival. As the field of islet transplantation continues to progress, it is foreseeable that a cure for type 1 diabetes mellitus is obtainable in the near future.

PREFACE

Dear Reader,

This thesis, titled "Advancing Engraftment and Cell Survival in Experimental and Clinical Islet Transplantation" is submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Experimental Surgery at the University of Alberta, Department of Surgery. The document is a compendium of various research projects aimed to improve multiples aspects of islet transplantation, providing a more comprehensive treatment modality for type 1 diabetes mellitus.

The thesis is organized in chapters containing individual pre-clinical and clinical projects where the author had a leading role within a team of collaborators. Chapters are presented in a logical order to provide better understanding for the rationale, the history, current results and future perspectives of islet transplantation. The document also includes complementary literature reviews contained in appendices to provide background information for the reader or to present the most updated clinical outcomes. The information presented in this thesis is especially prepared for publication. Most the chapters are currently published in journal articles or book chapters relevant to field of islet transplantation. Others are currently submitted and under revision for final dissemination.

Chapter 1 provides a general introduction to the topic of diabetes, its associated risks and the role of islet transplantation in the current treatment algorithm. The chapter includes a first

introductory section, followed by a general review on islet transplantation, published as a Special Review in the ***Juntendo Medical Journal*** (Gala-Lopez BL, Kawahara T, Pepper AR, and Shapiro AMJ. Islet Transplantation for Type 1 Diabetes. Juntendo Med J 2015, 61(2) reproduced here with permission from the publishers. My role for this publication was designing and performing the bibliographic review and writing 80% of the manuscript. TK, ARP wrote the remaining 20% of the manuscript and provided revisions. AMJS performed final edits as senior author. This section presents overall information on the history of islet transplantation, a summarized description of the process and information on the current results, the main limitations and the future directions of this therapeutic modality.

Chapter 2 approaches the islet isolation process and discusses a potential method to avoid islet attrition due to inflammation and oxidative stress. The chapter examines the impact of supplementing the isolation and culture phase with BMX-010, a metalloporphyrin with antioxidative properties. This experimental work was aimed to enhance islet viability and to improve engraftment and long-lasting islet function after transplant in a first-in-human pilot clinical trial performed within the University of Alberta Clinical Islet Transplantation program. Permission for that study was obtained from the Health Research Ethics Board of the University of Alberta, Edmonton, Alberta, Canada (protocol number: Pro00045961) and Health Canada (HC Control No.185631). The work included in this chapter was recently accepted for publication in ***Cell R^4, the official journal of the Cure Alliance*** (Gala-Lopez BL, Kin T, O'Gorman D, Malcolm A, Pepper AR, Pawlick RL, Bruni A, Abualhassn N, Bral M, Jones C, Piganelli J, Crapo J and Shapiro AMJ. The metalloporphyrin BMX-010 in human islet isolation and clinical transplantation. CellR4 2016; 4(3): e2066). My contribution in this research project

was to participate in performing approximately 50% of the characterization of control and treated islets (recovery, viability and insulin release), performing approximately 70% of the transplant procedures, performing all data analysis and writing 90% of the manuscript. TK, DO and AM performed all islet isolations, culture, participated in all transplant procedures and wrote the rest of the manuscript. ARP, RLP, AB, NA and MB collaborated during 50% of in vitro testing, including oxygen consumption rate assay. CJ, JP and JC are collaborators from BioMimetix. They provided the BMX-010 for this trial, supporting data from previous studies and quality tests for the BMX-010 lot used in this study. All coauthors reviewed the manuscript and provided corrections. AMJS designed and led the study, participated in all transplant procedures and performed final edits to the manuscript.

Chapter 3 continues the trail of enhancing islet viability *in vitro* to improve engraftment. New research is presented evaluating the benefits of treating islets with an Anti-aging Glycopeptide (AAGP), a synthetic anti-freeze protein with proven cytoprotective capabilities. The drug is used here specifically to prevent injury from tacrolimus, a calcineurin-inhibitor widely used in transplantation. AAGP was successful in protecting islets both, mouse and human, *in vitro* and *in vivo,* from the dysfunctional toxic effect of tacrolimus. Results from this experimental work were published in ***Diabetes*** (Gala-Lopez BL, Pepper AR, Pawlick RL, O'Gorman D, Kin T, Bruni A, Abualhassan N, Bral M, Bautista A, Manning-Fox JE, Young LG, MacDonald PE, and Shapiro AMJ. Antiaging Glycopeptide protects human islets against tacrolimus-related injury and facilitates engraftment in mice. Diabetes 2016; 65:451-462; reproduced here with permission from the publishers) and has now initiated a planned clinical trial to be launched at University of Alberta within the next months. My role in this project was to design 80% of the experiments, to

perform 70% of the *in vitro* characterization of islets (recovery, viability, insulin release, mixed lymphocyte reaction), to perform 50% of transplant procedures with human and mouse islets with subsequent monitoring, to prepare and study 80% of histology samples, to complete 80% of the data analysis and to write all the manuscript. ARP also participated in the design of the experiments, performed 30% of mouse islet isolations, 25% of transplant procedures, contributed in TUNEL assay and completed 20% of the data analysis. RLP performed 70% of mouse isolations, 25% of transplant procedures, contributed to most *in vitro* testing and animal monitoring. DO and TK performed all human islet isolations and contributed to dynamic insulin release testing. An.Br. performed Redox in vitro testing and, with NA and MB, contributed to mice monitoring. Au.Ba., JMF and PM performed intracellular calcium imaging and membrane capacitance studies. JGY represents ProtoKinetics, helped designing the studies, and provided the AAGP for these experiments. All coauthors reviewed the manuscript and provided corrections. AMJS supervised the study design and all experimental studies, and performed final edits to the manuscript.

Chapter 4 examines the potential influence of microbial contamination in islet transplantation. This chapter reports the results of a clinical investigation towards quality assurance of the islet preparation as a therapeutic biological agent. Despite islets being processed under current Good Manufacturing Practice, undiagnosed microbial contamination may be present at the time of infusion. The inherited risk of systemic infection in the presence of potent immunosuppression is explored, along with potential influence upon short and long-term graft function. The results from our investigation were published in ***Diabetes Technology & Therapeutics*** (Gala-Lopez BL, Kin T, O'Gorman D, Pepper AR, Senior PA, Humar A, and Shapiro AMJ. Microbial

contamination of clinical islet transplant preparations is associated with very low risk of infection. Diabetes Technol Ther 2013; 15(4): 323 – 27; reproduced here with permission from the publishers). My role in this clinical investigation was to design the research protocol, to perform some of the transplant procedures, to create 50% of databases and complete all data analysis and to write the manuscript. TK and DO performed all islet isolations, islet culture and participated in most transplant procedures. AH participated in the study design and interpreted all microbiology testing. PS participated in most of the transplant procedures. Al coauthors reviewed the manuscript and provided corrections. AMJS designed the experiments, participated in all transplant procedures and performed final edits to the manuscript.

Chapter 5 shifts focus to the potential influence of anatomic site in islet transplantation. We argue the need for an alternative site to intraportal transplant to safely accommodate co-transplantation for infusion of surrogate insulin-producing cells. This chapter discusses the subcutaneous space as a convenient location for this purpose and defines the need for prevascularization to allow islet engraftment. In this chapter, we evaluated the Sernova Cell Pouch^{TM} as a potential technology to allow islet engraftment and function, implanted under the skin. This device had been extensively tested in pre-clinical *in vivo* models. We herein report our first in-human phase I/II clinical trial performed at the University of Alberta's Clinical Islet Transplantation Program, to evaluate safety and efficacy of this device in type I diabetic patients (Clinical Trials.gov NCT01652911). This trial was authorized by the Health Research Ethics Board of the University of Alberta (Protocol number PRO00028097). Results from this clinical investigation are now under review for publication with the ***American Journal of Transplantation*** (Gala-Lopez BL, Pepper AR, Senior PA, Dinyari Parastoo, Malcolm A, Kin T,

Pawlick RL and Shapiro AMJ. Clinical subcutaneous islet transplantation into a pre-vascularized subcutaneous device – Experience in the first three cases. Am J Transplant May 2016). My role in this clinical investigation was to participate in the design of the research protocol, to participate in all the transplant procedures, to complete the data analysis and to write 80% of the manuscript. ARP provided supporting information for this technology and participated in the transplant procedures. PD and AM performed regulatory tasks for the trial and facilitated patient clinical information. RLP performed tissue sample preparation for histology analysis. PAS performed patient evaluations. All coauthors reviewed the manuscript and provided corrections. AMJS designed and led the trial, performed all transplant procedures and edited the final manuscript.

Following the analysis on alternative sites for islet transplantation, **Chapter 6** presents our works towards the development of a proprietary methodology to infuse and allow engraftment of different types of cells (including islets) in the subcutaneous space. We herein report our methodology to perform a prevascularized subcutaneous "device-less" transplantation, primarily validated with islets in a mouse model, but now extended to embryonic stem cells and cancer cells for personalized chemotherapy. This work was published in ***Nature Biotechnology*** (Pepper AR, Gala-Lopez BL, Pawlick RL, Merani S, Kin T, and Shapiro AMJ. A prevascularized subcutaneous device-less site for islet and cellular transplantation. Nature Biotechnol 2015; 33(5): 518 – 23; reproduced here with permission from the publishers).

This chapter presents the developmental phases and all *in vivo* testing where Dr. Andrew Pepper and I had leading roles in the design of the experiments (40% and 20%), performing material testing (60% and 20%), performing transplant procedures (60% and 20%), providing animal care

and monitoring (60% and 40%), and completing data analysis (80% and 20%). ARP performed all histology analysis and wrote the manuscript. I contributed to 20% of the bibliographic review to prepare the manuscript and review the manuscript at various stages. RLP performed mouse islet isolations, participated in 20% of transplant procedures and contributed to animal monitoring. SM participated in initial material testing (10%). TK performed all human islet isolations. AMJS designed and supervised the studies and performed final edits to the manuscript. Despite being second author in this publication, the experiments associated with this project became an important component of my PhD work.

Chapter 7 evaluates the "device-less" technique presented in the previous chapter as an adequate milieu to facilitate engraftment of pancreatic endoderm cells derived from embryonic stem cells. The pre-vascularized subcutaneous space allowed successful engraftment of these cells and permitted maturation into endocrine multihormonal cells after more than 20 weeks. The resulting collagen wall from the device-less technique was also able to contain benign cysts arising during histogenesis. Results from these exciting experiments were submitted and accepted for publication in the ***Journal of Stem Cell and Transplantation Biology*** (Gala-Lopez BL, Pepper AR, Pawlick RL, Bruni A, Abualhassan N, Kin T, Keller G, Nostro MC, and Shapiro AMJ. A novel pre-vascularized subcutaneous site safely accommodates stem cell derived therapies for treating diabetes J Stem Cell Transplant Biol March 2016). My contribution to this investigation was to participate in the design the experiments, to perform 60% of the transplant procedures with pancreatic endoderm cells and human islets, with subsequent monitoring, to provide 70% of animal care and monitoring, to perform 80% of blood C-peptide measurements, to prepare and study histology samples, to complete 80% of the data analysis and to write the

manuscript. ARP and RLP contributed in performing 40% of transplant procedures and providing animal care in 30%, AB and NA contributed in performing 20% of the blood C-peptide measuring and helped in histology analysis. TK performed all human islet isolations, GK and MCN helped in planning experiments and provided PE cells. All coauthors reviewed the manuscript and performed corrections. AMJS designed and supervised all experiments and performed final edits to the manuscript.

Chapter 8 provides a general overview of the topics discussed throughout. This section is followed by a personal analysis of new strategies to empower durable islet survival after transplantation, discussing some of the most recent contributions to improve islet isolation and post-transplant management. This section is followed by a brief analysis of the current status of stem cell transplantation and the feasibility to be fully implemented in clinical practice. Finally, a closing section provides general conclusions to this thesis highlighting potential future direction of this therapy.

Complementary information is provided as appendices related to the body of the thesis but not necessarily linked to a particular chapter. These appendix chapters represent major academic contributions of published manuscripts first-authored by myself with the collaboration of colleagues, and of specific relevance to both experimental and clinical islet transplantation. I have included them in the Appendix for their direct relevance to the experimental studies of my PhD thesis, and they further serve to place my academic contributions within a larger clinical context of knowledge in the islet transplantation, immunosuppression and patient management fields.

Appendix A - shifts focus to clinical outcomes of islet transplantation. In this section, I provide an extensive literature review of the role of immunity in preventing islets to last long-term, along with new strategies using more efficient immunosuppressants to avoid decline of islet function. This review will be published as a book chapter (Gala-Lopez BL, Pepper AR and Shapiro AMJ) named New Immunosuppression Therapies in: ***Islets, Biology, Immunology and Clinical Transplantation***, edited by F. Kandeel in April 2016. My role was to design the chapter structure and perform 60% of the bibliographic review, to write 60% of the chapter. ARP performed 40% of the bibliographic review and wrote 30% of the chapter. AMJS was the senior author and edited the final manuscript. The information from this book chapter is reproduced with permission from the publishers.

Appendix B - continues the debate on new immunosuppression strategies to prolong graft function after islet transplantation. Herein, the discussion focuses upon the evolution of biological agents and their role in preventing allo- and autoimmunity, and their contribution to improve current clinical outcomes in selected highly specialized centers. New, more potent biological agents are also presented as potential candidates to selectively avoid immune response to allografts and eventually allow durable single donor islet transplant. The information in this appendix was published as a literature review in ***Current Diabetes Report*** (Gala-Lopez BL, Pepper AR and Shapiro AMJ. Biologic agents in islet transplantation. Curr Diab Rep 2013 Oct; 13(5): 713 – 22; reproduced here with permission from the publishers). My role was to design the chapter structure and perform 60% of the bibliographic review, to write 60% of the chapter.

ARP performed 40% of the bibliographic review and wrote 30% of the chapter. AMJS was the senior author and edited the final manuscript.

Appendix C - focuses in islet autotransplantation as treatment modality after total pancreatectomy. This type of islet transplant was practiced early in the history of the procedure and popularized for patients undergoing surgery due to chronic pancreatitis. In this occasion, we revive the debate of expanding the indication of islet autotransplantation in the presence of malignancies, by exploring the brief reported cases and by presenting our own clinical experience with a first case of metastatic renal cell carcinoma. Results from this clinical experience were published in the ***American Journal of Transplantation*** (Gala-Lopez BL, Semlacher E, Manoucheri N, Kin T and Shapiro AMJ. Am J Transplant 2013; 13(9):2487-91; reproduced here with permission from the publishers. My contribution for this case report was to participate in the clinical evaluation of the patient, participate in the pancreatectomy and transplant procedure, to perform 100% of the bibliographic review and to write 100% of the manuscript. ES participated in the patient clinical evaluation. NM participated in the surgical procedure and prepared intraoperative photographs. TK participated in the surgical procedure and performed the islet isolation. AMJS was the main surgeon of the procedure and edited the final manuscript.

Appendix D – Provides supporting information for **Chapter 2**. The specific BMX-010 lot used in this clinical trial was further tested to assure that the study molecule was stable and functional. This supporting information includes a copy of the Certificate of Analysis with multiple stability tests for this specific lot, released by Ricerca Biosciences (Concord, OH, USA). We also report

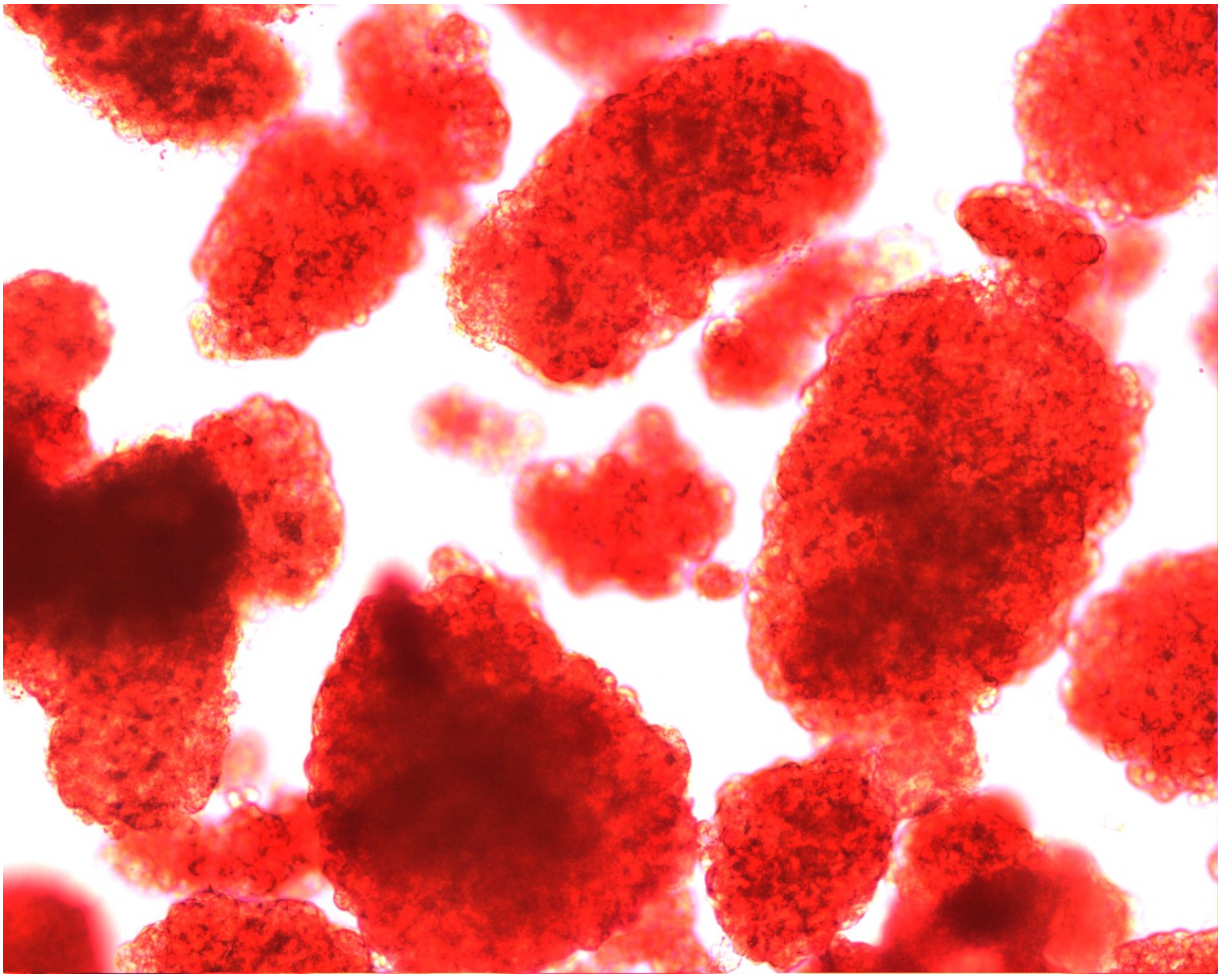
the results from in-house experimental work measuring protective capabilities of this molecule against oxidative stress in human islets, and immunomodulatory characteristics demonstrated by Jon Piganelli and collaborators, at University of Pittsburgh.

It is my most sincere desire that you find this work of interest and valuable for the practice of islet transplantation.

Boris L. Gala-Lopez.

DEDICATION

This thesis is dedicated to my wife Lisette and to my children, Daniel and Amanda for following me to the end of the world and always provide me with unconditional love and support through adversity. Thank you for being there…



"…If there ever was a solid-organ transplant that could be replaced by a cellular transplant, pancreas for islet transplantation was it. If there was ever an indication for a wholesale transfer from major to minimally invasive surgery, this is it."

David E. R. Sutherland, MD, PhD.

Foreword in *Islet Transplantation and Beta Cell Replacement Therapy*.

Shapiro AMJ and Shaw JAM, eds. Informa Healthcare 2007.

ACKNOWLEDGEMENTS

I would like to take this opportunity to acknowledge and thank all individuals who contributed to my education and research during my graduate studies in Alberta.

My most sincere gratitude for my PhD supervisor, Dr. James Shapiro for his invaluable guidance, encouragement and support since the first day we met back in the busy summer of 2010. His outstanding work in the field of islet transplantation was one of the reasons that brought me to Edmonton and I was genuinely honored when he offered me a PhD at his laboratory. Under Dr. Shapiro's supervision, I have learned first-hand the potential of this therapeutic modality and the many opportunities for improvement through high-quality translational research. Dr. Shapiro has also looked after my career as a transplant and hepatobiliary surgeon, and his tireless support and advice has now turned me into a more complete surgeon-scientist.

I want to thank the members of my Supervisory Committee. Dr. Colin Anderson for his continued support of my research and the fruitful insight he always provided to my experimentation. Dr. Darren Freed, for providing a fresh, out-of-the-box thinking to my view of islet transplantation, and for showing me the incredible opportunities of *ex vivo* organ perfusion. Although Dr. Thomas Mueller only sat on my committee for a year, I am grateful for his constructive feedback into my research project.

I extend my gratitude to Dr. Camillo Ricordi for making time in his busy schedule and generously accepting to serve as my External Examiner for my final dissertation. It is indeed an honor to have him review my thesis and participate in my oral defense examination.

My sincere gratitude for my clinical mentors, Dr. Norman Kneteman and Dr. David Bigam. Along with Dr. Shapiro, they allowed me to be part of the University of Alberta Multi-Organ Transplant Program and provided me with continuous training and support throughout my clinical fellowship and beyond.

I especially want to thank my colleagues in the pre-clinical experimental laboratory. Dr. Andrew Pepper who has served as a Post-Doctoral fellow for more than 4 years, sharing many of my scientific debates, experimental work and tireless writing. Mrs. Rena Pawlick, who has provided incredible support for my research and guided me through innumerable experimental techniques along these years. Mr. Antonio Bruni, a vivacious and hard-working scientist who always supported me in every possible way. Dr. Mariusz Bral, a brilliant surgeon who was always ready to share the burden of long surgical nights and provided significant insight to my work and personal life, and Dr. Nasser Abualhassan, my office mate, who provided unconditional support for my work and a tranquil work atmosphere.

I have been privileged to work with some of the members of the Clinical Islet Laboratory. Dr. Peter Senior, who always provided constructive guidance, Dr. Andrew Malcolm, who provided incredible support for most of my projects, Dr. Tatsuya Kin, who have been a true

research and clinical ally along these years, and Mr. Doug O'Gorman, a tireless scientist, always willing to support my work.

I want thank all our collaborators, which provided indispensable support to my research. Mr. Lachlan G. Young from *ProtoKinetix*, who kindly provided the AAGP molecule to perform multiple studies on islet protection. Dr. James Crapo, Dr. Jon Piganelli and Ms. Carissa Jones from *BioMimetix Pharmaceutical Inc*., for their continuous support to our pre-clinical and clinical experimentation with BMX molecules. Dr. M.Cristina Nostro and Dr. Gordon Keller from the *McEwen Centre for Regenerative Medicine*, for supporting our work with insulin-producing stem cells. Dr. Philip M. Toleikis, Mrs. Delfina M. Siroen and Mrs. Amanda MacGillivary from *Sernova Corp.* for their participation in our clinical trial. A special acknowledgement to the *Macdonald Islet biology Laboratory* (Dr. Patrick Macdonald, Dr. Jocelyn Manning-Fox, Dr. Kunimasa Suzuki, James Lyon and Austin Bautista) for their collaboration with our AAGP experiments.

My sincere gratitude to Dr. Tom Churchill, the Graduate Coordinator in the Department of Surgery, who provided continuous teaching and support for the wellbeing of my program. I especially value his open-door style for addressing all students. I also want to thank Dr. Gregory Korbutt for his continued scientific advice, for his research collaboration and for accepting to be my External Examiner during my Candidacy Exam.

My most sincere acknowledgement for the administrative support provided by Ms. Cherry Robinson, Ms. Carolyn Harrison, Mrs. Donna Fedorow, Mrs. Rosemary Henley, Mrs.

Colleen Ruptash, Mrs. Christina Smith and Ms. Lisa Brick. Their support allowed efficient communication and documentation toward the successful completion of my program.

I want to thank all the donor, recipient and research coordinators who made most of my clinical work possible. I want to extend my appreciation to all the personal in the surgical suite that provided indispensable assistance during surgeries, and to the organ donors and their families for their altruistic contribution to the organ transplantation in Edmonton and also to my experimental work.

A special acknowledgement to all my funding agencies that made this PhD possible through their generous financial support. The Transplant Fellowship Fund, the Alberta Diabetes Institute Research Studentship and Travel Award, the Blanch family for provide funds for the University of Alberta Blanch Award, the University of Alberta Graduate Student Recruitment Studentship, the Alberta Innovates Health solutions CRIO team award and the AIHS Clinician Fellowship, the Killam Trusts for their Izaak Walton Killam Memorial Scholarship, The Alberta Transplant Institute Research Fellowship and the Canadian National Transplant Research Program.

Of course, the work conducted here would not have been possible without the continuous support and assistance of my family and friends. I can never thank them enough for the vital assistance and love, especially from my wife Lisette and children Daniel and Amanda, who always showed me the value of every effort. To my all family in Cuba, my parents, my brother, my aunts and uncles, my family-in-law and those who are no longer among us, my infinite

thanks for lifetime of sacrifice. Finally, to all my friends, especially the ones in Edmonton, I sincerely thank you all for providing me with a new “family” to share happiness and grieve.

TABLE OF CONTENTS

CHAPTER 1.INTRODUCTION. **1**

1.1 - GENERAL INTRODUCTION **2**

1.1.2 – REFERENCES **5**

1.2 - ISLET TRANSPLANTATION FOR TYPE 1 DIABETES **6**

1.2.1. - ABSTRACT 8

1.2.2. – introduction 9

1.2.3. - Historical Perspective 10

1.2.4. -The Process of Islet Transplantation 11

1.2.5. - Sites for transplantation 18

1.2.6. - The Main Obstacles 18

1.2.7. - Graft function monitoring 19

1.2.8. - Future directions for islet transplantation 20

1.2.9. – Conclusion 21

1.3 – REFERENCES **22**

CHAPTER 2. THE METALLOPORPHYRIN BMX-010 IN HUMAN ISLET ISOLATION AND CLINICAL .....

TRANSPLANTATION **27**

2.1. – ABSTRACT **30**

2.2. – INTRODUCTION **31**

2.3.1. - Islet isolation and BMX-010 supplementation 32

2.3.2. - In vitro recovery and functional assessment 33

2.3.3. - Fractional beta cell viability 34
2.3.4. - Oxygen consumption rate 34
2.3.5. - Transplant procedures 35
2.3.6. - Statistical analysis 35
2.4. – RESULTS 37
2.5. – DISCUSSION 42
2.6. – REFERENCES 45
CHAPTER 3. 48
ANTIAGING GLYCOPEPTIDE PROTECTS HUMAN ISLETS AGAINST TACROLIMUS-RELATED INJURY AND FACILITATES ENGRAFTMENT IN MICE 48
3.1. – ABSTRACT 51
3.2. – INTRODUCTION 52
3.3. - RESEARCH DESIGN AND METHODS 53
3.3.1. - Human islet isolation, purification and culture 53
3.3.2. - In vitro evaluation of AAGP 54
3.3.3. - In vivo evaluation 57
3.3.4. - Statistical analysis 61
3.4. - RESULTS 61
3.4.1. - AAGP enhances human islet potency in culture and protects islets against acute exposure to tacrolimus 61
3.4.2. - Acute exposure to tacrolimus decreases insulin secretion but not biosynthesis 62
3.4.3. - AAGP reduces oxidative stress but does not inhibit tacrolimus function 62

3.4.4. - Tacrolimus effect on islet intracellular calcium responses and exocytosis. 67

3.4.5. - AAGP prevents islet apoptosis resulting from in-vitro exposure to tacrolimus..... 67

3.4.6. - AAGP ameliorates inflammatory response immediately post-transplant 70

3.4.6. - Post-transplant apoptosis 70

3.4.7. - AAGP-supplementation improves human islet transplant function despite tacrolimus exposure 71

3.4.8. - AAGP-supplementation improves islet transplant function despite continuous recipient treatment with tacrolimus 73

3.5. - DISCUSSION..... 77

3.6. – REFERENCES..... 80

CHAPTER 4. 85

MICROBIAL CONTAMINATION OF CLINICAL ISLET TRANSPLANT PREPARATIONS IS ASSOCIATED WITH VERY LOW RISK 85

4.1. - ABSTRACT 88

4.2. - INTRODUCTION 89

4.3. - MATERIALS AND METHODS..... 90

4.3.1. – Patients..... 90

4.3.2. - Transplant procedures..... 90

4.3.3. - Microbiological testing..... 91

4.3.4. - Antibiotic coverage..... 92

4.3.5. - Immunosuppression protocols..... 92

4.3.6. - Management of patients receiving contaminated islet preparation 93

4.3.7. - Graft function 94

4.3.8. - Statistical analysis..... 94

4.4. – RESULTS 95

4.5. – DISCUSSION 98

4.6. – REFERENCES..... 102

CHAPTER 5. 105

CLINICAL SUBCUTANEOUS ISLET TRANSPLANTATION INTO A PRE-VASCULARIZED SUBCUTANEOUS DEVICE - EXPERIENCE IN THE FIRST THREE CASES..... 105

5.1. – ABSTRACT..... 108

5.2. - INTRODUCTION 109

5.3. - CASE 1..... 112

5.4. - CASE 2..... 117

5.5. - CASE 3..... 118

5.6. - SERIAL C-PEPTIDE MONITORING AND SUBSEQUENT COURSE 120

5.7. – DISCUSSION 128

5.8. – REFERENCES..... 131

CHAPTER 6. 134

A PREVASCULARIZED SUBCUTANEOUS DEVICE-LESS SITE FOR ISLET AND CELLULAR TRANSPLANTATION 134

6.1. – ABSTRACT..... 137

6.2. – INTRODUCTION..... 138

6.3. – METHODS 144

6.3.1. - Creation of the DL subcutaneous transplant site 144
6.3.2. - Proinflammatory cytokine and chemokines measurements 146
6.3.3. - Mouse pancreatectomy and islet isolation 149
6.3.4. - Human islet isolation 150
6.3.5. - Diabetes induction and islet transplantation 151
6.3.6. - Evaluation of islet graft function 152
6.3.7. - Long-term islet graft retrieval 153
6.3.8. - Histological assessment 153
6.3.9. - Statistical analysis 154
6.4. – RESULTS 155
6.4.1. - Design and testing of the subcutaneous DL site 155
6.4.2. - Long-term function and vascularization of DL islet grafts 161
6.4.3. - Efficacy of DL transplantation in additional mouse models 169
6.5. - DISCUSSION 175
6.6. – REFERENCES 179
CHAPTER 7. 184
A NOVEL PRE-VASCULARIZED SUBCUTANEOUS SITE SAFELY ACCOMMODATES STEM CELL DERIVED THERAPIES FOR TREATING DIABETES 184
7.1. - ABSTRACT 187
7.2. – INTRODUCTION 188
7.3. - MATERIALS AND METHODS 189
7.3.1. - Human Embryonic Stem Cells-derived Pancreatic Endoderm 189

7.3.2. - Transplantation of PE cells 189
7.3.3. - C-peptide Measurements 190
7.3.4. - Histology 191
7.3.5. - Transplantation of Human Islets 191
7.3.6. - Statistical Analysis 192
7.4. – RESULTS 194
7.5. – DISCUSSION 201
7.6. – REFERENCES 204
CHAPTER 8 208
CONCLUSIONS AND FUTURE DIRECTIONS 208
8.1. - OVERVIEW 209
8.2. – EMPOWERING DURABLE ISLET SURVIVAL 213
8.2.1. - Donor selection 213
8.2.2. - Pancreas preservation prior to islet isolation 217
8.2.3. – Islet culture 220
8.2.4. - Assessment of islet preparations 223
8.2.5. - Cytoprotective strategies during islet isolation 227
8.2.6. – ISLET ENGRAFTMENT 232
8.2.7. - IMMUNOSUPPRESSIVE STRATEGIES TOWARDS TOLERANCE 238
8.3. – USING STEM CELLS IN CLINICAL TRANSPLANTATION. HOW CLOSE ARE WE? 244
CONCLUSIONS 251
GENERAL BIBLIOGRAPHY 275

APPENDIX A. 335

NEW IMMUNOSUPPRESSION THERAPIES 335

A.1. – INTRODUCTION 337

A.2. - HISTORICAL PERSPECTIVE 338

A.3. - ISLET IMMUNOBIOLOGY AND ENGRAFTMENT 341

A.3.1. - Immunobiology of the Islets of Langerhans 341

A.3.2. - Islet Engraftment 343

A.4. - CURRENT IMMUNOSUPPRESSION PROTOCOLS 348

A.5. - PATIENT CONSIDERATIONS 352

A.5.1. - Combined Islet-Kidney Transplant 352

A.5.2. - Risk of Sensitization 353

A.6. - THERAPEUTIC TARGETS & STRATEGIES 354

A.6.1. - Immunosuppressive Targets 355

A.6.2. - Antigen-Specific Vaccines 363

A.6.3. - Combination Strategies 363

A.7. – SUMMARY 364

A.8. – REFERENCES 366

APPENDIX B. 374

BIOLOGICAL AGENTS IN ISLET TRANSPLANTATION 374

B.1. – ABSTRACT 377

B.2. - INTRODUCTION 377

B.3. - HISTORICAL PERSPECTIVE OF BIOLOGICS IN TRANSPLANTATION .. 379

B.4. - CURRENT EXPERIENCE WITH BIOLOGICAL AGENTS IN THE FIELD OF TRANSPLANT 380

B.4.1. - Antithymocyte Globulins 382

B.4.2. - Anti-CD3 383

B.4.3. - Anti-CD25 383

B.4.4. - Alemtuzumab 384

B.4.5. – Belatacept 385

B.4.6. - Efalizumab 386

B.5. - FUTURE BIOLOGIC PROSPECTS FOR ISLET TRANSPLANTATION 387

B.5.1. - Induction Agents: Depleting Antibodies 387

B.5.2. - B-Cell Therapeutics 388

B.5.3. - Biologic Combination Strategies 392

B.6. – SUMMARY 394

B.7. – REFERENCES 395

APPENDIX C. 405

AUTOLOGOUS ISLET TRANSPLANTATION AFTER TOTAL PANCREATECTOMY FOR RENAL CELL CARCINOMA METASTASES 405

C.1. - ABSTRACT 408

C.2. - INTRODUCTION 409

C.3. - CASE REPORT 410

C.4. – DISCUSSION 416

C.5. – REFERENCES 418

APPENDIX D. 421

SUPPORTING EVIDENCE FOR STRUCTURAL AND FUNCTIONAL INTEGRITY OF BMX-010 USED IN CLINICAL TRIALS AT UNIVERSITY OF ALBERTA 421

D.1. – INTRODUCTION 422

D.2. – CERTIFICATE OF ANALYSIS 422

D.3. - ANTIOXIDANT PROPERTIES 426

D.4. - IMMUNOMODULATORY EFFECT 427

D.5. - CONCLUSIONS 429

D.6. - REFERENCES 431

LIST OF TABLES

Table 2.1 Baseline characteristics of donors allocated to BMX-010 and control group….. 36

Table 4.1 - Microbial species cultured from 19 contaminated human islet preparations 96

Table 4.2 Summary of 18 patients receiving islet transplant with microbial contamination in the final preparation.. 97

Table 5.1 Summarized demographic, glycemic and transplant characteristics of three subjects receiving subcutaneous Cell Pouch^{TM} islet transplantation. 113

Table 6.1 Translation of subcutaneous islet transplantation therapies. 140

Table 6.2 Islet transplantation sites. 142

Table 6.2 Islet transplantation sites. Continued 143

Table 7.1 Outcome summary of 50 animals transplanted with insulin-producing stem cells**.** .. 202

Table 8.1 Oxygen tension in different transplant sites and their ability to oxygenate transplanted islet. 236

Table B.1. Biologic agents currently being examined in transplantation……..…….....381

LIST OF FIGURES

Figure 1.2-1 Timeline with the most important milestones in the transition of islet transplantation from experimental to clinical therapy. 12

Figure 1.2-2 Pancreatic intraductal distension during the process of islet isolation. 14

Figure 1.2-3 Pancreatic digestion using the Ricordi® Chamber during the process of islet isolation. 15

Figure 1.2-4 Pancreatic purification using the COBE 2991 during the process of islet isolation. 16

Figure 1.2-5 Microphotograph of isolated mouse islets. 17

Figure 2.1. Human islet isolation with addition of BMX-010. 38

Figure 2.2 Function and viability of human islets treated with BMX-010 during isolation and culture. 40

Figure 2.3 Transplant efficacy of human islets treated with or without BMX-010. 41

Figure 3.1 AAGP improves human islet potency in culture and protects against acute exposure to tacrolimus. 64

Figure 3.2. Islets treated with AAGP have decreased oxidative stress. 65

Figure 3.3 AAGP preserves insulin release by maintaining islet exocytosis. 68

Figure 3.4 AAGP decreases islet loss in culture due to apoptosis, even in the presence of tacrolimus. 69

Figure 3.5 AAGP ameliorates inflammatory response immediately post-transplant. 72

Figure 3.6. AAGP-supplementation improves islets transplant function despite tacrolimus exposure. 74

Figure 3.7 AAGP-supplementation improves islets transplant function despite continuous (short and long duration) tacrolimus treatment. 76

Figure 4.1 Kaplan Meier Graft survival curves comparing the maintenance of stimulated C-peptide levels in subjects who did or did not receive contaminated islet preparations. 99

Figure 5.1 . Illustration of the Sernova Cell Pouch™. 111

Figure 5.2 Histology showing surviving islets surrounded new blood vessels in areas of the therapeutic devices explanted from Subjects 1 and 2. 116

Figure 5.3 Illustrative diagram and photograph showing surgical sites for implantation of Cell Pouch™ devices in subject three.. 119

Figure 5.4 Selected histology and immunohistochemistry from the sentinel devices explanted at 30 days post-transplant in subject 3 121

Figure 5.5 A. Masson's trichrome stain of a selected histology image of a Cell Pouch™ explanted after day 30 post-transplant from Subject 3 showing no evident T-cell infiltrate. 122

Figure 5.6 Evidence for survival of pancreatic exocrine tissue and ductal elements (CK19 positive) within the sentinel devices explanted from Subject 3. 123

Figure 5.7 Early post-transplant basal levels of C-peptide. 125

Figure 5.8 Pre and post-transplant function of the 3 study subjects as determined by stimulated C-peptide response to a mixed meal challenge (Ensure™), daily insulin requirement and glycated hemoglobin over time. 127

Figure 6.1 Design and characteristics of the subcutaneous DL cellular transplant site.. 145

Figure 6.2 Overview of the subcutaneous, 'device-less' transplant approach. 147

Figure 6.3 Histological analysis of islets transplanted long-term into the DL space**.** 148

Figure 6.4 Rate of diabetes reversal, defined as percent euglycemic, in mouse recipients of syngeneic BALB/c islet grafts.. 158

Figure 6.5 Intraperitoneal glucose tolerance tests in syngeneic BALB/c islet recipients**.**..... 160

Figure 6.6 The proinflammatory response elicited by angiocatheters implanted subcutaneously. 163

Figure 6.7 Long-term function of syngeneic islet grafts transplanted into the DL space..... 164

Figure 6.8 IPGTTs of syngeneic mouse islets transplanted under the KC or into the DL site, 100 days after transplant. 166

Figure 6.11 Vascular density of islet grafts post-transplantation..... 170

Figure 6.12 Time to normoglycemia in C57BL/6 vs. BALB/c mouse strains..... 172

Figure 6.13 Impact of pre-existing diabetes before placement of the device-less (DL) catheter upon subsequent islet engraftment. 173

Figure 6.14 Impact of an allogeneic barrier upon diabetes reversal using the device-less (DL) subcutaneous site. 174

Figure 7.1 Early graft visualization demonstrating formation of ductal structures in the DL space..... 193

Figure 7.2 Stimulated C-peptide secretion from transplanted mice..... 196

Figure 7.3 Blood glucose profiles from study groups..... 197

Figure 7.4 Engrafted PE after 22 weeks of transplantation using the pre-vascularized subcutaneous DL site 198

Figure 7.6 Benign cystic formations in one of the study animals..... 200

..... 214

Figure 8.1 The evolution of pancreatic islet transplantation..... 214

Figure 8.2 Gas permeable G-Rex culture flasks used in experimental and clinical transplantation 224

Figure 8.3 Schematic representation of pathways implicated in the effect of tacrolimus on islet cell function. 231

Figure 8.4 Different differentiation protocols to reach endocrine cells.. 249

Figure 8.5 Emulating pancreatic development. 251

Figure A.1 Islet transplantation: From infusion to engraftment.. 345

Figure A.2 Cellular pathways mediating the T-Cell induced auto- and alloimmune response.. 347

Figure A.3 Survival analysis for C-peptide secretion and insulin dependence post-transplantation at University of Alberta. 351

Figure A.4 Cellular targets for immunosuppressive drug intervention.. 356

Figure C.1 Computed tomography imaging pre-operatively demonstrating hypervascular metastatic renal cell carcinoma deposits. 411

Figure C.2 Intraoperative photograph of renal cell carcinoma in the pancreatic tail. 413

Figure C.3 A. Intraoperative photograph of surgical steps and back-table cannulation of the pancreatic duct prior to digestion and islet isolation. 414

Figure D.1A. Certificate of analysis for BMX-010, lot No. 60220-09-001 provided by Ricerca Biosciences. 423

Figure D.2. In vitro assessment of human islets pre-treated with BMX-010 and exposed to hydrogen peroxide (H_2O_2). 428

Figure D3. Effects of this lot of BMX-010 on interferon gamma (IFN-γ) expression from pre-stimulated murine spleen cells.. 430

LIST OF ABBREVIATIONS

AAGP	Anti-Aging Glycopeptide
AFPs	Anti-Freeze Proteins
AIT	Autologous Islet Transplantation
ANOVA	Analysis of Variance
APCs	Antigen Presenting Cells
ATG	Anti-T-Lymphocyte Globulin
AUC	Area Under the Curve
BMI	Body Mass Index
cGMP	Current Good Manufacturing Practice
CIT	Clinical Islet Transplant Consortium
CITR	Collaborative Islet Transplant Registry
CMRL	Connaught Medical Research Laboratories
CMV	Cytomegalovirus
CNI	Calcineurin Inhibitors
ConA	Concanavalin A
CT	Computed Tomography
CTLA-4	Cytotoxic T-Lymphocyte associated Antigen 4
D	Donor
DAPI	4',6-diamidino-2-phenylindole
DCD	Donation after Cardiac Death
DCs	Dendritic cells
d-GSIS	Dynamic Glucose-Stimulated Insulin Secretion
DL	Device-less
DNA	Deoxyribonucleic Acid
DRI	Donor Risk Index
ELISA	Enzyme-Linked Immunosorbent Assay
FC	Facilitating Cells
FDA	Federal Drug Administration

FDAc	Fluorescein Diacetate
FEP	Fluorinated Ethylene Propylene
GLP-1	Glucagon-Like Peptide 1
GVHD	Graft-Versus-Host Disease
H_2O_2	Hydrogen Peroxide
H&E	Hematoxylin & Eosin
HbA1C	Glycated – Glycosylated hemoglobin
hESC	Human Embryonic Stem Cells
HLA	Human Leukocyte Antigen
HTK	Histidine-Tryptophan-Ketoglutarate
IAPP	Islet Associated Polypeptide
IBMIR	Instant Blood-Mediated Inflammatory Reaction
ICAM	IntraCellular Adhesion Molecule
IEQ	Islet Equivalents
IFN	Interferon
IgG1	Immunoglobin G1
IL-	Interleukin
IPGTT	Intra-Peritoneal Glucose Tolerance Test
iPSCs	Induced Pluripotent Stem Cells
IPTR	International Pancreas Transplant Registry
IS	Immunosuppression
IT	Islet Transplantation
JDRF	Juvenile Diabetes Research Foundation
KC	Kidney Capsule
KC	Keratinocyte Chemokine
LFA	Leukocyte Function Antigen
mAb	Monoclonal Antibody
MAGE	Mean Amplitude Glycemic Excursions
MHC	Major Histocompatibility Complex
MLR	Mixed Lymphocyte Reaction
MMF	Mycophenolate Mofetil

MR	Magnetic Resonance
MSC	Mesenchymal Stem Cells
mTOR	Mammalian Target of Rapamycin
OCR	Oxygen Consumption Rate
OPTN	Organ Procurement and Transplantation Network
Ova	Ovalbumin
PBS	Phosphate Buffered Saline
PCV	Packed Cell Volume
PE	Pancreatic Endoderm
PRA	Panel Reactive Antibody
PTA	Pancreas Transplant Alone
PTDM	Post-Transplant Diabetes Mellitus
QALYs	Quality of Life units
R	Recipient
RCC	Renal Cell Carcinoma
ROS	Reactive Oxygen Species
SEM	Standard Error of the Mean
s-GSIS	Static Glucose-Stimulated Insulin Secretion
SI	Stimulation Index
SIK	Simultaneous Islet-Kidney transplant
SOD	Superoxide Dismutase
SPK	Simultaneous Pancreas-Kidney transplant
SRTR	Scientific Registry of Transplant Recipients
STZ	Streptozotocin
SubQ	Subcutaneous
SUITO	Secretory Units of Islet Transplant Objects
T1DM	Type 1 Diabetes Mellitus
Tac	Tacrolimus
TLM	Two-Layer Method
TNF-α	Tumor Necrosis Factor alpha
Tregs	Regulatory T cells

TUNEL	Terminal deoxynucleotidyl transferase dUTP Nick End Labeling
UNOS	United Network for Organ Sharing
US	United States
US	Ultrasound
UW	University of Wisconsin
vWF	Von Willebrand

CHAPTER 1.

INTRODUCTION

1. - INTRODUCTION

1.1 - GENERAL INTRODUCTION

Islet transplantation (IT) is today one of the most rapidly evolving fields within transplant medicine. It went from being a distant dream more than a century ago to be a very valuable treatment option for diabetic patients by compiling the continuous efforts from scientists all over the world, the courage of patients and the dedication of entire teams to turn an experimental treatment into an established therapy.

The principles of IT were born in the minds of pioneers like Watson Williams and Paul E. Lacy but decades went by before a viable islet isolation method was developed by Camillo Ricordi (1). Extensive experimentation was required to successfully extract the precious islets from pancreases to make them available for cell therapy, in times where cell therapy was not even a standard procedure.

Significant efforts resulted in well-established transplant models and soon the goal was to translate all this knowledge into acceptable clinical practice. Once islets were successfully transplanted in humans, the main objective was to keep cells alive and functional for longer periods. Many factors were soon identified as potential obstacles for engraftment and immunity was definitely one of the greatest adversaries. Clinicians used the available anti-rejection drugs and results slowly improved over time but it was not until the year 2000 when a significant sustained long-term graft function was achieved by introducing a new and more efficient protocol to select patients, along with a novel combination of immunosuppressants (2).

The Edmonton Protocol was also a tool to disseminate this treatment modality in countries all over the world. Soon, more patients were benefiting from this new approach and the attention

was then focused in prolonging graft function, reducing the diabetes-related complications and avoiding multiple donor procedures. Despite the growing success with IT, results have not been durable in time and many patients return to insulin therapy and require further transplants to attain normoglycemia (3).

Multiple studies are now being conducted to understand the mechanisms for graft function decline over time and potential strategies are continuously being examined to overcome this limitation by decreasing islet attrition after transplantation (3). Some of these strategies include the addition of promising molecules to the isolation and culture phase, the use of especially designed treatments to decrease immediate inflammatory response after transplant, the use of new and more potent immunosuppressive agents to decrease allo- and autoimmunity, as well as new strategies to protect beta cells from the adverse effects of anti-rejection drugs.

Islet transplantation like other transplant modalities suffers from the disparity between the number of available donors and the ever-growing number of type 1 diabetic patients potentially suitable for the procedure. This is one of the main reasons scientists have been chasing alternative sources to human pancreases. Potential ways to substitute human islets include utilizing of animal islets, reprograming other pancreatic cells to produce insulin and the use of human induced pluripotent or embryonic stem cells to mature into beta cells (4). The feasibility and the outcomes with each of these variants have evolved in time and today, new scientific breakthroughs have made these treatments a reality for the imminent future.

This thesis presents a compendium of history, principles and current outcomes of IT, along with some of our strategies to improve the long-term results with this procedure at the Edmonton Clinical Islet Transplant Program. We hypothesize that the inclusion of some or all of these strategies during the process of isolation, culture or immediately after transplant may result in

significant graft preservation and ultimately, in long-term diabetes reversal. These strategies specifically refer to supplementing the islets with potent antioxidants like metalloporphyrins. Another hypothesis we evaluated is that islets are severely impaired when exposed to tacrolimus at a therapeutic dose, and the Antiaging Glycopeptide is able to protect beta cell in vitro and in vivo from this deleterious effect. We also believe that implementing transplantation of surrogate insulin-producing stem cells provides a safe and viable alternative to traditional IT, with comparable results, using newly designed transplant sites more adequate for this treatment technique.

The document comprises of various chapters describing our view from the IT process exposed in the form of manuscripts published or being considered for publication in journals or books, relevant to this field.

1.1.2 – REFERENCES

1. Piemonti L. PA. 25 Years of the Ricordi Automated Method for Islet Isolation. CellR4 2013;1(1):e128.

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. Shapiro AM. Strategies toward single-donor islets of Langerhans transplantation. Curr Opin Organ Transplant 2011;16(6):627-631.

4. Bruin JE, Rezania A, Kieffer TJ. Replacing and safeguarding pancreatic beta cells for diabetes. Sci Transl Med 2015;7(316):316ps323.

1.2 - ISLET TRANSPLANTATION FOR TYPE 1 DIABETES

Special Reviews

Juntendo Medical Journal
2015. 61 (2), 000-000

Islet Transplantation for Type 1 Diabetes

BORIS GALA-LOPEZ*1), TOSHIYASU KAWAHARA*2),
ANDREW R. PEPPER*1), A.M. JAMES SHAPIRO*1)

*1)Clinical Islet Transplant Program, Department of Surgery, University of Alberta, *2)Division of Gastroenterological and Transplant Surgery, Department of Surgery, Asahikawa Medical University, Asahikawa, Japan

Decades of research have brought islet transplantation from the dream of few to the reality of many. This procedure started as an experimental method to treat type 1 diabetes mellitus, which features β-cell dysfunction due to auto immunity. The burden of complication in these patients is cumulative and has become a major health problem, despite the availability of insulin therapy. The procedure relies on a sequence of finely orchestrated procedures starting in the donor and followed by several steps to isolate high quality islets. Extensive research is nowadays focussed in improving islet engraftment by providing a more beneficial environment to newly transplanted cells, coupled by more effective immunosuppressive drugs to avoid allo and auto immunity. Excellent results are now a reality in the most specialized centers. Yet, further steps are required to transform this low-risk treatment in a widely available and long-lasting therapy for diabetics worldwide.

Key words: islet transplantation, diabetes mellitus, graft survival

Introduction

Type 1 diabetes Mellitus (T1DM) is a chronic, autoimmune disease resulting from the destruction of the insulin-producing β-cells within the pancreatic islets. This disease is characterized by impaired glucose metabolism leading to chronic micro and macrovascular complications[1) 2)]. Despite ongoing advances in monitoring and treatment of diabetes, morbidity and mortality remains increased when compared with non-diabetic populations[1) 2)].

Current treatment for T1DM mainly relies on the use of insulin replacement to attain normoglycemia. However, the metabolic control resulting from insulin therapy may not be accurate and is not sufficient to prevent long-term complications[3)]. Transplants have therefore become a valid alternative for these patients given the current results and despite the risk associated with the procedure and the long-term immunosuppression[4) 5)].

Significant progress has occurred in the outcomes of clinical islet transplantation, due to significant development in immunosuppression and availability of high quality of islets preparations for transplantation. Since the introduction of the Edmonton Protocol, when sustained c-peptide production and successful insulin independence was reported after solitary islet transplantation, this procedure has become an accepted modality to stabilize frequent hypoglycemias or severe glycemic lability in highly selected subjects with poor diabetic control[6)-8)]. This new breakthrough was possible with the avoidance of corticosteroids, and high-quality islet preparations. This mini-review presents the important role of islet transplantation, some of the challenges it faces and potential improvements for the future.

Boris Gala-Lopez
Immunobiology & Islet Transplantation Research, Clinical Islet Transplant Program, Alberta Diabetes Institute, University of Alberta
5-040 Li Ka Shing Health Center for Research Innovation
116 St & 85 Avenue. Edmonton, AB. Canada T6G 2R3
TEL: +1-780-492-4656 (Lab) FAX: +1-780-492-2892
Special Reviews: Minimally Invasive Hepatobiliary and Pancreatic Surgery
〔**Received Jan. 7, 2015**〕

Note: A version of this section was published as a Special Review in the Juntendo Med J 2015; 61 (2).

MINI-REVIEW ARTICLE

Islet transplantation for type 1 diabetes

Authors: Boris Gala-Lopez[1], Toshiyasu Kawahara[2], Andrew R. Pepper[1] and A.M. James Shapiro[1]

Affiliation:

1. Clinical Islet Transplant Program. Department of Surgery, University of Alberta. Edmonton, Canada
2. Division of Gastroenterological and Transplant Surgery. Asahikawa Medical University. Hokkaido Japan

Correspondence:

Boris Gala-Lopez. MD, MSc.

Immunobiology & Islet Transplantation Research

Clinical Islet Transplant Program

Alberta Diabetes Institute, University of Alberta

5-040 Li Ka Shing Health Center for Research Innovation

116 St & 85 Avenue. Edmonton, AB. Canada T6G 2R3

Tel: 1 (780) 492 - 4656 (Lab). Fax: 1 (780) 492 - 2892

1.2.1. - ABSTRACT

Decades of research have brought islet transplantation from the dream of few to the reality of many. This procedure started as an experimental method to treat type 1 diabetes mellitus, which features β-cell dysfunction due to auto immunity. The burden of complication in these patients is cumulative and has become a major health problem, despite the availability of insulin therapy. The procedure relies on a sequence of finely orchestrated procedures starting in the donor and followed by several steps to isolate high quality islets. Extensive research is nowadays focussed in improving islet engraftment by providing a more beneficial environment to newly transplanted cells, coupled by more effective immunosuppressive drugs to avoid allo- and auto immunity. Excellent results are now a reality in the most specialized centers. Yet, further steps are required to transform this low-risk treatment in a widely available and long-lasting therapy for diabetics worldwide.

1.2.2. – introduction

Type 1 diabetes Mellitus (T1DM) is a chronic, autoimmune disease resulting from the destruction of the insulin-producing β-cells within the pancreatic islets. This disease is characterized by impaired glucose metabolism leading to chronic micro and macrovascular complications (1, 2). Despite ongoing advances in monitoring and treatment of diabetes, morbidity and mortality remains increased when compared with non-diabetic populations (1, 2). Current treatment for T1DM mainly relies on the use of insulin replacement to attain normoglycemia. However, the metabolic control resulting from insulin therapy may not be accurate and is not sufficient to prevent long-term complications (3). Transplants have therefore become a valid alternative for these patients given the current results and despite the risk associated with the procedure and the long-term immunosuppression (4, 5).

Significant progress has occurred in the outcomes of clinical islet transplantation, due to significant developments in immunosuppression and availability of high quality of islet preparations for transplantation. Since the introduction of the Edmonton Protocol, when sustained c-peptide production and successful insulin independence was reported after solitary islet transplantation, this procedure has become an accepted modality to stabilize frequent hypoglycemia or severe glycemic lability in highly selected subjects with poor diabetic control (6-8). This new breakthrough was possible with the avoidance of corticosteroids, and high-quality islet preparations. This mini-review presents the important role of islet transplantation, some of the challenges it faces and potential improvements for the future.

1.2.3. - Historical Perspective

Clinical islet transplant was first attempted in the XIX century when early and rudimentary experimental treatments were performed by Dr. Watson-Williams and colleagues, consisting of subcutaneous implantation of a sheep's pancreas (9, 10). Several years later (1960) the vision changed to isolating cells rather than transplanting pancreas fragments. Paul E. Lacy pioneered experiments in mice, later refined by mechanical enzymatic digestion and by the use of dialyzed Ficoll for more efficient islet separation (9, 11, 12).

Various research groups were working on improving techniques that allow a larger yield and the intraductal injection of collagenase proved to be the most effective method for the successful isolation of islets from large animals, including humans (13). The introduction of a semi-automated dissociation chamber originally developed by Camillo Ricordi et al. in 1988 was definitely a major revolution in the process of obtaining high quality cells (14). The Ricordi® Chamber along with the COBE 2991 refrigerated centrifuge and a highly purified enzyme blend are today key elements to consistently achieve clinically relevant islet yield, with improved viability and function (9).

These studies allowed the establishment of successful transplantation in diabetic rodents and paved the way to eventually allow reversal of diabetes in the first human patient in 1989, followed by significant improvement when enhanced immunosuppression was introduced (15-17).

The Edmonton Protocol was definitely a milestone in the ultimate implementation of previous findings into a more efficient method to achieve lasting normoglycemia in a human cohort. James Shapiro and collaborators combined the use of sufficient islet mass (~11,500 islet

equivalent/kg) with a steroid-free immunosuppressive regime. Induction was achieved with daclizumab, followed by a combination of sirolimus and low-dose tacrolimus (6). This new protocol resulted in 100% insulin independence at 1 year in the first 7 treated patients, and was rapidly implemented in all existing islet transplant centers.

Figure 1.2-1 summarizes the most important milestones from this initial era in the development of islet transplantation from experimental to clinical therapy.

1.2.4. -The Process of Islet Transplantation

The process to obtain high quality islets for transplantation starts with the adequate selection of donors. Previous studies have identified various donor-related variables affecting islet isolation outcome; including donor age, cause of death, body mass index (BMI), cold ischemia time, length of hospitalization, use of vasopressors, and blood glucose levels (18, 19).

Preserved pancreases are then put through the digestion process via injection of an enzyme preparation to distend the main duct and elicit tissue separation (**Figure 1.2-2**) (20). To facilitate the isolation of the islets, enzyme blends are delivered to the islet-exocrine interface of which collagen is the major structural protein. (21, 22). As such, collagenase is a key component of the enzyme product for isolating islets. However, the use of collagenase alone results in inadequate tissue digestion (23, 24). Today, enzyme blends are used to allow efficient and gentle digestion the pancreatic tissue.

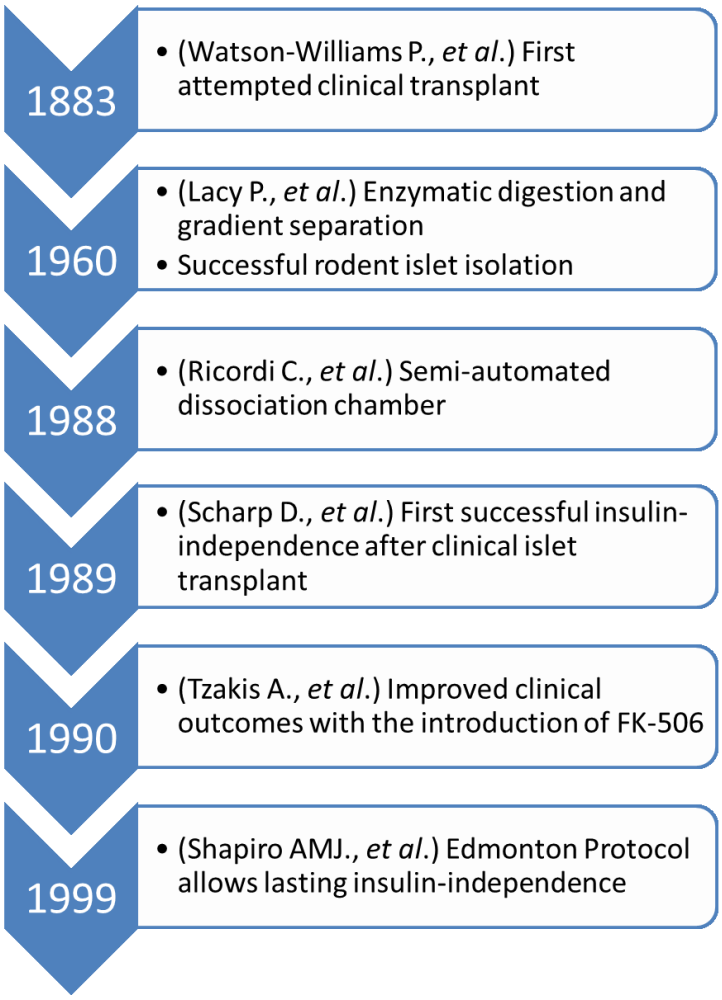


Figure 1.2-1 Timeline with the most important milestones in the transition of islet transplantation from experimental to clinical therapy.

The Ricordi® Chamber (Biorep Technologies, Miami Lakes, FL, USA) is an automated method for islet isolation developed by Dr. Camillo Ricordi in 1988. It's a crucial element for this step and allows a more optimal digestion through a combination of enzymatic and mechanical separation, with precise temperature control to allow proper preservation of the endocrine cell cluster integrity (**Figure 1.2-3**) (14). High purity islet fractions are then obtained after using Ficoll gradients, being this density dependent separation of islets from exocrine tissue is the simplest and most effective approach for islet purification (12, 20). The methodology is based on the principle that, during centrifugation, tissue will migrate and settle to the density that is equal to its own density (20). The use of COBE 2991 refrigerated centrifuge (COBE Laboratories Inc., Lakewood, CO, USA) has largely revolutionized this process, allowing highly improved yields (**Figure 1.2-4**). The COBE 2991 cell processor, originally developed for producing blood cell concentrates, is indispensable equipment in human islet processing facilities. It allows processing of a large volume of tissue in an enclosed sterile system. Moreover, it offers decreased operating time with an ease of generating continuous density gradients in conjunction with a two-chamber gradient maker. The final islet preparation is kept in culture until an adequate ABO-compatible recipient is identified (**Figure 1.2-5**).

The transplant procedure has evolved in time to allow current results. Today the intraportal access has become gold standard with minimal risk for the patients and the best engraftment possibility for diabetic patients (25). However, despite the latest refinements in the procedure and enhanced immunosuppression, islets must overcome significant obstacles to engraft, survive and function for a long time.

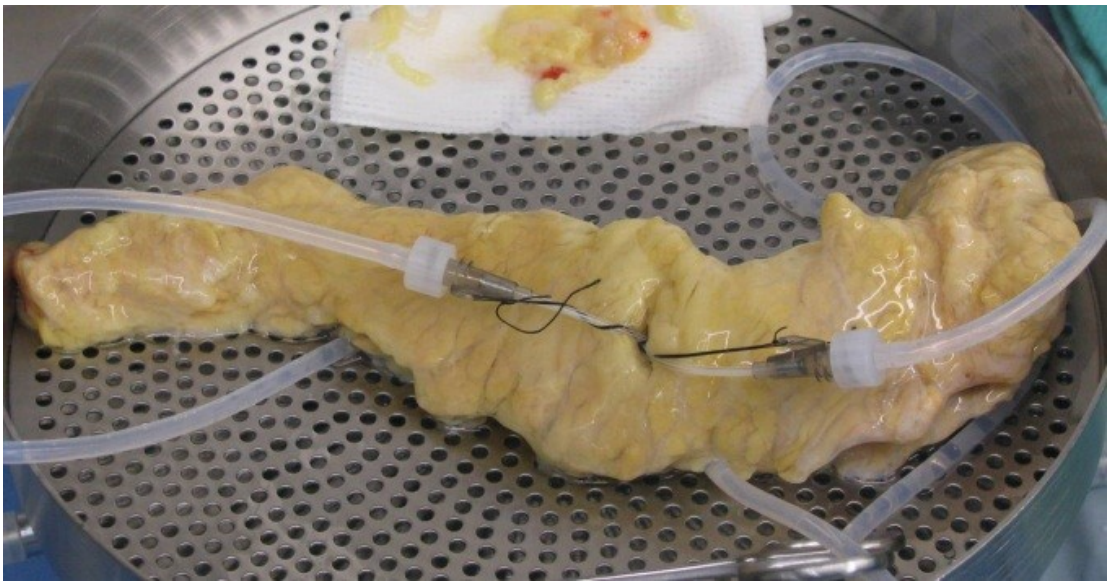


Figure 1.2-2 Pancreatic intraductal distension during the process of islet isolation. Photograph kindly provided by Dr. Tatsuya Kin from the University of Alberta Clinical Islet Laboratory. Reproduced with permission.

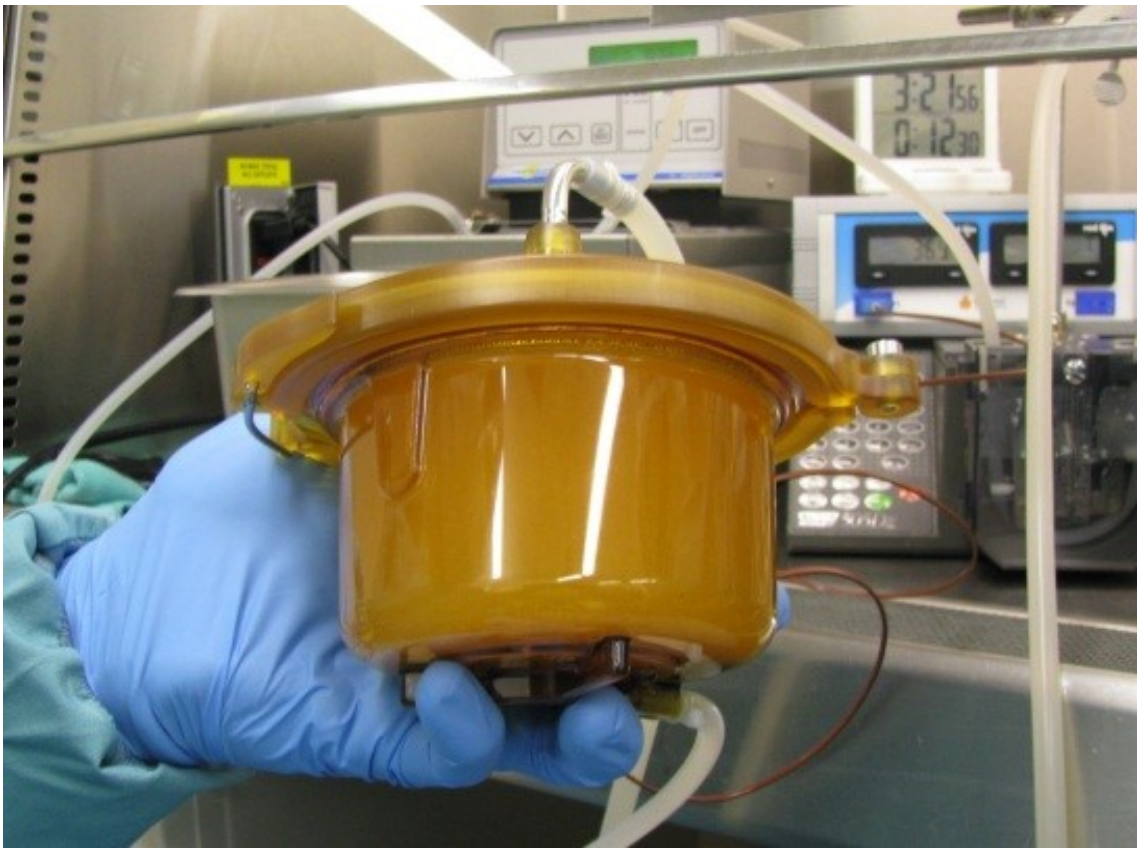


Figure 1.2-3 Pancreatic digestion using the Ricordi® Chamber during the process of islet isolation. Photograph kindly provided by Dr. Tatsuya Kin from the University of Alberta Clinical Islet Laboratory. Reproduced with permission.

Figure 1.2-4 Pancreatic purification using the COBE 2991 during the process of islet isolation. Photograph kindly provided by Dr. T. Kin from the University of Alberta Clinical Islet Laboratory. Reproduced with permission.

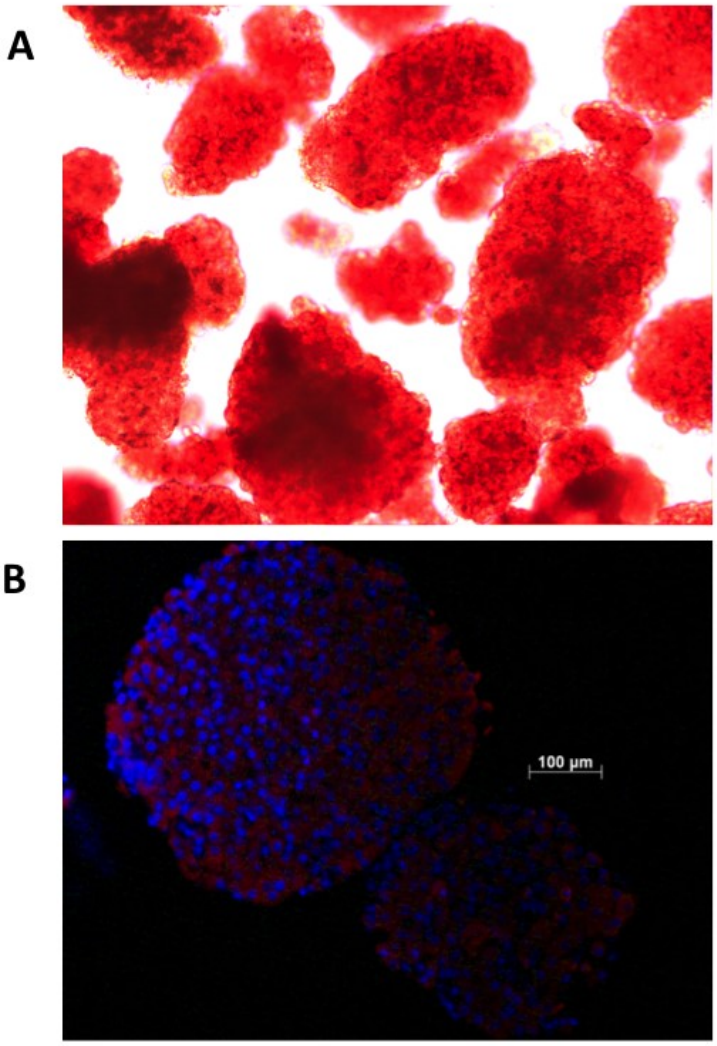


Figure 1.2-5 Microphotograph of isolated mouse islets. A. Islets stained with dithizone (DTZ, Sigma) under bright field microscopy (200X). Fluorescent microscopy of islets dually stained for insulin (red) and nuclei (4',6-diamidino-2-phenylindole, DAPI - blue) (400X magnification).

1.2.5. - Sites for transplantation

Success in islet engraftment is very much dependent on the availability of abundant vessels to allow a proper exchange between islets and the surrounding tissues (26). Multiple sites for transplantation have been studied with clear differences in performance and efficiency. These sites include the intraportal site, the kidney subscapular space, the splenic pulp, the omentum, testes, epididymal fat, the eye chamber, gastric submucosa, the subcutaneous space, vein sacs and small bowel, among others (26).

Intraportal infusion has been recognized as the most clinically efficient site for implantation given its high vascularity, the proximity to nutrient factors, and the physiological first pass insulin delivery to the liver (9). However, this site also carries potential risk for the recipient, including post-procedural hemorrhage or thrombosis, acute portal hypertension and arteriovenous fistula (25).

1.2.6. - The Main Obstacles

Islet transplantation through the portal vein is minimally invasive but results in islet entrapment within the sinusoids. This space provides an opportunity to acquire oxygen and nutritional support. However, the appropriate vascular connections are only formed around two weeks after infusion, leaving a large portion of the graft in ischemic conditions (26). The presence of tissue factor associated with the instant blood-mediated inflammatory reaction (IBMIR) and subsequent platelet activation, clot formation, and lymphocyte recruitment, may also negatively influence initial islet survival and overall transplant outcome (27, 28).

There are immunological challenges to islet survival too. Alloimmune rejection is a very important factor characterized by a full-blown response upon transplantation. However, autoimmune destruction is also present due to the inner nature of the disease featuring β-cell autoantibodies and β-cell-specific autoreactive T-cells. This type of response demands biological strategies to overcome immune response and eventually elicit tolerance in the host (26). The agents used vary in nature and are permanently needed after transplantation.

This continuous exposure to immunosuppressants may have an adverse impact on islet function and revascularization (29). Tacrolimus and sirolimus are two well-known and widely used drugs in the settings of transplant in general and also in islet transplantation, with proven deleterious (diabetogenic) effects on β-cell mass over time (30-33).

1.2.7. - Graft function monitoring

The overall results of clinical islet transplantation have significantly improved over the years reaching up to 50% graft survival after 5 years for selected centers. Yet, the profile of β-cell function of these individuals does not compare to healthy counterparts (4, 34). Multiple factors still account for the loss of islet mass, including immunological events, islet exhaustion and drug cytotoxicity. Thus, continuous monitoring of the graft function is paramount to secure long-term success.

The main tools to positively confirm islet health are relatively frequent clinical indicators. Levels of blood glucose stimulated C-peptide, glycated hemoglobin (Hb1AC) and insulin requirements are among the parameters evaluated in recipients. These variables may be analyzed individually or combined as scoring systems such as: the Beta Score, the Mean Amplitude Glycemic Excursions (MAGE), Lability Index, Hypo Score, Arginine Stimulation Score, the Secretory

Unit of Islet Transplant Object (SUITO) Index, etc. They all provide objective and quantitative information about the graft function and serve as important decision-making tools for the clinician (9, 35-37).

Complementary imaging studies may also be used to visualize the islets, with a high value when correlated with scoring systems. Techniques like optical imaging, magnetic resonance imaging and positron emission tomography are currently evaluated experimentally and have demonstrated potential for clinical application in the near future (38, 39).

1.2.8. - Future directions for islet transplantation

Islet transplant has evolved in time and today is performed in various centers with long-term results comparable to whole organ transplant. However, a significant number of patient undergoing this procedure still require multiple infusions to achieve normoglycemia and at some point some of them return to insulin supplementation. Yet, being insulin-dependent again does not necessarily equate with complete loss of graft function, as many of these patients remain C-peptide positive.

Several pre-clinical and clinical projects are now focused in finding improved results in islet transplantation by introducing tolerance-inducing medication. New potent biological agents like humanized anti-CD154, anti–CD28 or anti–CD52, among others have shown very encouraging results in solid organ transplant and are expected to produce enhanced islet survival after transplant (26).

Another stream of investigation is co-transplantation of islets with cells providing immune-protection and/or anti-inflammatory effects; such as Sertoli cells and mesenchymal stem cells.

An important alternative is the use of surrogate insulin-producing stem cells or xenotransplantation as a source of unlimited supply of islets (4).

1.2.9. – Conclusion

Islet transplantation is a treatment option for patients with type I diabetes, allowing glucose homeostasis without exogenous insulin administration. The procedure is very safe and currently provides extensive protection from hypoglycemic episodes for as much as 5 years. However, approximately 60% of the graft is lost soon after infusion due to various mechanisms (40). Preventing this significant post-transplant cell death is strategic and would have an immediate impact on the procedure.

Opportunities for intervention are endless to diminish the damage associated with the isolation, preservation, engraftment and immune-protection. These strategies would reduce the amount of islets required per patient to attain normoglycemia and would open up the avenue for using surrogate cells in clinical practice.

Preventing allo- and auto-immunity in islet transplantation demands lifelong immunosuppression. Many of the new developments in the field are devoted to immunoprotection and to induction of tolerance with new and more potent biological agents. Other technologies, like the use of bio-engineered devices or encapsulation are also used, looking to avoid the need for immunosuppression altogether.

Evidence shows that success may come from the combination of different approaches to achieve a synergistic solution to the current limitations of islet transplantation.

1.3 – REFERENCES

1. Forbes JM, Cooper ME. Mechanisms of diabetic complications. Physiol Rev 2013;93(1):137-188.

2. Vazeou A. Continuous blood glucose monitoring in diabetes treatment. Diabetes Res Clin Pract 2011;93 Suppl 1:S125-130.

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

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

5. Chhabra P, Brayman KL. Current status of immunomodulatory and cellular therapies in preclinical and clinical islet transplantation. J Transplant 2011;2011:637692.

6. 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.

7. Shapiro AM, Ricordi C, Hering BJ, Auchincloss H, Lindblad R, Robertson RP et al. International trial of the Edmonton protocol for islet transplantation. N Engl J Med 2006;355(13):1318-1330.

8. 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.

9. Shapiro AMJ. A historical perspective on experimental and clinical islet transplantation. In: A.M. James Shapiro, James AM Shaw, (ed). Islet Transplantation and beta cell replacement therapy New York. New York: Informa helthcare, 2007.

10. Watson-Williams, P. Notes on diabetes treated with extract and by grafts of sheep's pancreas. Br Med J 1894(2):1303.

11. Lacy PE, Kostianovsky M. A method for th isolation of intact islets of Langerhans from the rat pancreas. Diabetes 1967;15(35).

12. Scharp DW, Kemp CB, Knight MJ, Ballinger WF, Lacy PE. The use of Ficoll in the preparations of viable islet of Langerhans from the rat pancreas. Transplantation 1973;16(6):686.

13. Lakey JR, Warnock GL, Shapiro AM, Korbutt GS, Kneteman NM, Rajotte RV. Intraductal collagenase delivery into the human pancreas using syringe loading or controlled perfusion. Cell Transplant 1999;8(3):285-292.

14. Ricordi C, Lacy PE, Scharp DW. Automated islet isolation from human pancreas. Diabetes 1989;38 Suppl 1:140-142.

15. Scharp DW, Lacy PE, Santiago JV, McCullough CS, Weide LG, Falqui L et al. Insulin independence after islet transplantation into type I diabetic patient. Diabetes 1990;39(4):515-518.

16. Tzakis AG, Ricordi C, Alejandro R, Zeng Y, Fung JJ, Todo S et al. Pancreatic islet transplantation after upper abdominal exenteration and liver replacement. Lancet 1990;336(8712):402-405.

17. Ricordi C, Tzakis AG, Carroll PB, Zeng YJ, Rilo HL, Alejandro R et al. Human islet isolation and allotransplantation in 22 consecutive cases. Transplantation 1992;53(2):407-414.

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

19. O'Gorman D, Kin T, Murdoch T, Richer B, McGhee-Wilson D, Ryan E et al. The standardization of pancreatic donors for islet isolation. Transplant Proc 2005;37(2):1309-1310.

20. Pepper A, Gala-Lopez BL, Kin T. Advances in Clinical Islet Isolation. In: Islam MS, (ed). Islets of Langerhans. Dordrecht: Springer, 2014: 1165-1187.

21. Hughes SJ, Clark A, McShane P, Contractor HH, Gray DW, Johnson PR. Characterisation of collagen VI within the islet-exocrine interface of the human pancreas: implications for clinical islet isolation. Transplantation 2006;81(3):423-426.

22. Van Deijnen JH, Van Suylichem PT, Wolters GH, Van Schilfgaarde R. Distribution of collagens type I, type III and type V in the pancreas of rat, dog, pig and man. Cell Tissue Res 1994;277(1):115-121.

23. Wolters GH, Vos-Scheperkeuter GH, Lin HC, van Schilfgaarde R. Different roles of class I and class II Clostridium histolyticum collagenase in rat pancreatic islet isolation. Diabetes 1995;44(2):227-233.

24. 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.

25. Kawahara T, Kin T, Kashkoush S, Gala-Lopez B, Bigam DL, Kneteman NM et al. Portal vein thrombosis is a potentially preventable complication in clinical islet transplantation. Am J Transplant 2011;11(12):2700-2707.

26. 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.

27. Hawthorne WJ, Salvaris EJ, Phillips P, Hawkes J, Liuwantara D, Burns H et al. Control of IBMIR in neonatal porcine islet xenotransplantation in baboons. Am J Transplant 2014;14(6):1300-1309.

28. Hwang JW, Jung HS, Lee DY. Inhibition of platelet adhesion onto intrahepatically transplanted islets using PEGylation for attenuating instant blood-mediated inflammatory reaction (IBMIR). J Control Release 2011;152 Suppl 1:e213-214.

29. Nishimura R, Nishioka S, Fujisawa I, Shiku H, Shimada M, Sekiguchi S et al. Tacrolimus inhibits the revascularization of isolated pancreatic islets. PloS One 2013;8(4):e56799.

30. Hernandez-Fisac I, Pizarro-Delgado J, Calle C, Marques M, Sanchez A, Barrientos A et al. Tacrolimus-induced diabetes in rats courses with suppressed insulin gene expression in pancreatic islets. Am J Transplant 2007;7(11):2455-2462.

31. Johnson JD, Ao Z, Ao P, Li H, Dai LJ, He Z et al. Different effects of FK506, rapamycin, and mycophenolate mofetil on glucose-stimulated insulin release and apoptosis in human islets. Cell Transplant 2009;18(8):833-845.

32. Li F, Li Q. Effects of different dose of FK506 on endocrine function of pancreatic islets and damage of beta cells of pancreatic islets in a Wistar rat model. Immunopharmacol Immunotoxicol 2010;32(2):333-338.

33. Rostambeigi N, Lanza IR, Dzeja PP, Deeds MC, Irving BA, Reddi HV et al. Unique cellular and mitochondrial defects mediate FK506-induced islet beta-cell dysfunction. Transplantation 2011;91(6):615-623.

34. McCall M, Shapiro AM. Update on islet transplantation. Cold Spring Harb Perspect Med 2012;2(7):a007823.

35. Caumo A, Maffi P, Nano R, Luzi L, Hilbrands R, Gillard P et al. Comparative evaluation of simple indices of graft function after islet transplantation. Transplantation 2011;92(7):815-821.

36. Ryan EA, Shandro T, Green K, Paty BW, Senior PA, Bigam D et al. Assessment of the severity of hypoglycemia and glycemic lability in type 1 diabetic subjects undergoing islet transplantation. Diabetes 2004;53(4):955-962.

37. Matsumoto S, Noguchi H, Hatanaka N, Kobayashi N, Jackson A, Naziruddin B et al. Evaluation of engraftment after single islet transplantation from a brain-dead donor by the secretory unit of islet transplant objects (SUITO) index. Transplant Proc 2008;40(2):364-366.

38. Borot S, Crowe LA, Toso C, Vallee JP, Berney T. Noninvasive imaging techniques in islet transplantation. Curr Diab Rep 2011;11(5):375-383.

39. Speier S, Nyqvist D, Cabrera O, Yu J, Molano RD, Pileggi A et al. Noninvasive in vivo imaging of pancreatic islet cell biology. Nat Med 2008;14(5):574-578.

40. Shapiro AM. Strategies toward single-donor islets of Langerhans transplantation. Curr Opin Organ Transplant 2011;16(6):627-631.

CHAPTER 2.

THE METALLOPORPHYRIN BMX-010 IN HUMAN ISLET ISOLATION AND CLINICAL TRANSPLANTATION

CELLR4

WWW.THECUREALLIANCE.ORG THE CURE ALLIANCE

CellR4 2016; 4 (3): e2066

The metalloporphyrin BMX-010 in human islet isolation and clinical transplantation

B.L. Gala-Lopez[1,3], T. Kin[1,2], D.O'Gorman[1,2], A.J. Malcolm[2], A.R. Pepper[1,3], R.L. Pawlick[1], A. Bruni[1,3], N. Abualhassan[1,3], M. Bral[1], C. Jones[4], J.D. Piganelli[5], J.D. Crapo[4,6,7], A.M. James Shapiro[1,2,3]

[1]Alberta Diabetes Institute, University of Alberta, Edmonton, Alberta, Canada
[2]Clinical Islet Transplant Program, University of Alberta, Edmonton, Alberta, Canada
[3]Canadian Transplant Research Program (CNTRP), Edmonton, Alberta, Canada
[4]BioMimetix Pharmaceutical Inc., Englewood, USA
[5]Department of Immunology, University of Pittsburgh, Pittsburgh, USA
[6]University of Colorado Health Sciences Center, Denver, CO, USA
[7]National Jewish Health, Denver, CO, USA

Corresponding Author: A.M. James Shapiro, MD Ph.D FRCS (Eng) FRCSC MSM; e-mail: amjs@islet.ca

Keywords: Islet transplantation, Oxidative stress, Catalytic antioxidants, Viability.

Abstract

Introduction: **Despite the success of islet transplantation, islet loss during isolation and culture remains an enduring obstacle.**
Background: **Islets are known to have poor defense mechanisms against the accumulation of free radicals. Antioxidants have proven to be beneficial for improving islet viability and function during culture.**
This pilot study evaluates the benefits of the metalloporphyrin BMX-010 for clinical islet transplantation.
Materials and Methods: **Islets were isolated from 6 human pancreases in the presences of BMX-010 (34 µmol/L) supplementation. Treatment isolations were matched with 14 comparable non-research clinical isolation controls. All islet preparations were assessed for viability and function and subsequently transplanted into patients.**
Results: **Both groups showed similar yield (BMX: 511,581 IEQ *vs*. Controls: 395,021 IEQ, p=0.28) and comparable insulin release (stimulation index 4.48 ± 1.8 *vs*. 3.3 ± 0.7, p=0.45) after a median culture period of 33 hours. Oxygen consumption rate and fractional viability were also similar before transplant (p=0.14 and p=0.68, respectively). Isolations were more likely to be used in transplant when supplemented with BMX-010 (5/6; 83% *vs*. 8/14; 57%, p=0.26). Post-transplant graft function was also similar for both groups.**
Discussion: **BMX-010 did not impair human islet function but did not provide detectable benefit to cell yield or transplant efficacy compared to controls. Conversely, pre-clinical studies were encouraging. This may suggest that contrary to prior studies, cell death activation pathways may be less activated in clinical islet transplantation than previously estimated; alternatively, dose delivery or other parameters may be suboptimal.**
Conclusions: **We demonstrate herein that addition of BMX-010 across the islet isolation process does not affect human islet yield, post culture survival or beta cell function.**

Introduction

Islet transplantation has evolved substantially as a treatment modality for control of brittle type 1 diabetes complicated by frequent hypoglycemia. Since its introduction in the late 1990's there have been major modifications in islet isolation techniques, infusion procedures, post-transplant immunosuppression and medical care, resulting in substantial improvements in clinical outcomes[1,2]. However, there still remain critical steps in the

Note: A version of this section was published as an Original Manuscript in *Cell R4*, June 2016; 4(3): e2066

ORIGINAL PAPER

TITLE: The metalloporphyrin BMX-010 in human islet isolation and clinical transplantation

AUTHORS: Boris L. Gala-Lopez[1,3], Tatsuya Kin[1,2], Doug O'Gorman[1,2], Andrew J. Malcolm[2], Andrew R. Pepper[1,3], Rena L. Pawlick[1], Antonio Bruni[1,3], Nasser Abualhassan[1,3], Marius Bral[1], Carissa Jones[4], Jon D. Piganelli[5], James D. Crapo[4,6,7], A.M. James Shapiro[1,2,3]

AFFILIATIONS:

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

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

[3]Canadian Transplant Research Program (CNTRP)

[4]BioMimetix Pharmaceutical Inc.

[5]Department of Immunology. University of Pittsburgh

[6]University of Colorado Health Sciences Center

[7]National Jewish Health

CORRESPONDING AUTHOR:

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

Fellow of the Royal Society of Canada

Canada Research Chair in Transplant Surgery and Regenerative Medicine

Professor of Surgery, Medicine and Surgical Oncology

AHS Director Clinical Islet and Living Donor Liver Transplant Programs

Clinical Senior Scholar Alberta Innovates - Healthcare Solutions (AIHS)

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

tel. (780) 407 7330 fax. (780) 407 8259 Email: amjs@islet.ca

2.1. – ABSTRACT

Despite the success of islet transplantation, islet loss during isolation and culture remains an enduring obstacle. Islets are known to have poor innate defense mechanisms against the accumulation of free radicals. The use of antioxidants has proven to be beneficial for improving islet viability and function during culture. This pilot study evaluates the benefits of the metalloporphyrin BMX-010 for clinical islet transplantation. Islets were isolated from 6 human pancreases in the presences of BMX-010 (34 μmol/L) supplementation. Treatment isolations were matched with 14 comparable non-research clinical isolation controls. All islet preparations were assessed *in vitro* for viability and function and subsequently transplanted into patients. Both groups showed similar yield (BMX: 511,581 IEQ vs. Controls: 395,021 IEQ, p=0.28) and comparable insulin release (stimulation index 4.48 ± 1.8 vs. 3.3 ± 0.7, p=0.45) after a median culture period of 33 hours. Oxygen consumption rate and fractional viability were also similar before transplant (p=0.14 and p=0.68, respectively). Isolations were more likely to be used in transplant when supplemented with BMX-010 (5/6; 83% vs. 8/14; 57%, p=0.26). Post-transplant graft function was also similar for both groups. BMX-010 did not impair human islet function but did not provide detectable benefit to cell yield or transplant efficacy compared to controls. Conversely, pre-clinical studies were encouraging. This may suggest that contrary to prior studies, cell death activation pathways may be less activated in clinical islet transplantation than previously estimated; alternatively, dose delivery or other parameters may be suboptimal. We demonstrate herein that addition of a potent metalloporphyrin, BMX-010 across the islet isolation process does not affect human islet yield, post culture survival or beta cell function.

2.2. – INTRODUCTION

Islet transplantation has evolved substantially as a treatment modality for control of brittle type 1 diabetes complicated by frequent hypoglycemia. Since its introduction in the late 1990's there have been major modifications in islet isolation techniques, infusion procedures, post-transplant immunosuppression and medical care, resulting in substantial improvements in clinical outcomes (1, 2). However, there still remain critical steps in the isolation, culture and transplant process that result in islet cell death (3-5). These deleterious events may be associated with increased oxidative stress and triggered pro-inflammatory cascade resulting in cellular dysfunction and death, impaired clinical islet function and potential need for repeated transplants to achieve sustained insulin-independent normoglycemia (2).

Oxidative stress normally occurs when the balance between free radical production and elimination is disrupted. Islets are especially sensitive to hypoxia due to decreased innate antioxidant capacity, and the antioxidant gene *MnSOD* was previously shown to be under expressed in islets (6). Islets are therefore prone to dysfunction and death. As a consequence, antioxidant approaches have generated considerable interest including therapeutic agents such as metabolites, vitamins, trace elements, herbal products and enzymatic antioxidants that could potentially improve islet survival and function (7-9).

Metalloporphyrins are one potent approach that has been applied successfully to enhance superoxide dismutase (SOD) function. These compounds have demonstrated beta cell protection against diabetogenic agents with marked protection against autoimmune-mediated diabetes in mice (6). Furthermore, the metalloporphyrin analogue BMX-010 (AEOL10113) has markedly

enhanced islet preservation during culture through mitigation of reactive oxygen species (ROS)-induced damage and modulated inflammatory response (8, 10).

Despite overwhelming evidence associated with use of antioxidants to preserve beta cells experimentally, limited information is available reflecting clinical application in islet transplantation. The aim of this study was to assess whether supplementation with BMX-010, a metalloporphyrin analogue derived from the group of Mangano Porphyrin Antioxidant Mimetics, is safe and beneficial in clinical islet transplantation.

2.3. - MATERIALS AND METHODS

A non-randomized prospective, open label, pilot study (Clinical Trials.gov NCT02457858) was performed at the University of Alberta Clinical Islet Transplantation program with permission from Health Research Ethics Board of the University of Alberta, Edmonton, Alberta, Canada (protocol number: Pro00045961) and Health Canada (HC Control No.185631). Only donation after brain death was considered for this trial and study cases were subsequently age-matched to standard of care control donors at a ratio of 1:2.

2.3.1. - Islet isolation and BMX-010 supplementation

Human pancreata were procured from consenting multi-organ deceased donors and flushed *via* superior mesenteric and splenic artery with 500 mL of University of Wisconsin solution (SPS-1; Organ Recovery Systems. Itasca, IL, USA) containing BMX-010 (BioMimetix, Greenwood

Village, CO, USA), at 34 μmol/L, followed by pancreatic duct distension with a collagenase blend supplemented with BMX-010 at the same concentration.

Islets were isolated as previously described using the modified Ricordi chamber, and purified with refrigerated centrifugation and continuous gradient density separation (11). All islet preparations were cultured on Connaught Medical Research Laboratories (CMRL) media supplemented with 34 μmol/L of BMX-010 for a median period of 33 hours (range 14.2 – 53.7) before infusion in patients, to allow for administration of the corresponding immunosuppressive protocol.

2.3.2. - In vitro recovery and functional assessment

Islets were assessed *in vitro* for recovery, insulin release, fractional viability and oxygen consumption rate (OCR). Recovery rate was calculated as the proportion of live islets after culture compared to the initial count. The secretory function was evaluated by both static glucose-stimulated insulin secretion (s-GSIS) performed at low (2.8 mmol/L) and high (28 mmol/L) glucose concentrations, followed by measurement of insulin concentration in supernatants using ELISA (Mercodia, Uppsala, Sweden). A stimulation index (SI) was subsequently calculated as the ratio of stimulated to basal insulin secretion. In another experiment triplicate samples were taken from one isolation and cultured with or without BMX supplementation. s-GSIS was performed after culture to evaluate functional differences in paired samples.

2.3.3. - Fractional beta cell viability

Beta cell viability was assessed as previously reported (12, 13). Briefly, dissociated islets were incubated with 3 mM Newport Green (NG; Molecular Probes, Eugene, OR, USA) and 0.2 nM of Tetramethylrhodamine ethyl ester (TMRE; Molecular Probes) for 45 min at 37°C in PBS. After washing, 1 mg/ml of 7-aminoactinomycin D (7-AAD; Molecular Probes) was added. Cell suspension was analyzed by flow cytometry using a BD Laser Scanning Cytometer II (LSR II; BD Biosciences, Missisauga ON, Canada). Data were analyzed using the FCS Express 3 software (DeNovo Software, Los Angeles CA, USA). Results are expressed as a proportion of live and non-apoptotic beta cells within the cell population.

2.3.4. - Oxygen consumption rate

To further characterize the islet preparations before transplantation, OCR was measured (before and after culture) as an indirect indicator of cell potency and a predictor of post-transplant function (14). *In vitro* OCR was performed as previously reported (14, 15). Aliquots of 3,000 IEQ were split into triplicate samples and introduced into pre-calibrated, water-jacketed, titanium chambers outfitted with fiber optic patches (175 µl FOL oxygen monitoring system, Instech Laboratories Inc., Plymouth Meeting, PA, USA). Crude OCR measurements were normalized to the islet DNA content per chamber, assessed using a dsDNA fluorescent dye (Quant-iTPicoGreendsDNA Assay Kit, Invitrogen, Life Technologies Corporation, Grand Island, NY, USA). Results are expressed as OCR/DNA (nmol O_2/min•mg DNA).

2.3.5. - Transplant procedures

Islet preparations were used for clinical transplantation when release criteria were met (16). Recipients were adult patients listed for islet infusion at the University of Alberta Hospital. Immunosuppressive induction therapy was accomplished with a combination of alemtuzumab (MabCampath, Genzyme Corp., Mississauga, ON, Canada), anakinra (Kineret; Amgen Canada Inc., Mississauga, ON, Canada) and etanercept (Enbrel; Amgen Canada Inc., Mississauga, ON, Canada), followed by a maintenance immunomodulation with tacrolimus (Prograf; Astellas Pharma Canada Inc., Markham, ON, Canada) and mycophenolate mofetil (CellCept; Hoffmann-La Roche Ltd., Mississauga, ON, Canada)

Patients were followed post-transplant, according to our standard clinical protocol and graft function was evaluated periodically with various tests including blood concentrations of C-peptide before and after standard mix meal stimulation, as well as daily insulin requirements (17).

2.3.6. - Statistical analysis

Data are represented as means ± standard error of the mean (SEM). Differences between groups were analyzed using unpaired t-test and Z-score test was used to compare proportions between groups. All comparisons between groups were performed with a 95% confidence interval and a two-tailed p-value <0.05 was considered significant. Analysis was performed using GraphPad Prism (GraphPad Software, La Jolla, CA, USA).

Table 2.1 Baseline characteristics of donors allocated to BMX-010 and control group. Data shows comparable donor age and pancreas weight resulting in similar isolation yield and islet preparation purity. There is a slight superiority for isolation success in the BMX-010 group, although differences are not statistically significant (p=0.26). Variables expressed as means (95% confidence interval).

	BMX-010	Controls	p-value
Sample size	6	14	-
Mean donor age (years)	51.3 ± 7	46.8 ± 14	0.47
Mean pancreas weight (g)	93.7 ± 43	89.8 ± 18	0.77
Mean cold ischemia time (h)	9.7 ± 4	10.1 ± 4	0.76
Mean isolation yield (IEQ)	511,580 ± 220,939	395,021 ± 213,405	0.28
Preparation purity (%)	57.5 ± 20	51.0 ± 14	0.43
Mean culture time (h)	33.5 ± 17	34.3 ± 10	0.92
Isolation Success	5/6 (83.3%)	8/14 (57.1%)	0.26

2.4. – RESULTS

Six human islet isolations were performed with BMX-010 supplementation within this pilot study and 14 non-research clinical islet isolations served as controls. **Table 2.1** summarizes the baseline characteristics for both groups, comparable for donor age, pancreas weight and cold ischemia time.

The isolation and purification process resulted in a slightly higher absolute islet yields for the treatment group although not significant compared to controls (BMX: 511,581 ± 220,939 IEQ vs. Controls: 395,021 ± 213,405 IEQ, p=0.28). Similarly, islet yield adjusted per pancreas weight was slightly better for the BMX group (BMX: 5,614 ± 554 IEQ/g vs. Controls: 4,357 ± 551 IEQ/g, p=0.19) with comparable preparation purity (BMX: 57% ± 20 vs. Controls: 51% ± 14, p=0.43) (**Figure 2.1A and B**).

Islets were maintained in culture with or without BMX-010 for similar duration (BMX: 33.5h ± 17 vs. Controls: 34.3h ± 10, p=0.92) while the transplant was allocated to the corresponding recipient and immunosuppressive induction was given. Recovery after culture was higher in the BMX group, although differences were not statistically significant (BMX: 87% ± 6 vs. 86% ± 4, p=0.78) (**Figure 2.1C**).

The functional assessment of islets in both groups showed a slightly higher stimulation index in the BMX group (4.5 ± 1.8) compared to controls (3.3 ± 0.7, p=0.45) (**Figure 2.2A**). There was also a trend in improved insulin secretion comparing stimulation indices of paired samples after culture with or without antioxidant, but were non-significant (p=0.19) (**Figure 2.2B**).

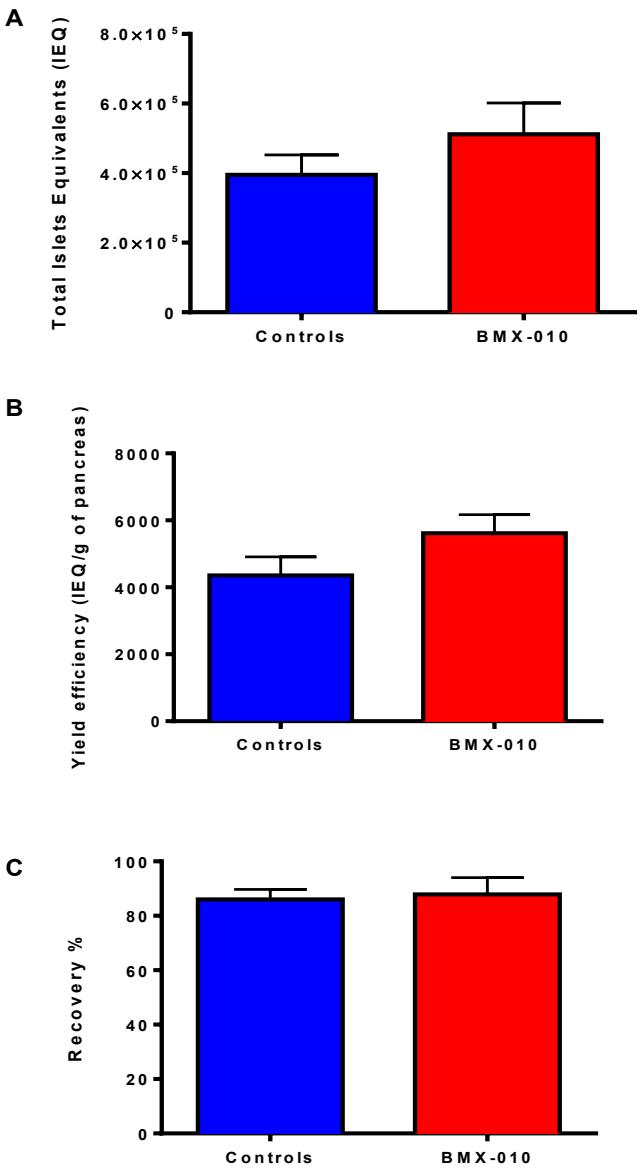


Figure 2.1. Human islet isolation with addition of BMX-010. A. Similar total islet mass resulting from isolation with or without BMX supplementation (p=0.28). **B**. Yield is also similar for both groups when islet mass is adjusted by initial pancreas weight (p=0.19). **C**. Islet recovery refers to the number of surviving islets after culture. No statistically significant difference is seen between groups. Data expressed as means ± SEM (95% confidence interval).

OCR was measured in islet preparations from both groups. Pre and post culture measurements were considered adequate and similar for both groups with no significant variations before (BMX: 112 ± 14nmol/min*mgDNA vs. Controls: 94.5 ± 7.7nmol/min*mgDNA, p=0.25) and after culture period (BMX: 89.5 ± 10.6nmol/min*mgDNA vs. 112.3 ± 9.1nmol/min*mgDNA, p=0.14) (**Figure 2.2C and D**).

Fractional viability of islets was assessed immediately before transplantation. Both groups exhibited similar viability profiles with comparable percentages of live beta cells (BMX: 54% ± 4 vs. Controls: 57% ± 6.1, p=0.68) as well as proportions of live non-apoptotic beta cells (BMX: 26% ± 3.6 vs. Controls: 41% ± 5.5, p=0.08) (**Figure 2.2E and F**).

Of the BMX-supplemented islet preparations, 5 of 6 (83%) were successfully used for transplantation, whereas only 8 of 14 (57%) were utilized in the control group (p=0.26). The islet dose was similar for both groups of recipients (BMX: 6,562 ± 830 IEQ/Kg vs. Controls: 6,989 ± 571 IEQ/Kg, p=0.67) (**Figure 2.3A**) and the 45-day follow up assessment showed adequate graft function in both groups, with significant decrease in recipient's daily insulin requirement post-transplant (BMX: 0.55 ± 0.08 units/Kg vs. 0.08 ± 0.03 units/Kg, p<0.0001; Controls: 0.61 ± 0.06 units/Kg vs. 0.03 ± 0.02 units/Kg, p<0.0001) paired with a significant increase in the blood concentration of C-peptide after high glucose stimuli (BMX: 0.02 ± 0.004 nmol/L vs. 1.71 ± 0.13 nmol/L, p<0.0001. Controls: 0.02 ± 0.004 nmol/L vs. 1.79 ± 0.16 nmol/L, p<0.0001) (**Figure 2.3B and C**). Further stability and functional testing for this specific lot of BMX-010 is provided in **Appendix D**

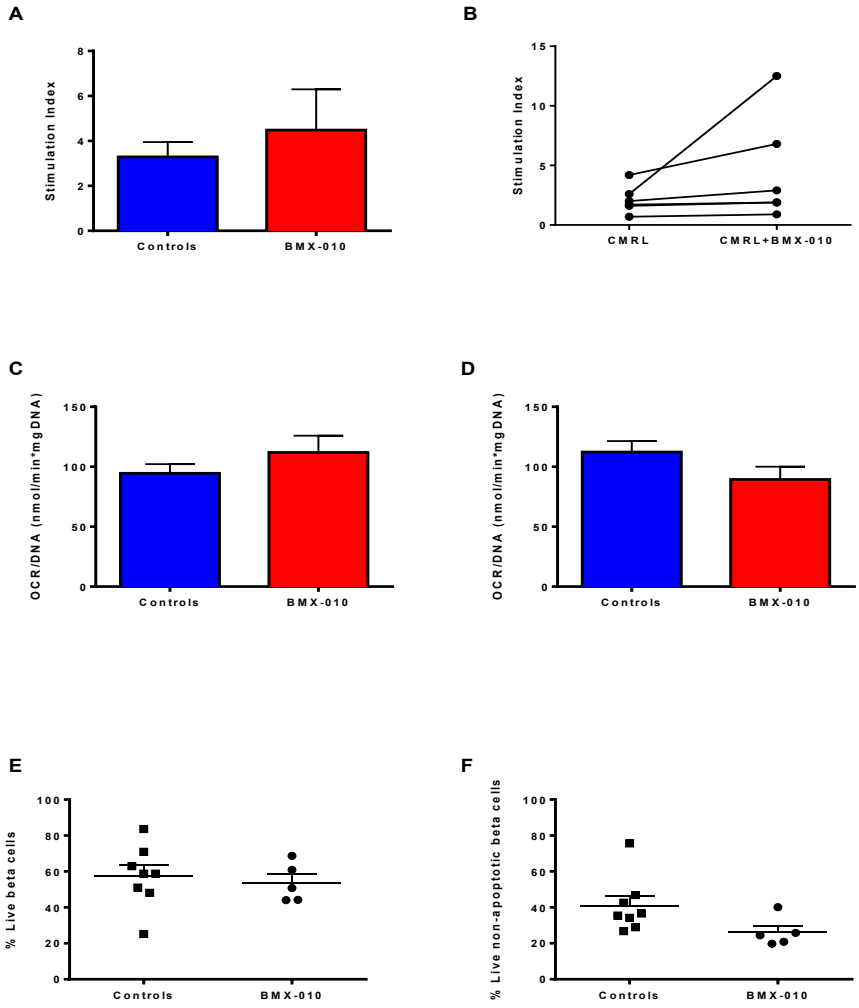


Figure 2.2 Function and viability of human islets treated with BMX-010 during isolation and culture. A. Static Glucose-stimulated insulin secretion (s-GSIS) is similar for both groups after culture (p=0.19). **B.** Post-culture s-GSIS analysis in paired samples of islets cultured with or without BMX-010 showing little variation in insulin secretion, expressed as Stimulation Index (SI) (p=0.45). **C and D**. Oxygen consumption rate (OCR) was measured as a direct indicator of cell viability and a predictor of function. **C.** Pre-culture measurements showing no inter-group differences (p=0.25). **D.** Similarly, no significant difference is found after the culture period (p=0.14). Data expressed as means ± SEM (95% confidence interval). CMRL: Connaught Medical Research Laboratories media. **E and F**. Fractional beta cell viability of human islets after BMX-010 supplementation. The proportion of live beta cells and non-apoptotic beta cells remains unchanged in both groups despite the use of the catalytic antioxidant (p=0.68 and p=0.08, respectively). Data expressed as means ± SEM (95% confidence interval).

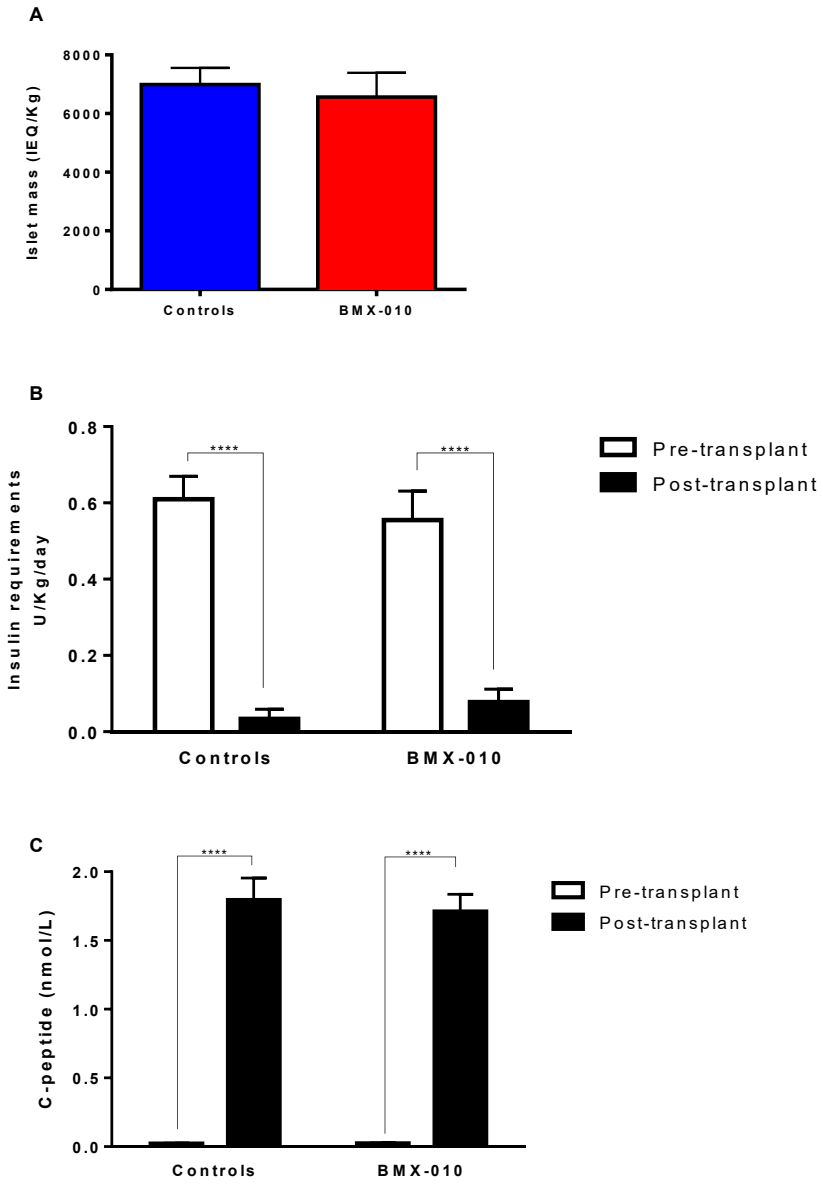


Figure 2.3 Transplant efficacy of human islets treated with or without BMX-010. (**A**). The islet dose was similar for both groups (p=0.67) and within our clinical protocol. Early graft function is similar for both groups, expressed as significant reduction on daily insulin requirements (**B**), as well as a significant increase in blood concentrations of C-peptide after a high glucose stimulus (**C**). Data expressed as means ± SEM (95% confidence interval).

2.5. – DISCUSSION

Previous studies have demonstrated that beta cells are especially susceptible to oxidative stress and to injuries resulting from accumulation of free radicals. Pancreatic islets contain low concentrations of antioxidant enzymes (catalases, superoxide dismutase and glutathione peroxidase) and respond weakly to increasing levels of ROS (7, 8). The natural mechanism for cells to neutralize harmful ROS include combinations of enzymes, minerals and vitamins, which are found in lower concentration in islets. Antioxidants supplementation during the islet transplant process has been shown to be beneficial through scavenging of locally generated ROS, thereby improving islet engraftment (6).

In the current study, we evaluated the potential benefits of BMX-010 for clinical islet transplantation. This compound is a synthetic member of the Mangano Porphyrin Antioxidant Mimetics group, which have been extensively studied and previously characterized (10). BMX-010 has clearly demonstrated immunomodulatory effects, as well as cytoprotective effects in the field of experimental islet transplantation (18-21). BMX-010 supplementation *in vitro* has been associated with a significant decrease of NF-κB activation with subsequent protection of islets against oxidative stress (18).

We incorporated BMX-010 into the vascular flush of human pancreata immediately prior to isolation, with the goal being to deliver this drug to the intact islet microcirculation before islets are disrupted from their basement membranes. Furthermore, we supplemented the enzymatic digestion media and culture media, to ensure active compound was present during critical steps of potential injury. *In vitro* viability and functional measurements exhibited similar outcomes for BMX-010 and control groups, suggesting no overt added benefit for this therapy. However, these

results also imply that the reagent is non-toxic at least when delivered at a local concentration of 34 μmol/L.

Furthermore, we observed a higher islet utilization ratio for clinical transplantation was observed when BMX-010 treatment was used. However, the sample size was underpowered to reflect a clear advantage. Post-transplant graft function was equally comparable in both groups importantly indicating no harm in use of this compound when exposed to human islets.

A possible explanation for lack of potent effect in the clinical setting compared to small animal supportive studies published previously may relate to the design of pre-clinical experiments, which may have inadequately replicated the more complex human islet isolation process. The findings may further highlight the limited translatability of preclinical catalytic antioxidant studies for human experimentation.

Another explanation may be that the dose used in our experiments was suboptimal for clinical settings. We used BMX-010 at a concentration of 34 μmol/L, which corresponds to the physiologic levels of SOD in most cells (8). Previous experimental work with this drug has demonstrated that this concentration is sufficient to successfully protect islets against oxidative stress (10). However, other studies have used doses as high as 68 μM without toxic events (8).

Finally, we cannot discount the possibility that given the fact that we did deliver a potent antioxidant compound directly to the islet microvasculature before isolation, that in fact oxidative stress may not be the dominant pathway leading to obligate islet demise in the clinical setting.

Despite the overall results of no added benefit by supplementing islet isolation and culture with BMX-010, the important observation that BMX-010 was non-toxic to human islets should not be discounted, and therefore does not preclude further work utilizing SOD mimetics in experimental

or clinical islet isolation and transplantation. Further evidence demonstrating functional and structural integrity of the BMX-010 lot used in this study is now included in **Appendix D**. We are now designing a more dose-efficient study in humans to explore the full capabilities of this BMX family molecules, including its latest addition, BMX-001, which has shown a more potent antioxidant effect at lower doses (22).

2.6. – REFERENCES

1. Shapiro AM. Immune antibody monitoring predicts outcome in islet transplantation. Diabetes 2013;62(5):1377-1378.

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

3. Hanley SC, Paraskevas S, Rosenberg L. Donor and isolation variables predicting human islet isolation success. Transplantation 2008;85(7):950-955.

4. 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.

5. 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.

6. Delmastro MM, Piganelli JD. Oxidative stress and redox modulation potential in type 1 diabetes. Clin Dev Immunol 2011;2011:593863.

7. 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.

8. Sklavos MM, Bertera S, Tse HM, Bottino R, He J, Beilke JN et al. Redox modulation protects islets from transplant-related injury. Diabetes 2010;59(7):1731-1738.

9. 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.

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. Andres A, Kin T, O'Gorman D, Livingstone S, Bigam D, Kneteman N 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.

12. Ichii H, Inverardi L, Pileggi A, Molano RD, Cabrera O, Caicedo A et al. A novel method for the assessment of cellular composition and beta-cell viability in human islet preparations. Am J Transplant 2005;5(7):1635-1645.

13. Miki A, Ricordi C, Yamamoto T, Mita A, Barker S, Khan A et al. Effect of human islet rescue gradient purification on islet yield and fractional Beta cell viability. Transplant Proc 2008;40(2):360-361.

14. Papas KK, Colton CK, Nelson RA, Rozak PR, Avgoustiniatos ES, Scott WE, 3rd et al. Human islet oxygen consumption rate and DNA measurements predict diabetes reversal in nude mice. Am J Transplant 2007;7(3):707-713.

15. Kitzmann JP, Pepper AR, Gala-Lopez B, Pawlick R, Kin T, O'Gorman D et al. Human islet viability and function is maintained during high-density shipment in silicone rubber membrane vessels. Transplant Proc 2014;46(6):1989-1991.

16. Kin T, O'Gorman D, Schroeder A, Onderka C, Richer B, Rosichuk S et al. Human islet distribution program for basic research at a single center. Transplant Proc 2011;43(9):3195-3197.

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

18. 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.

19. 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.

20. Sklavos MM, Tse HM, Piganelli JD. Redox modulation inhibits CD8 T cell effector function. Free Radic Biol Med 2008;45(10):1477-1486.

21. 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.

22. Tanaka T, Fujita M, Bottino R, Piganelli JD, McGrath K, Li J 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.

CHAPTER 3.

ANTIAGING GLYCOPEPTIDE PROTECTS HUMAN ISLETS AGAINST TACROLIMUS-RELATED INJURY AND FACILITATES ENGRAFTMENT IN MICE

3. - Antiaging Glycopeptide Protects Human Islets against Tacrolimus-Related Injury and Facilitates Engraftment in Mice

Diabetes Volume 65, February 2016 451

Boris L. Gala-Lopez,[1,2] Andrew R. Pepper,[1,2] Rena L. Pawlick,[1] Doug O'Gorman,[3] Tatsuya Kin,[3] Antonio Bruni,[1,2] Nasser Abualhassan,[1,2] Mariusz Bral,[1] Austin Bautista,[1,4] Jocelyn E. Manning Fox,[1,4] Lachlan G. Young,[5] Patrick E. MacDonald,[1,4] and A.M. James Shapiro[1,2,3]

Antiaging Glycopeptide Protects Human Islets Against Tacrolimus-Related Injury and Facilitates Engraftment in Mice

Diabetes 2016;65:451–462 | DOI: 10.2337/db15-0764

Clinical islet transplantation has become an established treatment modality for selected patients with type 1 diabetes. However, a large proportion of transplanted islets is lost through multiple factors, including immunosuppressant-related toxicity, often requiring more than one donor to achieve insulin independence. On the basis of the cytoprotective capabilities of antifreeze proteins (AFPs), we hypothesized that supplementation of islets with synthetic AFP analog antiaging glycopeptide (AAGP) would enhance posttransplant engraftment and function and protect against tacrolimus (Tac) toxicity. In vitro and in vivo islet Tac exposure elicited significant but reversible reduction in insulin secretion in both mouse and human islets. Supplementation with AAGP resulted in improvement of islet survival (Tac^+ vs. Tac+AAGP, 31.5% vs. 67.6%, $P < 0.01$) coupled with better insulin secretion (area under the curve: Tac^+ vs. Tac+AAGP, 7.3 vs. 129.2 mmol/L/60 min, $P < 0.001$). The addition of AAGP reduced oxidative stress, enhanced insulin exocytosis, improved apoptosis, and improved engraftment in mice by decreasing expression of interleukin (IL)-1β, IL-6, keratinocyte chemokine, and tumor necrosis factor-α. Finally, transplant efficacy was superior in the Tac+AAGP group and was similar to islets not exposed to Tac, despite receiving continuous treatment for a limited time. Thus, supplementation with AAGP during culture improves islet potency and attenuates long-term Tac-induced graft dysfunction.

Islet transplantation outcomes have improved significantly in highly specialized centers, with a 5-year insulin independence rate exceeding 50% (1). However, a proportion of patients require reintroduction of insulin at delayed time points (2). Numerous factors contribute to limited durability of both short- and long-term glycemic control. Exposure to diabetogenic immunosuppressive agents is associated with islet functional impairment and graft loss, especially that linked to corticosteroid exposure or use of calcineurin inhibitors (CNIs), including tacrolimus (Tac) (3,4). Tac is the most potent mainstay immunosuppressant in most clinical protocols, although diabetogenicity is well documented, accounting for an increased incidence of posttransplant diabetes mellitus (5–8). Early peak Tac levels in portal blood after islet transplantation after oral administration may further increase islet exposure, thereby magnifying toxicity (9,10). Although undesirable, Tac has become necessary to preventing rejection and autoimmune recurrence after islet transplantation and may be partly responsible for limited islet durability and a need for multiple donors for each recipient (11).

Antifreeze proteins (AFPs) have generated considerable interest for their ability to protect cells under a variety of conditions. They naturally occur in Arctic and Antarctic fish as well as in other cold climate–dwelling invertebrates and are responsible for maintaining cell and tissue function at subzero temperatures (12,13). AFPs were successfully isolated in the 1950s and have demonstrated an ability to noncolligatively lower the freezing temperature of body fluids by binding to ice crystals (12,14). Early experiments with AFPs in the

[1]Alberta Diabetes Institute, University of Alberta, Edmonton, Alberta, Canada
[2]Canadian National Transplant Research Program, University of Alberta, Edmonton, Alberta, Canada
[3]Clinical Islet Transplant Program, University of Alberta, Edmonton, Alberta, Canada
[4]Department of Pharmacology, University of Alberta, Edmonton, Alberta, Canada
[5]ProtoKinetix Inc., Vancouver, Vancouver, British Columbia, Canada

Corresponding author: A.M. James Shapiro, amjs@islet.ca.

Received 8 June 2015 and accepted 10 November 2015.

© 2016 by the American Diabetes Association. Readers may use this article as long as the work is properly cited, the use is educational and not for profit, and the work is not altered.

Note: A version of this section was published as an Original Manuscript in *Diabetes* 2016; 65: 451 - 462.

ORIGINAL ARTICLE

Anti-aging Glycopeptide Protects Human Islets Against Tacrolimus-related Injury and Facilitates Engraftment in Mice

Authors: Gala-Lopez Boris L[1,5], Pepper Andrew R[1,5], Pawlick Rena L[1], O'Gorman Doug[2], Kin Tatsuya[2], Bruni Antonio[1,5], Abualhassan Nasser[1,5], Bral Mariusz[1], Bautista Austin[1,4], Manning Fox Jocelyn E[1,4], Young Lachlan G[3], MacDonald Patrick E.[1,4] and Shapiro AMJ[1,2,5]

Affiliations:

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

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

[3]ProtoKinetix Inc. Vancouver, British Columbia, Canada

[4]Department of Pharmacology, University of Alberta, Edmonton, Alberta, Canada

[5]Canadian Transplant Research Program (CNTRP)

Corresponding author contact information:

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

Fellow of the Royal Society of Canada

Canada Research Chair in Transplant Surgery and Regenerative Medicine

Professor of Surgery, Medicine and Surgical Oncology

AHS Director Clinical Islet and Living Donor Liver Transplant Programs

Clinical Senior Scholar Alberta Innovates - Healthcare Solutions (AIHS)

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

tel. (780) 407 7330 fax. (780) 407 8259 Email: amjs@islet.ca

Running title: AAGP protects transplanted human islets

3.1. – ABSTRACT

Clinical islet transplantation has become an established treatment modality for selected patients with Type 1 diabetes. However, a large proportion of transplanted islets is lost through multiple factors including immunosuppressant-related toxicity, often requiring more than one donor to achieve insulin independence. Based on the cytoprotective capabilities of antifreeze proteins (AFP) we hypothesize that supplementation of islets with synthetic AFP analogue, Anti-aging Glycopeptide (AAGP) would enhance post-transplant engraftment and function, and would protect against tacrolimus (Tac) toxicity. *In vitro* and *in vivo* islet Tac exposure elicited significant but reversible reduction in insulin secretion in both mouse and human islets. Supplementation with AAGP resulted in improvement of islet survival (Tac+ vs. Tac+AAGP, 31.6 vs. 67.7, $p<0.001$) coupled with better insulin secretion (AUC: Tac+ vs. Tac+AAGP, 8.2 vs. 129.3, $p<0.01$). The addition of AAGP reduced oxidative stress, enhanced insulin exocytosis and improved engraftment in mice by decreasing expression of IL-1β, IL-6 and keratinocyte chemokine in Tac+AAGP, resulting in less graft apoptosis. Finally, transplant efficacy was superior in the Tac+AAGP group, and was similar to islets not exposed to tacrolimus, despite receiving continuous treatment. Thus, supplementation with AAGP during culture improves islet potency and attenuates long-term tacrolimus induced graft dysfunction.

3.2. – INTRODUCTION

Islet transplantation outcomes have improved significantly in highly specialized centers, with 5-year insulin-independence rate exceeding 50% (1). However, a proportion of patients require reintroduction of insulin at delayed time-points (2). Numerous factors contribute to limited durability of glycemic control both acutely and chronically. Exposure to diabetogenic immunosuppressive agents is associated with islet functional impairment and graft loss, especially linked to corticosteroid exposure or use of calcineurin-inhibitors (CNI), including tacrolimus (Tac) (3, 4). Tac is used as the most potent mainstay immunosuppressant in most clinical protocols, although diabetogenicity is well documented, accounting for increased incidence of post-transplant diabetes mellitus (PTDM) (5-8). Early peak tacrolimus levels in portal blood after islet transplantation after oral administration may further increase islet-exposure thereby magnifying toxicity (9, 10). Tacrolimus has become 'a necessary evil' to prevent rejection and autoimmune recurrence after islet transplantation, but may in part be responsible for limited islet durability, and need for multiple donors for each recipient (11).

Anti-freeze proteins (AFPs) have generated considerable interest for their ability to protect cells under a variety of conditions. They naturally occur in Arctic and Antarctic fish as well as other cold-climate dwelling invertebrates, and are responsible for maintaining cell and tissue function at sub-zero temperatures (12, 13). AFPs were successfully isolated in the 1950s and have demonstrated an ability to non-colligatively lower the freezing temperature of body fluids by binding to ice crystals (12, 14). Early experiments with AFP in the field of organ and tissue transplantation showed promising results, making them attractive therapeutic candidates to protect cells against harmful conditions associated with the process of procurement-preservation-

reperfusion (14). Moreover, benefits have also been demonstrated during cryopreservation of different cells, including islets of Langerhans, with significant improvements in their viability and function when supplemented with AFP during cryostasis (15, 16).

Anti-Aging Glycopeptide (AAGP) is a 580 dalton synthetic AFP analog initially developed by Dr. Geraldine Castelot-Deliencourt-Godefroy (Rouen, France) and later manufactured by ProtoKinetix Inc. (Vancouver, Canada). This new compound has improved stability, is water soluble and has proven to be more potent in terms of cytoprotective capabilities under extreme conditions (pH variations, sudden temperature changes, nutrient deprivation, oxidative stress, UV radiation and inflammation) (17).

In light of this evidence, significant attention is now being directed towards AFP and their potential use in reparative and regenerative medicine, particularly in the field of transplantation. We herein evaluate the cytoprotective capacity of AAGP to protect against the diabetogenic effect of tacrolimus resulting in improved islet engraftment.

3.3. - RESEARCH DESIGN AND METHODS

3.3.1. - Human islet isolation, purification and culture

Human islet preparations were isolated from consenting multi-organ deceased donors, as previously described (18) 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 for these studies was granted by the Health Research Ethics Board of the University of Alberta, Edmonton, Alberta, Canada, and after written permission was obtained from donor families.

3.3.2. - In vitro evaluation of AAGP

3.3.2.1. - Islet recovery, viability and secretory function

Experiments with human islets *in vitro* included four groups: a. Control (islets cultured in medium alone) (Tac^-); b. Islets cultured in medium containing AAGP (AAGP); c. Islets cultured in medium containing tacrolimus (Prograf, Astellas Pharma Canada Inc., Markham, ON, Canada) (Tac^+), and; d. Islets cultured in medium supplemented with AAGP (ProtoKinetix) and tacrolimus (Tac+AAGP). Islets were cultured for 24 hours with ± 3 mg/mL AAGP before addition of tacrolimus at clinically relevant concentration of 10ng/mL. All four groups were then cultured for an additional 24 hours.

Islets were assessed for recovery, viability, insulin release, oxidative stress and cell death. Recovery rate was calculated as the percentage of surviving islets after culture in comparison to the initial count for each condition. Viability was assessed using a fluorescent membrane integrity assay with counter-stains using SYTO® 13 Green and Ethidium bromide (Life Technologies, Burlington, ON, and Sigma-Aldrich, ON) (19-22).

Hormonal islet secretory function was assessed by both static glucose-stimulated insulin secretion (s-GSIS), sequentially performed at low (2.8 mmol/L) and high (16.7 mmol/L) glucose concentrations, and with dynamic islet perifusion (d-IP), as described by Cabrera *et al* (23). D-IP was performed at 16 min intervals using low (2.8 mmol/L), high x 2 (28 mmol/L), followed by low glucose concentration. Glucose was infused at 100µL/min and results are expressed as fold-change of insulin secretion compared to the low glucose stimulation baseline, normalized for 100 IEQ. For s-GSIS, insulin concentrations in supernatants were measured by ELISA (Mercodia,

Uppsala, Sweden). A stimulation index was calculated as the ratio of stimulated to basal insulin secretion normalized by DNA. These insulin secretion studies were always performed in vitro, on cultured human islets.

Apoptosis was assayed by determining quantity of cleaved caspase-3 in the frozen lysates from fixed islets cultured under the above mentioned conditions using a spectrophotometric assay (EMD Millipore. Billerica, MA, USA). Results are expressed as fold-change increase compared to controls.

Islet apoptosis was also examined by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining (DeadEnd Apoptosis Detection System, Promega, Madison, WI), following formalin fixation, processing and paraffin-embedded. Co-staining with insulin (1:200 concentration of anti-insulin antibody (Dako, Mississauga, ON) and DAPI (Invitrogen Molecular Probes. Eugene, Oregon) was performed to identify graft and nuclei present, respectively. Islet apoptosis was quantified by percentage of positive TUNEL stained area using ImageJ software (freeware ImageJ v1.33 and Cell Counter plug-in, [http://rsb.info.nih.gov/ij]).

3.3.2.2. - Reactive oxygen species analysis

Frozen samples from *the* study groups were assayed for reactive oxygen species (ROS) released into the culture medium, using the Acridan Luminogen PS-3 assay (Amershan ECL Plus kit, Fisher Scientific Inc. Ottawa, ON, Canada) (24). Acridan Luminogen PS-3 is excited by reactive oxygen and nitrogen species in the presence of hydrogen peroxide, producing chemiluminescense at 430 nm. Media samples were flash-frozen in liquid nitrogen and stored

until analyzed. CMRL culture medium alone served as a control, and results were expressed as fold-change increase compared to control.

3.3.2.3. - Mixed Lymphocyte Reaction (MLR)

To rule out direct drug inhibition of AAGP and tacrolimus a one-way MLR assay was performed to assess proliferation of responder T-cells against antigens present on allogeneic stimulator cells. Briefly, BALB/c (stimulators) and C57BL/6 (responders) mice were euthanized and spleens were removed through midline incision for tissue homogenization and filtration (70μm) on ice. Red blood cells were removed from the homogenate using lysis buffer and the remaining cells were washed and resuspended in 0.3% BSA/Dulbecco's phosphate buffered saline mix.

Proliferation was measured by loss of fluorescence intensity using fluorochrome 5,6-carboxyfluorescein diacetate succinimidyl ester (CFSE, Invitrogen. Eugene, OR), which spontaneously binds to intracellular proteins shared between daughter cells. Responder cells were stained with 5 μM of CFSE and cultured in complete RPMI (RPMI+10%FBS+1%L-glutamine+1%HEPES+1%Pen/Strep) in the presence of gamma-irradiated (3000 cGy) stimulator cells. Cultured cells were allocated into three different study groups by supplementing medium with tacrolimus at 10 ng/mL (Tac^{+}), AAGP 3 mg/mL (AAGP), tacrolimus at 10 ng/mL and AAGP at 3 mg/mL (Tac+AAGP) or no supplementation (Control),

After 7 days of culture, cells were washed and stained for cell surface antibody with anti-mouse TCR-beta eFluor450, CD4 APC and CD8a APC-eFluor780 (eBioscience, San Diego, CA, USA) to characterize different subpopulations and acquisition was performed on a BD LSR II flow

cytometer (BD Biosciences, San Jose, CA, USA), followed by analysis with FCS Express Data software (De Novo Software, Los Angeles, CA).

3.3.2.4. - Calcium imaging

Measurements of intracellular calcium concentration ($[Ca^{2+}]_i$) for individual human islets from the different treatment groups were carried out by previously described methods (25, 26) at glucose concentrations of 2.5 mmol/L and 25 mmol/L. Glucose-stimulated increase in $[Ca^{2+}]_i$ was expressed as area under the curve.

3.3.2.5. - Capacitance studies

Measurement of membrane capacitance was performed on islets according to our previously established method (27, 28) to determine the effect of Tac and AGPP on β-cell exocytotic responses. Cells were stimulated with a series of ten depolarizations to activate voltage-dependent Ca^{2+}–channels. Whole-cell capacitance responses were normalized to initial cell size and expressed as femtofarad per picofarad (fF/pF).

3.3.3. - In vivo evaluation

All mice were housed under conventional conditions with access to food and water *ad libitum*. The care for mice was in accordance with guidelines approved by the Canadian Council on Animal Care.

3.3.3.1. - Transplants with human islets and inflammation analysis

Diabetes was induced chemically on 8-12 week immunodeficient B6.129S7-Rag1^{tm1Mom} recipient mice (Jackson Laboratory, Bar Harbor, ME, USA) by intraperitoneal injection of 180mg/kg streptozotocin (STZ, Sigma-Aldrich, ON, Canada). Mice were considered diabetic after two consecutive blood glucose measurements ≥11.3 mmol/L (350 mg/dL). Recipients (n=10 per group) received approximately 1,000 IEQ human islets from 3 different isolations. Islets from each isolation were randomly allocated to each group (Tac^{-}, AAGP, Tac^{+}, Tac+AAGP), to control for potential differences in each islet preparation. Islets were transplanted under the kidney capsule as described previously (29). A minimal islet dose was utilized to stress the model and maximize covert toxicity (30-32).

Three mice per group underwent acute graft explantation and were euthanized on day 1 and 7 post-transplant to determine proinflammatory cytokine concentrations (at both time points), cleaved caspase-3 and TUNEL (24h) within the graft. For cytokine and cleaved caspase-3 quantification the islet grafts were excised from the kidney, bisectioned, with one section flash frozen in liquid nitrogen and stored at -80°C, while the other was formalin fixed and processed for TUNEL quantification. Tissue samples were subsequently lysed in acid buffer as reported previously (30). Cytokine and cleaved caspase-3 determination was adjusted per gram of tissue.

3.3.3.2. - Pro-inflammatory cytokines and chemokines

Relevant cytokines and chemokines (IFN-γ, IL-1β, IL-6, IL-10, IL-12, Keratinocyte-derived Chemokine (KC), and TNF-α) were measured using a Multi-Spot Mouse ProInflammatory 7-

Plex Ultra-Sensitive kit and analyzed on a SECTOR Imager (Meso Scale Discovery®, Gaithersburg, MD, USA). Results are expressed as absolute values (pg/mL), and normal renal tissue lysate samples from a mouse receiving a sham operation are used as control.

3.3.3.4. – Apoptosis

Apoptosis was determined in the excised grafts 24h post-transplant by quantifying cleaved caspase-3 and analyzing percentage of dead cells (TUNEL) within the graft. Caspase-3 concentration was expressed as fold-change increase compared to normal renal tissue lysate samples from a naive mouse.

3.3.3.5. - Long-term human islet graft function after transplantation in immunodeficient mice

Non-fasting blood glucose was monitored in the remaining animals three times a week using a portable glucometer (OneTouch Ultra 2, LifeScan, Canada) over 60 days. Normoglycemia was defined as two consecutive readings <11.3 mmol/L.

Intraperitoneal glucose-tolerance tests (IPGTT) were conducted 60 days post-transplant to evaluate the capacity of islets to respond to a glucose bolus (3 g/kg) after overnight fast. Blood glucose levels were monitored at baseline time 0, 15, 30, 60, 90 and 120 minutes post-injection. All results were compared to blood glucose profiles of naive control non-diabetic mice.

Recovery islet-bearing nephrectomies were performed on day 65 to demonstrate graft-dependent euglycemia. Both cultured islets and recovered grafts were stored at -80°C and processed to

measure intracellular insulin content by acid-ethanol homogenization and ultrasonic lysis. The extract was neutralized and insulin measured with ELISA (30).

3.3.3.6. - Transplants with mouse islets under continuous treatment with tacrolimus

3.3.3.6.1. - Mouse islet isolation

Pancreatic islets were isolated from 8 to 12 week male BALB/c mice (Jackson Laboratories, Canada) as reported previously (18). Islets were counted and divided in three groups (Tac^-, Tac^+ and Tac+AAGP). All islets were incubated for 1h in conditions described above and Tac+AAGP islets were additionally supplemented with AAGP at 3 mg/mL during the incubation period. Recipient syngeneic BALB/c mice were also rendered diabetic with STZ and transplanted incubated with 500 ± 10 IEQ of 90% purity, under the renal capsule (18).

3.3.3.6.2. - Transplant Procedures

Subcutaneous Micro-Osmotic Pumps (Model 1002, Alzet, Cupertino, CA) were implanted in all mice at the time of islet transplantation to provide continuous delivery of tacrolimus. A first group (short duration) received a pump delivering Tac for 7 days, at a dose of 1mg/kg/day to the relevant groups (Tac^+, n=6 and Tac+AAGP, n=8), and a second group (long duration) received pumps delivering the CNI for 28 days (Tac^+, n=6 and Tac+AAGP, n=6). Tac^- group (n=10) received pumps loaded with normal saline as placebo. Steady state Tac levels were monitored selectively from the dorsal tail vein at day 5, and ranged from 10-20 ng/ml (clinically relevant

range, tandem liquid chromatography-mass spectroscopy, for continuously administered drug) (33).

Animals underwent IPGTT on day 7 (short duration), day 14 (long duration) during treatment course, and again, on day 30 and 40, respectively after CNI treatment cessation. Transplant islet-bearing nephrectomies were performed after tolerance tests to prove graft-dependent function.

3.3.4. - Statistical analysis

Data are represented as means ± standard error of the mean (SEM). Area under the curve was calculated for GSIS and D-IP, calcium imaging, capacitance measurements and IPGTT, and differences between groups were analyzed with one-way ANOVA with Tukey's post-hoc test. A p-value <0.05 was considered significant and all the analysis was performed using GraphPad Prism (GraphPad Software, La Jolla, CA, USA).

3.4. - RESULTS

3.4.1. - AAGP enhances human islet potency in culture and protects islets against acute exposure to tacrolimus

Isolated human islets from 6 different preparations were cultured in medium supplemented with or without AAGP and tacrolimus, as described above. After 48 hours of culture, all groups were characterized for *in vitro* survival, viability, function, oxidative stress and apoptosis.

After the study period, cells were counted resulting in a greater number of surviving islets in the AAGP supplemented group (71.1%). Exposure to tacrolimus clearly decreased survival, but islets were significantly protected when simultaneously supplemented with AAGP (Tac^+ vs. Tac+AAGP, 31.5% vs. 67.6%, $p<0.01$) (**Figure 3.1A**). There was no difference in cell viability by membrane integrity stain (**Figure 3.1B**). When comparing in-vitro function by D-IP, insulin release was completely suppressed after tacrolimus exposure (AUC: Tac^- vs. Tac^+, 131 vs. 7.3 mmol/L/60min, $p<0.001$). However, islet function was fully maintained after supplementation with AAGP and comparable to the other culture conditions (Tac^+ vs. Tac+AAGP, 7.3 vs. 129.2 mmol/L/60 min, $p<0.001$) (**Figure 3.1C**).

3.4.2. - Acute exposure to tacrolimus decreases insulin secretion but not biosynthesis

Aliquots of 100 IEQ human islets were collected for each group for s-GSIS assay and intracellular insulin content. The Tac+ group showed significant impairment of insulin secretion, which was not observed in the Tac+AAGP group (stimulation index 1.4 vs. 10.7, $p<0.01$) (**Figure 3.1D**). However, intracellular insulin content remained stable and comparable throughout groups (**Figure 3.1E**).

3.4.3. - AAGP reduces oxidative stress but does not inhibit tacrolimus function

Oxidative stress was observed in all groups, but Tac exposure resulted in substantial increase in ROS, which was ameliorated in the presence of AAGP (**Figure 3.2A**, $n=6$, $p<0.05$).

To confirm that AAGP did not inhibit Tac suppression of T cell proliferation, MLRs were completed with donor splenocytes. The assay measured T cell proliferative response by CFSE staining. As expected, T cell proliferation was significantly decreased in the Tac^{+} group compared to the control ($n=4$, $p<0.001$). Proliferation of CD8+ and CD4+ positive T cells was equally decreased in the presence of Tac alone or in combination with AAGP ($n=4$, $p<0.001$ in both cases), with no impediment to MLR suppression in the presence of AAGP (**Figure 3.2B, C and D**).

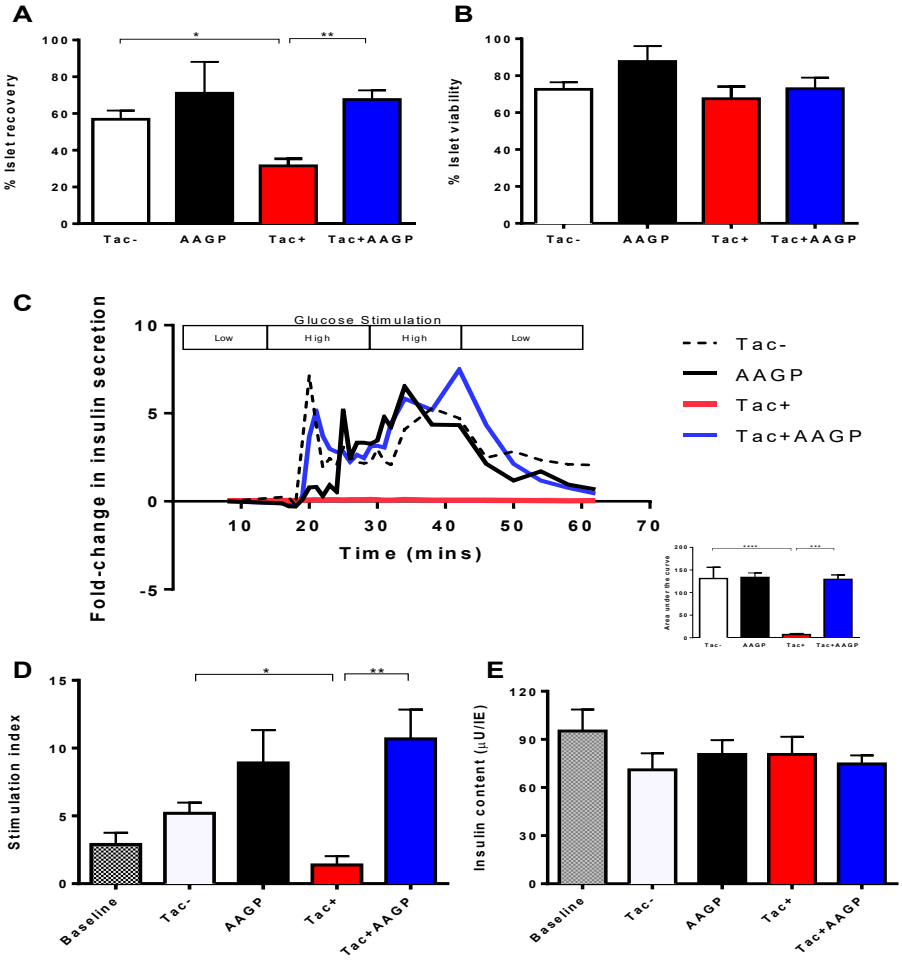


Figure 3.1 AAGP improves human islet potency in culture and protects against acute exposure to tacrolimus. *In vitro* assessment of human islets in culture with or without AAGP supplementation showed (A) significantly higher islet recovery rate after culture in the presence of AAGP. (B) No significant changes in cell viability were found after study period. (C) Perifusion curves comparing glucose-stimulated insulin secretion (GSIS) after stimulation with variable glucose concentrations (low=2.8 and high=28 mM),showing severely impaired islet function for the Tac^+ group and significantly better response for groups treated with AAGP, also seen in the corresponding area under the curve (inset) (n=6).

Tacrolimus impairs insulin secretion without affecting insulin content.

GSIS static assay and intracellular insulin content were simultaneously measured on human islets kept in culture. **(D)** Stimulation index (SI) for group Tac^+ is significantly decreased in comparison with controls. However, a significant improvement was observed in insulin secretion of Tac+AAGP islets ($p<0.01$), while no changes were seen in the intracellular content of insulin across the different groups **(E)**. Data points represent mean ± SEM, triplicates from four different preparations. *P<0.05, **p<0.01, ***p<0.001 and ****p<0.0001.

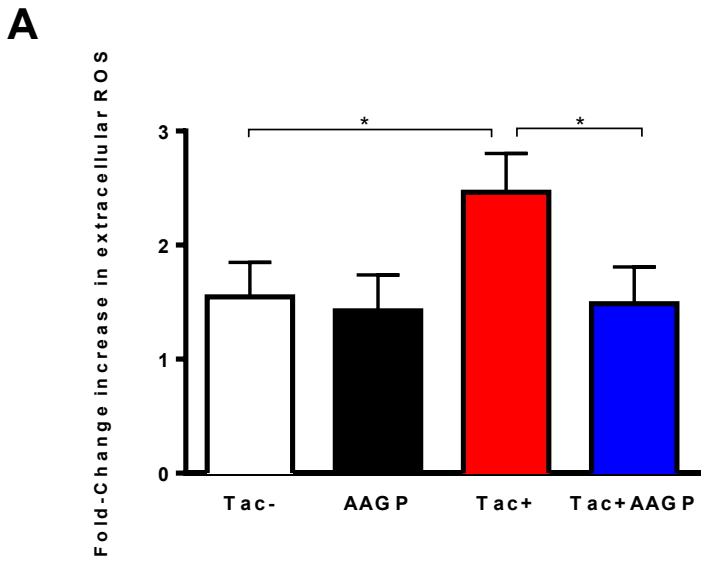


Figure 3.2.A Islets treated with AAGP have decreased oxidative stress. Human islets in culture had increased concentration of reactive oxygen species when treated with Tac. However, supplementation with AAGP significantly decreases this effect ($p<0.05$). Oxidative stress was measured by fold-increase in extracellular ROS analyzed with the Acridan Luminogen PS-3 assay. Data points represent mean ± SEM, n=5.

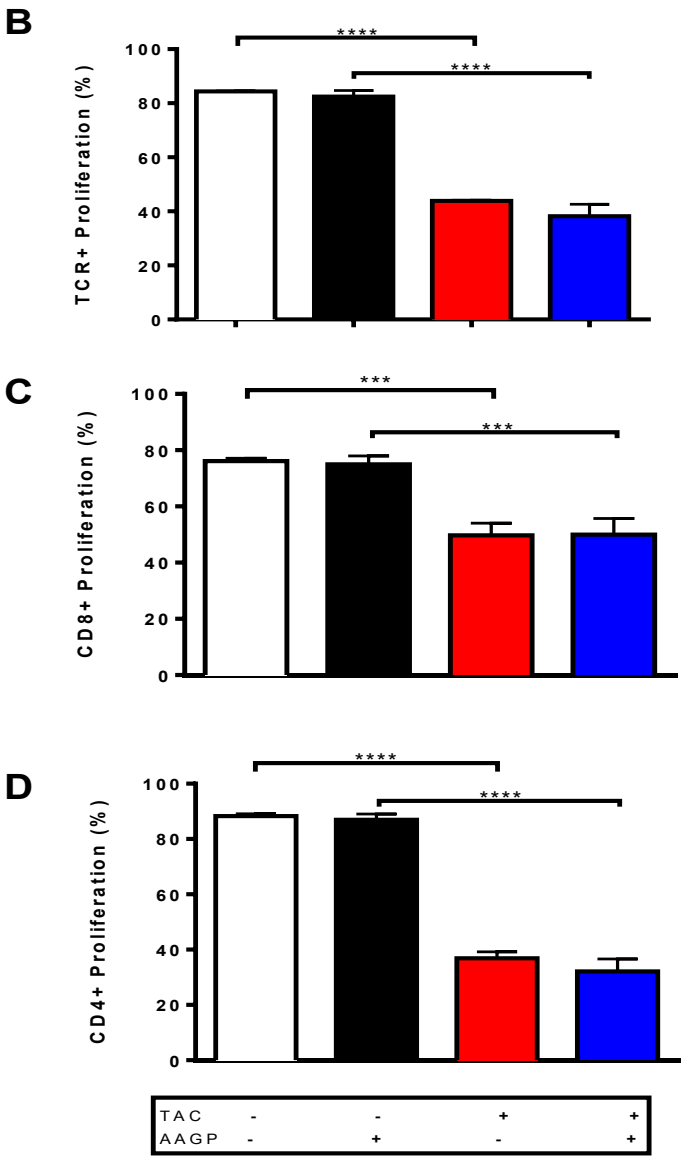


Figure 3.2.B-D. AAGP effect is not the result of direct drug inhibition with tacrolimus. Allogeneic mixed lymphocyte reaction (MLR) was used to evaluate direct drug inhibition. Results show a significant decrease of T cell proliferation in the presence of tacrolimus, AAGP and the combination of both. Hence, no direct inhibition of tacrolimus by AAGP. Data points represent mean ± SEM, n=6, ***p<0.001 and ****p<0.0001.

3.4.4. - Tacrolimus effect on islet intracellular calcium responses and exocytosis.

Various studies were performed on human islets to elucidate a potential mechanism of action for the AAGP by characterizing CNI-related injury and its minimization. Intracellular calcium concentrations were measured to determine possible influence of AAGP on glucose-stimulated calcium influx (34). No significant differences in calcium influx were observed between groups (AUC: Tac^- 209.4, Tac^+ 221.6, Tac+AAGP 208.7 fF/Treatment/2s. $p>0.05$), suggesting that the protective effect of AAGP was further downstream in the secretory pathway (data not shown). Complementary membrane capacitance studies were performed under similar conditions as an indirect indicator of insulin exocytosis. There was a decreased cumulative capacitance response for the Tac^+ group compared to other groups (**Figure 3.3A**), corresponding to a significantly lower area under the curve (AUC; Tac^+**:** 2.9 vs. Tac+AAGP: 10.5 fF/pF/treatment, $p<0.001$, **Figure 3.3B**).

3.4.5. - AAGP prevents islet apoptosis resulting from in-vitro exposure to tacrolimus

Exposure to tacrolimus during culture resulted in increased concentration of intracellular cleaved caspase-3 (fold change Tac- 1.9 vs Tac+ 4.3, $p<0.05$), which corresponded with increased percentage of apoptotic islets (Tac- 18.9% vs. Tac+ 48.6%, $p<0.01$). Conversely, pre-treatment with AAGP prevented tac-induced cell death, showing reduced levels of caspase-3 (Tac+ 4.3 vs. Tac+AAGP 2.2, $p<0.05$) and fewer number of apoptotic cells (Tac+ 48.6% vs. Tac+AAGP 26%, $p<0.05$) (**Figure 3.4A and B**). Representative slides from TUNEL histology are shown in **Figure 3.3C**.

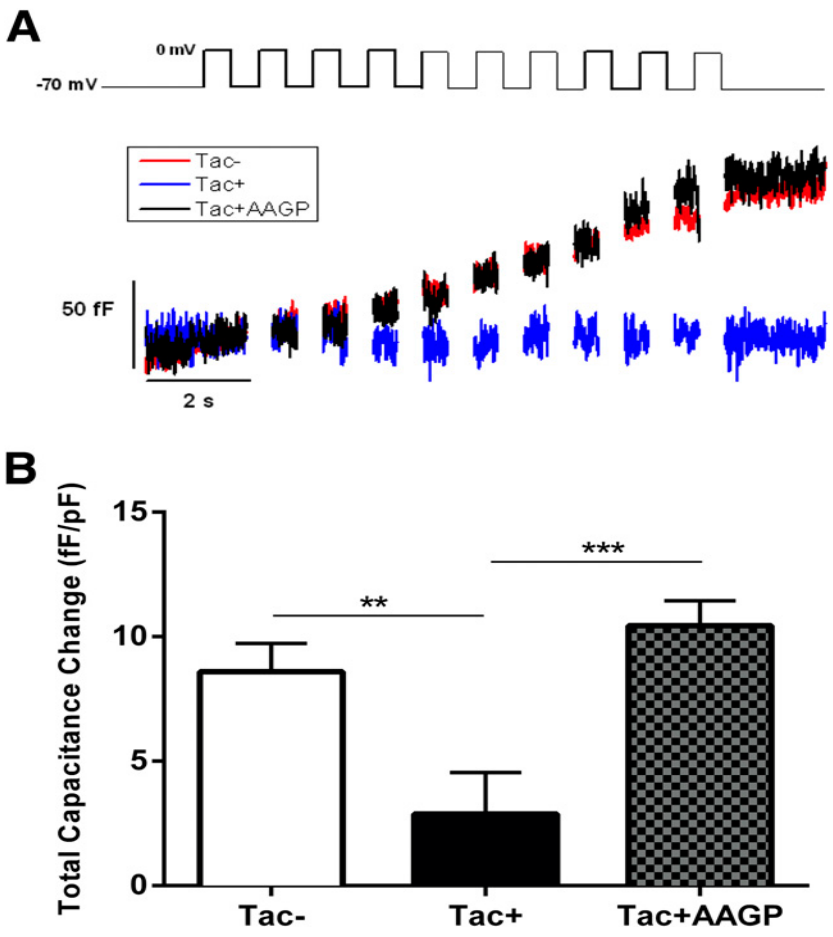


Figure 3.3 AAGP preserves insulin release by maintaining islet exocytosis. Human islets were cultured and treated accordingly with and without Tac/AAGP. (**A**) Insulin secretion impairment for Tac^{+} group was also met by a lower normalized membrane capacitance measurements (blue), indicating impaired exocytosis, whereas measurements were superior and comparable in the Tac^{-} (red) and Tac+AAGP groups (black). (**B**) Represents corresponding area under the curve ($p<0.01$ and $p<0.001$). Data points represent mean ± SEM, triplicates from two isolations.

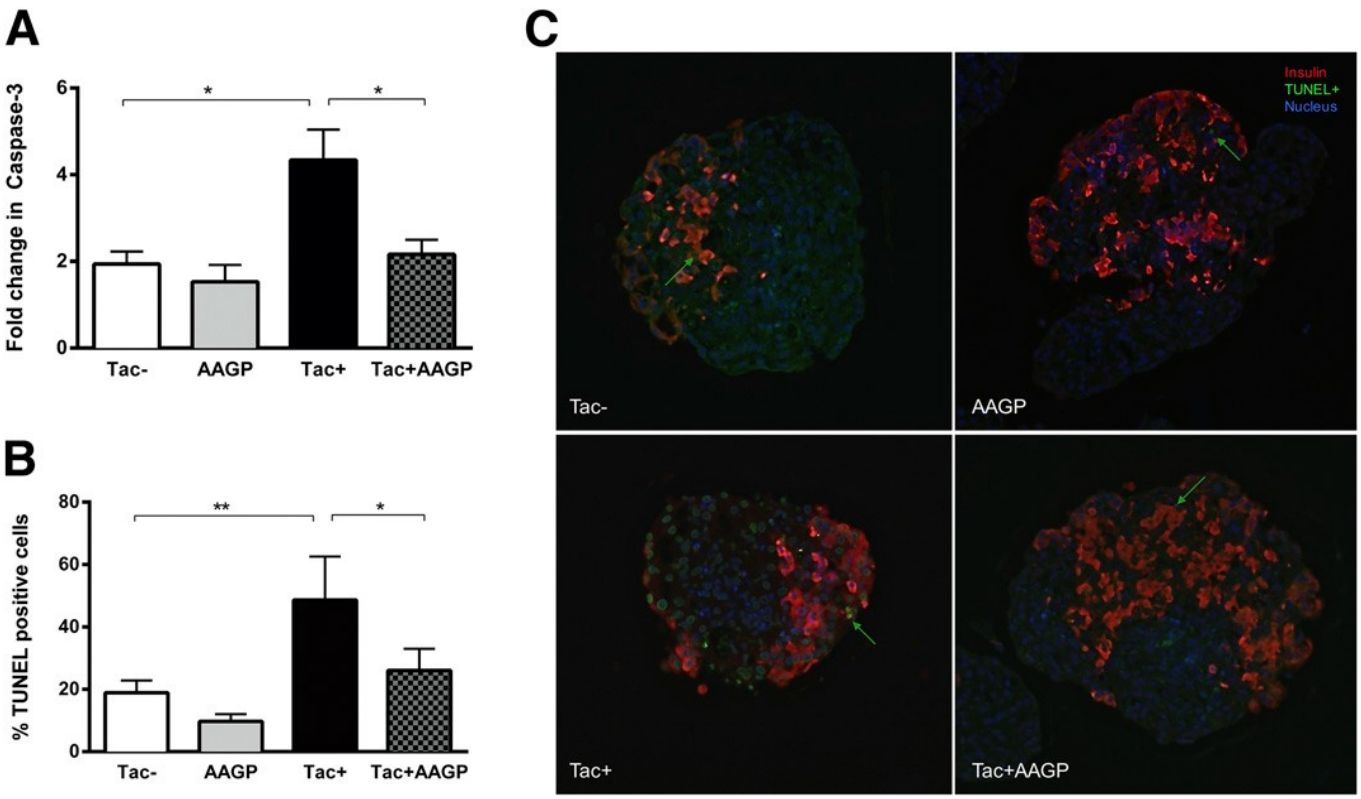


Figure 3.4 AAGP decreases islet loss in culture due to apoptosis, even in the presence of tacrolimus. Human islets were cultured and treated with Tac/AAGP, accordingly. Cell death due to apoptosis was significantly higher in Tac^{+} by quantification of intracellular cleaved caspase-3 (A) and by analysis of TUNEL staining (B). Supplementation of medium with AAGP was able to prevent loss of viability and significantly decrease cell death ($p<0.05$). (C) Representative microphotographs of TUNEL slides of fixed islets from different study groups. Green arrow points to TUNEL positive nuclei.

3.4.6. - AAGP ameliorates inflammatory response immediately post-transplant

Minimal mass (1,000 IEQ) human islet transplants were performed in diabetic immunodeficient mice. Grafts from three animals per group (day 1 and day 7 post-transplant) were homogenized and characterized for proinflammatory cytokines and chemokines.

Acute levels of IL-1β were significantly increased in Tac^+ with respect to those in the sham group (163.1 vs. 18.9 pg/g-tissue, $p<0.001$). Cytokine concentration was however considerably dampened in the Tac+AAGP group (163.1 vs 44.9, $p<0.001$), with similar excretion behavior on day 7 (269.5 vs. 121 pg /g-tissue, n=3, $p<0.001$) (**Figure 3.5A and B)**. Similarly, IL-6 was significantly increased in the Tac^+ group (1414 vs. 804.7 pg/ g-tissue, n=3, $p<0.001$) but differences were no longer apparent at later time points (**Figure 3.5C - D)**.

Among the chemokines measured acutely post-transplant, KC secretion, involved in neutrophil recruitment, was significantly overexpressed in Tac^+ and again, significantly reduced in the presence of AAGP (85.4 vs. 32 pg/ g-tissue, $p<0.001$, n=3). By day 7 the cytokine was clearing and differences were no longer evident (**Figure 3.5E and F)**.

Tumor necrosis factor levels on the other hand, were not significantly increased on day 1, but became notably different on day 7 (Tac^-, 33.9 pg/g-tissue; Tac^+,76.7 pg/g-tissue and Tac+AAGP, 48.7 pg/g-tissue. $P<0.001$) (**Figure 3.5G and H**).

3.4.6. - Post-transplant apoptosis

Intra-graft apoptosis showed an increasing trend in cleaved caspase-3 concentration and TUNEL positive cells in Tac^+ group, when compared to the rest of the groups, suggesting increased in-

vivo cell death after CNI treatment and subsequent AAGP protective effect. However, differences did reach statistical significance (Tac^+ vs Tac+AAGP: Fold-change in Caspase-3, 2.9 vs. 1.9, N.S.; TUNEL positive cells: 35.3 ± 26.7% vs 7.9 ± 8%, N.S.) (Data not shown).

3.4.7. - AAGP-supplementation improves human islet transplant function despite tacrolimus exposure

The remaining transplanted mice (n=7 per group) were followed beyond 60 days. Delayed engraftment was observed as expected in this marginal islet mass model. Blood glucose improved over time in Tac^- and Tac+AAGP groups, with the proportion of euglycemic animals being significantly higher when compared to the Tac^+ where all mice demonstrated poor function ($p<0.05$) (**Figure 3.6A and B**).

Thirty days post-transplant mice underwent IPGTT to evaluate transplant function. Tac^- and Tac+AAGP groups both responded appropriately, but Tac^+ remained hyperglycemic at 120 min (AUC: Tac^- vs. Tac+AAGP, 92.6 vs. 91.2, $p>0.05$.; Tac^- vs. naïve, 92.6 vs. 71.4, $p>0.05$.; Tac+AAGP vs. naïve, 91.2 vs. 71.4, $p>0.05$, and Tac^+ vs. Tac+AAGP, 149.8 vs. 91.2, $p<0.05$, **Figure 3.6C**). All mice reverted to their previous diabetic state following islet-bearing nephrectomy. Insulin content was assessed as a measure of residual islet mass after 30 days. **Figure 3.6D** shows significant differences between groups with reduced insulin content in grafts exposed to tacrolimus. Again, presence of AAGP was beneficial in islet protection despite exposure to tacrolimus (Tac^+ vs. Tac+AAGP, 30.9 vs. 100.8 ng/mL, $p<0.01$).

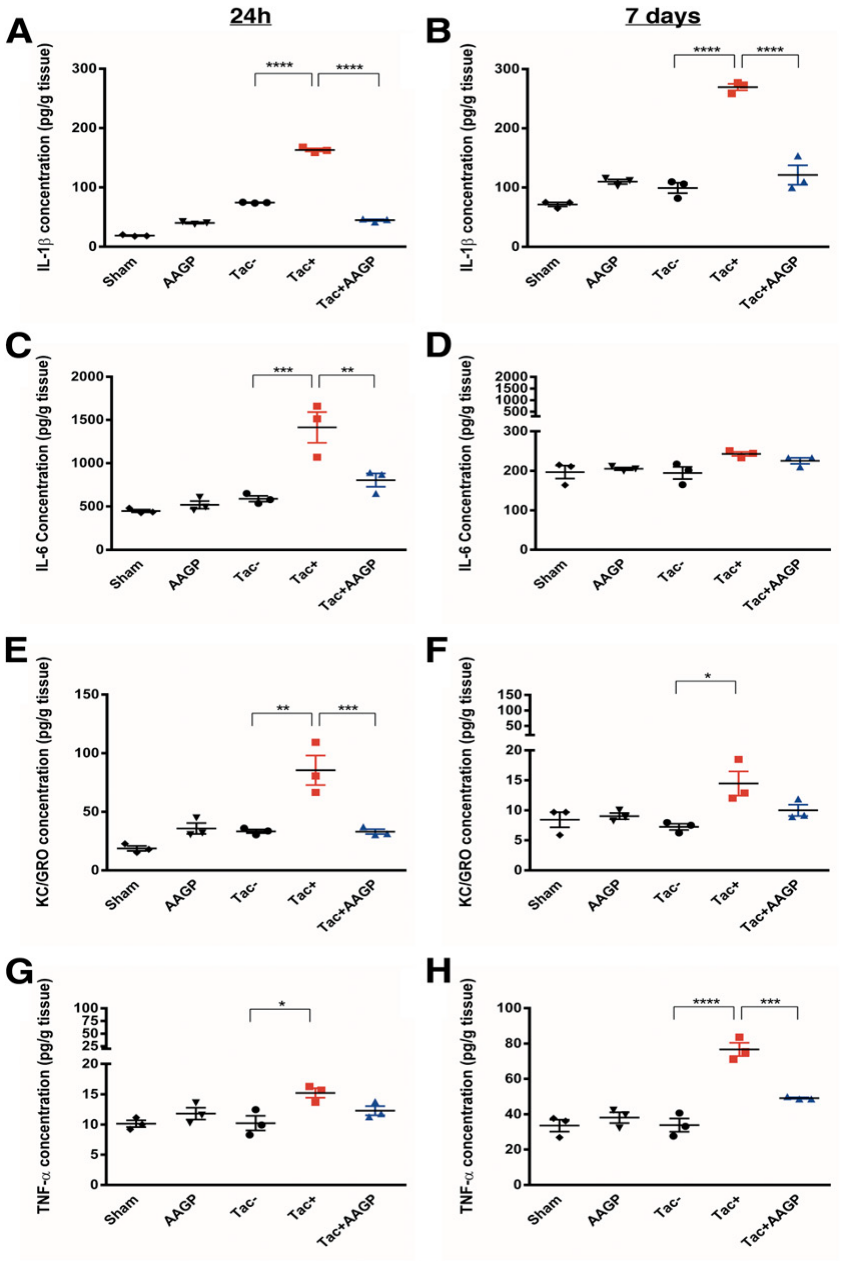


Figure 3.5 AAGP ameliorates inflammatory response immediately post-transplant. Proinflammatory cytokines and chemokines locally expressed (1 and 7 days after transplantation. (A) Concentrations of IL-1β, IL-6, Keratinocyte chemokine (KC/GRO) and Tumor Necrosis Factor (TNF) were significantly lower in the engrafted islets previously treated with AAGP. Cytokines were measured 24h and 7 days after transplantation, locally to the graft by homogenization (normalized per gram of tissue, n=3). Data points represent mean ± SEM adjusted per gram of tissue, n=3, *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001.

3.4.8. - AAGP-supplementation improves islet transplant function despite continuous recipient treatment with tacrolimus

In further support of the above findings, syngeneic diabetic mice were implanted with mini-osmotic pumps to model continuous post-transplant Tac treatment to resemble clinical practice. As with the *in vitro* findings, transplanted islets exposed to Tac were unable to effectively secrete insulin nor return mice to euglycemia during the treatment course. The additional presence of AAGP, however, prevented toxicity and mantained normal islet function despite Tac exposure, similar to controls (**Figure 3.7A and B,** $p<0.001$).

Similar findings were observed when utilizing subcutaneous pumps providing Tac treatment for a longer duration (28 days). Again, AAGP-supplemented islets functioned normally and rendered normoglycemia for all animals, despite a single 1h AAGP treatment to islets prior to transplant (**Figure 3.7E and F**, $p<0.01$)

Results were corroborated by IPGTTs performed in both treatment modalities (short and long duration). Tolerance tests performed under Tac treatment showed impaired glucose control in Tac+ group, whereas Tac+AAGP behaved similar to controls (**Figure 3.7C and G**, $p<0.001$).

Tacrolimus treatment cessation resulted in normalization of graft function and euglycemia in all animals. Repeat IPGTTs at this stage (30 and 45 days, respectively) showed no residual differences between groups (**Figure 3.7D and H**).

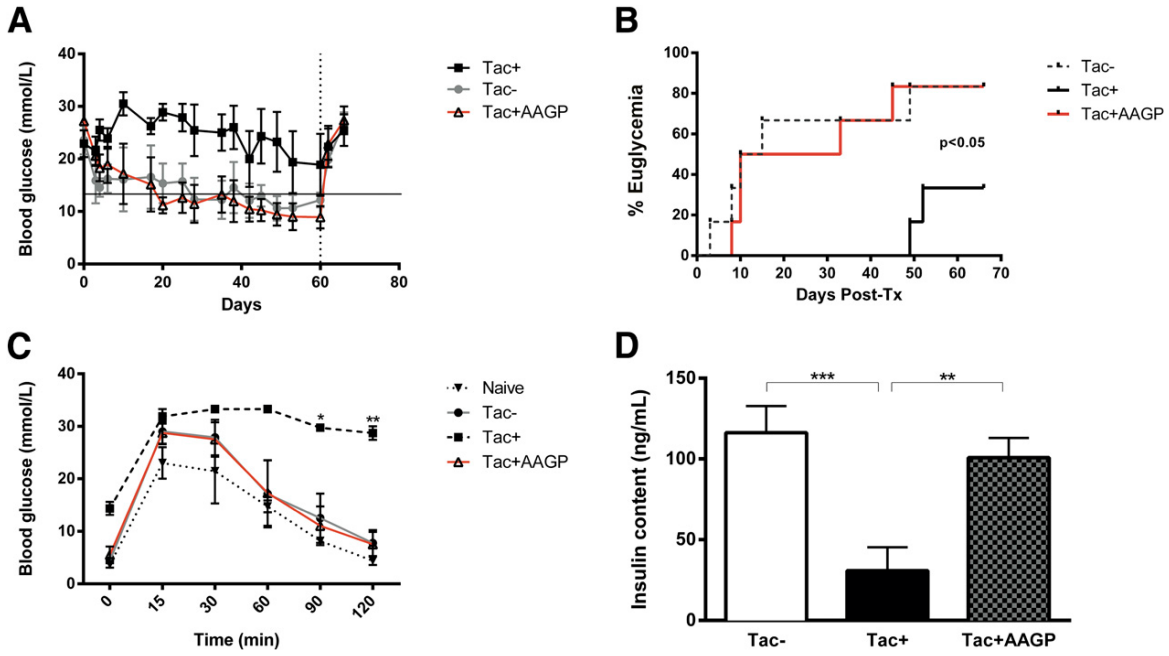


Figure 3.6. AAGP-supplementation improves islets transplant function despite tacrolimus exposure. Post-transplant graft function in immunodeficient mice receiving minimal human islet mass (1,000 IEQ). Islets were previously treated with or without AAGP and tacrolimus accordingly. Horizontal continuous line at 11 mM indicates the normoglycemia limit. **(A)** Pooled blood glucose profiles and **(B)** percent of mice reaching euglycemia, demonstrating long-term graft function (60 days) with a non-functioning graft for the Tac^+ group. Graft-bearing nephrectomy was performed on day 60 to demonstrate graft-dependent euglycemia. **(C)** Intraperitoneal glucose tolerance test (IPGTT) to evaluate metabolic response after receiving a glucose bolus. Tac^+ group mice were intolerant to high glucose corresponding also to less residual insulin content **(D)** when grafts were removed after 60 days of transplant. Data points represent mean ± SEM adjusted per gram of tissue, (Tac^- n=6, Tac^+ n=3 and Tac+AAGP n=7), *p<0.05, **p<0.01 and ***p<0.001.

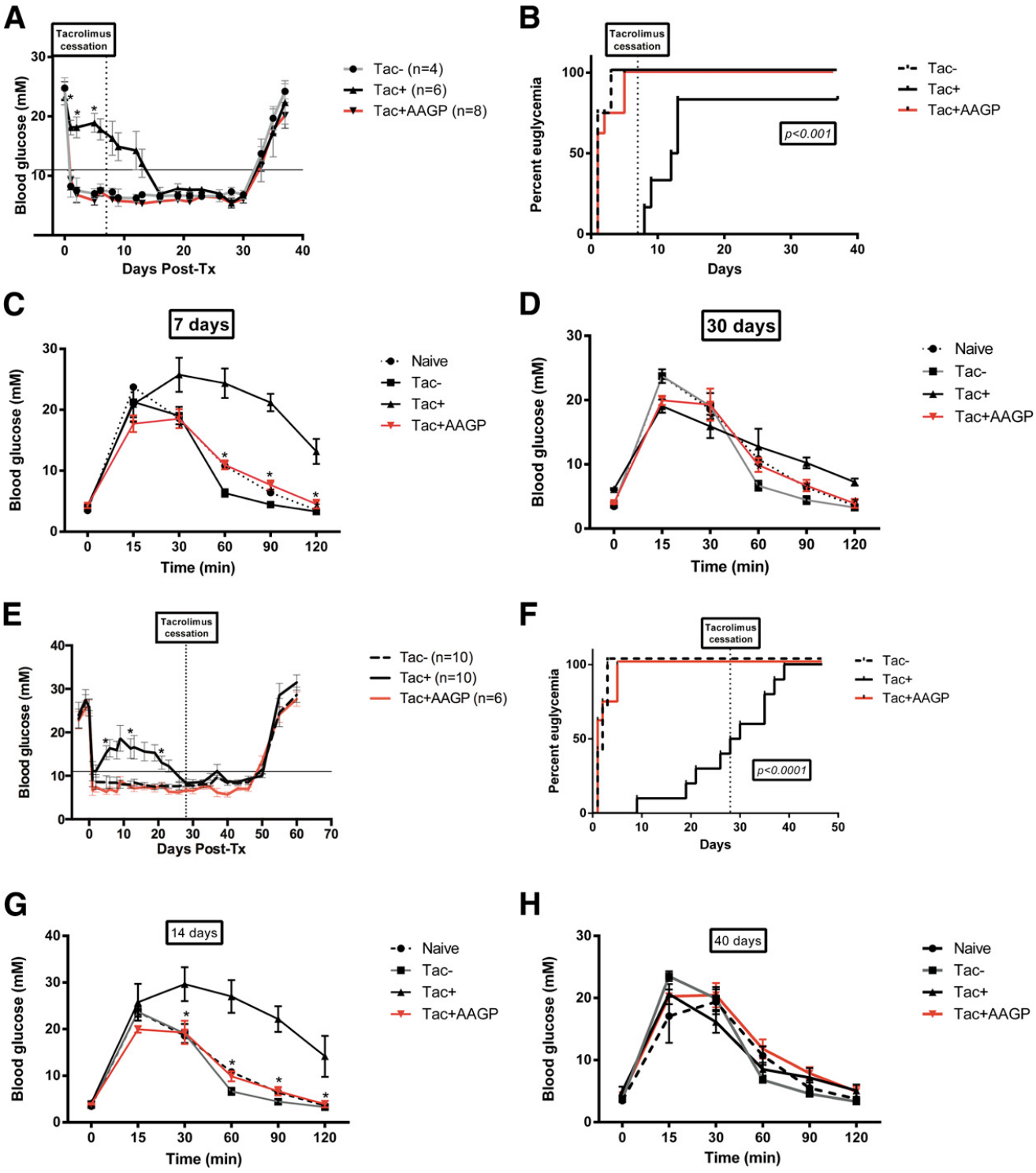

A
Tacrolimus cessation
Blood glucose (mM)
Days Post-Tx
Tac- (n=4)
Tac+ (n=6)
Tac+AAGP (n=8)
B
Tacrolimus cessation
Percent euglycemia
Days
p<0.001
Tac-
Tac+
Tac+AAGP
C
7 days
Blood glucose (mM)
Time (min)
Naive
Tac-
Tac+
Tac+AAGP
D
30 days
Blood glucose (mM)
Time (min)
Naive
Tac-
Tac+
Tac+AAGP
E
Tacrolimus cessation
Blood glucose (mM)
Days Post-Tx
Tac- (n=10)
Tac+ (n=10)
Tac+AAGP (n=6)
F
Tacrolimus cessation
Percent euglycemia
Days
p<0.0001
Tac-
Tac+
Tac+AAGP
G
14 days
Blood glucose (mM)
Time (min)
Naive
Tac-
Tac+
Tac+AAGP
H
40 days
Blood glucose (mM)
Time (min)
Naive
Tac-
Tac+
Tac+AAGP

Figure 3.7 AAGP-supplementation improves islets transplant function despite continuous (short and long duration) tacrolimus treatment. Post-transplant graft function in mice receiving syngeneic full mass (500 islets) islet transplant. AAGP was added to the culture media 1 hour prior to transplant and tacrolimus was administered via a subcutaneous osmotic pump (implanted during the same procedure) at a continuous rate of 1mg/kg/day. (**A**) Pooled blood glucose profiles of animal over 40 days with clear dysfunction for Tac^+ islets during the presence of Tac. Vertical dotted line indicates tacrolimus treatment cessation at day 7 and marks a gradual recovery of Tac^+ grafts. Horizontal continuous line at 11 mM indicates the normoglycemia limit. Graft recovery nephrectomy was again performed on day 30. (**B**) Mean time-to-euglycemia after transplant showing Tac+AAGP mice reversing diabetes earlier ($p<0.001$, log-rank, Mantel-cox test). Finally, glucose tolerance tests showed a significant difference in graft response for Tac^+ when mice were receiving tacrolimus (7 days) and when recipient were CNI free. These differences were not observed in the Tac+AAGP group. (**C**) Glucose tolerance tests of mice receiving continuous treatment with tacrolimus showing impairment for Tac+ group, which is fully reversed once the CNI treatment is ceased (**D**). (**E – H**) A similar experiment was conducted with long duration subcutaneous pumps providing tacrolimus during 28 days. Results show a consistent and significant difference in immediate post-transplant function for mice receiving AAGP supplemented islets

3.5. - DISCUSSION

We demonstrate herein that addition of a potent anti-freeze protein, AAGP only to the islet culture media for a 48-hour exposure affords considerable protection of human islet survival and *in vitro* function. This protective effect is especially pronounced when used to prevent Tac-induced islet toxicity.

Tacrolimus is currently regarded as a mainstay, potent immunosuppressant given to prevent both auto- and alloimmunity after clinical islet transplantation (35). Prolonged exposure to CNI-class immunosuppressants is strongly associated with nephrotoxicity and PTDM in all organ transplants (4).

Tacrolimus is known to impair insulin secretion in the native pancreas, after pancreas and especially after islet transplantation, and is characterized by impairment of early secretion and by decreased biosynthesis. Several associated mechanisms have been defined, including calcineurin/nuclear factor of activated T-cells signaling inhibition (36), insulin gene suppression (37), mitochondrial arrest (38) and decreased post-transplant vascularization (39). In our experimental model, the addition of Tac resulted in striking inhibition of insulin secretion and cell death *in vitro*, and impaired islet engraftment and function *in vivo*.

Furthermore, we confirmed islet function impairment after Tac exposure *in vitro* and we found that AAGP was able to re-establish insulin release despite acute exposure to high-dose Tac.

Increased loss of islets during culture associated with apoptosis was observed after *in vitro* exposure to tacrolimus. Increase in cleaved caspase-3 and TUNEL staining indicated significantly higher cell death in Tac^{+} group, however this was diminished with AAGP supplementation.

Islets are highly susceptible to hypoxia throughout all stages of cell procurement, preparation and intraportal transplantation, and relates to their intrinsic oxygen demand and size, especially related to islet seeding density in culture (40). Islets are prone to oxidative stress due to decreased antioxidant capacity (41). These elements contribute to islet loss during culture and post-transplant. Our findings confirm increase in oxidative stress after Tac exposure, with increased extracellular ROS. AAGP supplementation reduced oxidative stress in this model. Similar redox modulation findings have also been noted when using AAGP with other cell lines (17).

In vivo studies complemented all *in vitro* findings, which clearly demonstrated that AAGP supplementation suppressed early inflammation and improved islet engraftment with long-term efficacy. AAGP-supplemented islets showed significantly reduced expression of IL-1β and IL-6, along with decreased secretion of KC and TNF, despite exposure to Tac in culture. These cytokines and chemokines are key participants in the post-transplant inflammatory response and subsequent adaptive immunity activation (42), and a vital element in the early clinical post-transplant phase (30). These findings are consistent with previous experiments showing reduced expression of cyclooxygenase-2 expression in HeLa cells exposed to increasing concentrations of IL-1β, in the presence of AAGP (17).

Tac exposure was provided by continuous mini-osmotic pump for 7 and 28 days in syngeneic mice. We chose this approach in selected experiments, as twice-daily oral gavage of Tac would have been too stressful, but wished to maintain sustained clinically relevant drug exposure for transplanted islets. We observed marked impairment of transplanted islets occurred immediately following Tac exposure, which lasted throughout Tac exposure, but was reversible after withdrawal of Tac. Islets treated with AAGP however, were protected from Tac toxicity, and

functioned similar to controls both, in short and long duration treatment groups. Potentially, since a marked and prolonged post-transplant engraftment and functional benefit was observed consistently when AAGP treatment was confined only to the *in vitro* culture period, this treatment could be readily applied in clinical studies to enhance islet engraftment and function in patients receiving tacrolimus immunosuppression.

In exploring potential mechanisms of action of AAGP, we found no beneficial effect upon insulin synthesis or storage. Furthermore, we did not find an interactive impact of AAGP upon the immunosuppressive properties of Tac. We found that neither Tac nor AAGP affected glucose-stimulated calcium-influx in islets, which is a key element in the insulin secretion mechanism of beta cells. This information is supportive of recently published evidence pointing to a potential tacrolimus mechanistic site further downstream in the secretory pathway (43). Conversely, islet capacitance measurements in the current studies revealed significant differences between Tac^{+} and TAC+AAGP, findings suggestive of impaired insulin exocytosis in the presence of Tac, which was reversed by AAGP.

In conclusion, supplementation of islets with AAGP during culture enhanced both the quality and yield of post-culture human islets, which translated into improved engraftment, despite the presence of Tac. AAGP also protected islets continuously exposed to Tac post-transplant, with improved efficacy and decreased inflammatory response. Clinical translation of these findings could potentially offer a means to protect islets both *in vitro*, and *in vivo* from diabetogenic immunosuppression after transplantation, as a means to enhance single donor islet engraftment and durable long-term function.

3.6. – REFERENCES

1. Shapiro AM. Immune antibody monitoring predicts outcome in islet transplantation. Diabetes 2013;62(5):1377-1378.

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

3. Nir T, Melton DA, Dor Y. Recovery from diabetes in mice by beta cell regeneration. J Clin Invest 2007;117(9):2553-2561.

4. Chand DH, Southerland SM, Cunningham RJ, 3rd. Tacrolimus: the good, the bad, and the ugly. Pediatr Transplant 2001;5(1):32-36.

5. Yates CJ, Cohney SJ. Prediction and Diagnosis of Post Transplant Diabetes. Curr Diabetes Rev 2015.

6. Weng LC, Chiang YJ, Lin MH, Hsieh CY, Lin SC, Wei TY et al. Association between use of FK506 and prevalence of post-transplantation diabetes mellitus in kidney transplant patients. Transplant Proc 2014;46(2):529-531.

7. Niu YJ, Shen ZY, Xu C, Li CF, Liu XJ, Teng YQ et al. Establishment of tacrolimus-induced diabetes in rat model and assessment of clinical treatments for post-transplant diabetes mellitus in liver transplant recipients. Clin Lab 2013;59(7-8):869-874.

8. Therasse A, Wallia A, Molitch ME. Management of post-transplant diabetes. Curr Diab Rep 2013;13(1):121-129.

9. Desai NM, Goss JA, Deng S, Wolf BA, Markmann E, Palanjian M et al. Elevated portal vein drug levels of sirolimus and tacrolimus in islet transplant recipients: local immunosuppression or islet toxicity? Transplantation 2003;76(11):1623-1625.

10. Shapiro AM, Gallant HL, Hao EG, Lakey JR, McCready T, Rajotte RV et al. The portal immunosuppressive storm: relevance to islet transplantation. Ther Drug Monit 2005;27(1):35-37.

11. Shapiro AM. Strategies toward single-donor islets of Langerhans transplantation. Curr Opin Organ Transplant 2011;16(6):627-631.

12. Bang JK, Lee JH, Murugan RN, Lee SG, Do H, Koh HY et al. Antifreeze peptides and glycopeptides, and their derivatives: potential uses in biotechnology. Mar Drugs 2013;11(6):2013-2041.

13. DeVries AL. Antifreeze peptides and glycopeptides in cold-water fishes. Annu Rev Physiol 1983;45:245-260.

14. Amir G, Rubinsky B, Basheer SY, Horowitz L, Jonathan L, Feinberg MS et al. Improved viability and reduced apoptosis in sub-zero 21-hour preservation of transplanted rat hearts using anti-freeze proteins. J Heart Lung Transplant 2005; 24(11):1915-1929.

15. Matsumoto S, Matsusita M, Morita T, Kamachi H, Tsukiyama S, Furukawa Y et al. Effects of synthetic antifreeze glycoprotein analogue on islet cell survival and function during cryopreservation. Cryobiology 2006;52(1):90-98.

16. Deller RC, Vatish M, Mitchell DA, Gibson MI. Synthetic polymers enable non-vitreous cellular cryopreservation by reducing ice crystal growth during thawing. Nat Commun 2014;5:3244.

17. Inc. P. AAGPs Overview. 2014; Available from: http://www.protokinetix.com/aagp/overview/

18. 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.

19. T. K. Islet isolation for clinical transplantation. In: Shahidul. M, (ed). The islets of Langerhans. New York: Springer, 2010.

20. Ranuncoli A, Cautero N, Ricordi C, Masetti M, Molano RD, Inverardi L et al. Islet Cell Transplant: in vivo and in vitro functional assessment of nonhuman primate pancreatic islets. Cell transplant 2000;9(3):409-414.

21. Ricordi C, Gray DW, Hering BJ, Kaufman DB, Warnock GL, Kneteman NM et al. Islet isolation assessment in man and large animals. Acta Diabetologica Lat 1990;27(3):185-195.

22. Barnett MJ, McGhee-Wilson D, Shapiro AM, Lakey JR. Variation in human islet viability based on different membrane integrity stains. Cell transplant 2004;13(5):481-488.

23. Cabrera O, Jacques-Silva MC, Berman DM, Fachado A, Echeverri F, Poo R et al. Automated, high-throughput assays for evaluation of human pancreatic islet function. Cell Transplant 2008;16(10):1039-1048.

24. Uy B MS, 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.

25. Dezaki K, Kageyama H, Seki M, Shioda S, Yada T. Neuropeptide W in the rat pancreas: potentiation of glucose-induced insulin release and Ca2+ influx through L-type Ca2+ channels in beta-cells and localization in islets. Regul Pept 2008;145(1-3):153-158.

26. Yang YH, Manning Fox JE, Zhang KL, MacDonald PE, Johnson JD. Intraislet SLIT-ROBO signaling is required for beta-cell survival and potentiates insulin secretion. Proc Natl Acad Sci U S A 2013;110(41):16480-16485.

27. Dai XQ, Plummer G, Casimir M, Kang Y, Hajmrle C, Gaisano HY et al. SUMOylation regulates insulin exocytosis downstream of secretory granule docking in rodents and humans. Diabetes 2011;60(3):838-847.

28. Pigeau GM, Kolic J, Ball BJ, Hoppa MB, Wang YW, Ruckle T et al. Insulin granule recruitment and exocytosis is dependent on p110gamma in insulinoma and human beta-cells. Diabetes 2009;58(9):2084-2092.

29. Li D-s, Hao J, Yuan Y-H, Yun SH, Feng J-B, Dai L-J et al. Pancreatic islet transplantation to the renal subcapsule in mice. Protoc exch 2011. doi:10.1038/protex.2011.221

30. 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.

31. Emamaullee JA, Merani S, Toso C, Kin T, Al-Saif F, Truong W et al. Porcine marginal mass islet autografts resist metabolic failure over time and are enhanced by early treatment with liraglutide. Endocrinology 2009;150(5):2145-2152.

32. 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.

33. Streit F, Armstrong VW, Oellerich M. Rapid liquid chromatography-tandem mass spectrometry routine method for simultaneous determination of sirolimus, everolimus, tacrolimus, and cyclosporin A in whole blood. Clin Chem 2002;48(6 Pt 1):955-958.

34. Dou H, Wang C, Wu X, Yao L, Zhang X, Teng S et al. Calcium influx activates adenylyl cyclase 8 for sustained insulin secretion in rat pancreatic beta cells. Diabetologia 2015;58(2):324-333.

35. 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.

36. Oetjen E, Baun D, Beimesche S, Krause D, Cierny I, Blume R et al. Inhibition of human insulin gene transcription by the immunosuppressive drugs cyclosporin A and tacrolimus in primary, mature islets of transgenic mice. Mol Pharmacol 2003;63(6):1289-1295.

37. Hernandez-Fisac I, Pizarro-Delgado J, Calle C, Marques M, Sanchez A, Barrientos A et al. Tacrolimus-induced diabetes in rats courses with suppressed insulin gene expression in pancreatic islets. Am J Transplant 2007;7(11):2455-2462.

38. Rostambeigi N, Lanza IR, Dzeja PP, Deeds MC, Irving BA, Reddi HV et al. Unique cellular and mitochondrial defects mediate FK506-induced islet beta-cell dysfunction. Transplantation 2011;91(6):615-623.

39. Nishimura R, Nishioka S, Fujisawa I, Shiku H, Shimada M, Sekiguchi S et al. Tacrolimus inhibits the revascularization of isolated pancreatic islets. PloS One 2013;8(4):e56799.

40. Papas KK, Avgoustiniatos ES, Tempelman LA, Weir GC, Colton CK, Pisania A et al. High-density culture of human islets on top of silicone rubber membranes. Transplant Proc 2005;37(8):3412-3414.

41. Sklavos MM, Bertera S, Tse HM, Bottino R, He J, Beilke JN et al. Redox modulation protects islets from transplant-related injury. Diabetes 2010;59(7):1731-1738.

42. Kanak MA TM, Kunnatodi F, Lawrence MC, Levy M, Nazirudin B. Inflammatory Response in Islet Transplantation. Int J Endocrinol 2014.

43. Uchizono Y, Iwase M, Nakamura U, Sasaki N, Goto D, Iida M. Tacrolimus impairment of insulin secretion in isolated rat islets occurs at multiple distal sites in stimulus-secretion coupling. Endocrinology 2004;145(5):2264-2272.

CHAPTER 4.

MICROBIAL CONTAMINATION OF CLINICAL ISLET TRANSPLANT PREPARATIONS IS ASSOCIATED WITH VERY LOW RISK

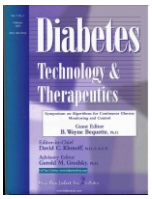
4. - Microbial Contamination of Clinical Islet Transplant Preparations is Associated with Very Low Risk

DIABETES TECHNOLOGY & THERAPEUTICS
Volume 15, Number 4, 2013
© Mary Ann Liebert, Inc.
DOI: 10.1089/dia.2012.0297

DTT
Diabetes Technology & Therapeutics

ORIGINAL ARTICLE

Microbial Contamination of Clinical Islet Transplant Preparations Is Associated with Very Low Risk of Infection



Boris Gala-Lopez, MD,[1] Tatsuya Kin, MD,[1] Doug O'Gorman,[1] Andrew R. Pepper, MD,[1] Peter Senior, MD, PhD,[1,2] Atul Humar, MD,[2] and A.M. James Shapiro, MD, PhD, FRCS (Eng), FRCSC, MSM[1,3]

Abstract

Background: Several published studies have analyzed microbial contamination rates of islet products, ranging from 0% to 16%. However, few studies make reference to potential clinical consequences for transplant recipients and possible impact on islet survival.
Materials and Methods: The current study defines rates of microbiological contamination of islet products under current good manufacturing practice conditions in 164 patients receiving 343 transplants at a single institution.
Results: Nineteen (5.5%) islet preparations showed positive microbial growth with a majority (79.4%) due to Gram-positive organisms. The most frequently identified microorganism was coagulase-negative *Staphylococcus* (nine of 19 [47.3%]), followed by polymicrobial organisms (eight of 19 [42.1%]). No patient developed signs of clinical infection, and there were no hepatic abscesses evident on imaging by ultrasound or magnetic resonance imaging (none of 19 [0%]), despite the use of potent T-depletional induction. Finally, we could not demonstrate any negative impact of microbiological contamination on long-term islet graft survival.
Conclusions: Microbiological contamination of the final islet preparation appears to have little or no effect on patients or on islet survival when appropriate antibiotics are given. However, preparation sterility should be guaranteed at all cost in order maximize patient safety and avoid potential complications in immunosuppressed patients.

Introduction

Clinical islet transplantation is an accepted treatment modality to stabilize frequent hypoglycemia or severe glycemic lability in highly selected subjects with type 1 diabetes and poor glycemic control that cannot be stabilized by other means.[1,2] Established final islet product release criteria must be met prior to clinical transplantation and must include adequate islet yield, purity, tissue volume, viability, negative Gram stain, and post hoc confirmation of microbiological sterility, an important consideration in the setting of immunodepletion and immunosuppression for transplant recipients.[3]

Several studies have reported microbial contamination rates of islet products, ranging from 0% to 16% during pancreatic retrieval, in transport media, in islet isolation, and during islet culture.[4–8] It is generally believed that the major source of bacterial contamination arises from the retrieved duodenal segment of small bowel attached to the pancreas. However, few studies have explored the potential clinical consequences for transplant recipients or the potential impact on islet survival. The objective of the study was to monitor the rate of microbiological contamination of islet products under current good manufacturing practice (cGMP) conditions at a large-volume transplant center and the clinical consequences for patients, in terms of both infectious complications and graft function.

Subjects and Methods

Patients

Between March 1999 and July 2012, the clinical islet transplant program in Edmonton, AB, Canada, has carried out 358 islet transplants procedures in 171 subjects with type 1 diabetes mellitus under a series of evolving induction and maintenance immunosuppressive protocols. Patients received a median of two procedures (range, one to four). Seven

[1]Clinical Islet Transplant Program and Departments of [2]Medicine and [3]Surgery, University of Alberta, Edmonton, Alberta, Canada.
This study is registered with ClinicalTrials.gov under Clinical Trial Agreements NCT00014911, NCT00175253, NCT00175266, NCT00434811, and NCT00468403.

Note: A version of this section was published as an Original Paper in Diabetes Technol Ther 2013; 15(4): 323 - 27.

ORIGINAL PAPER

Title: Microbial contamination of clinical islet transplant preparations is associated with very low risk of infection.

Authors: Boris Gala-Lopez[1], Tatsuya Kin[1], Doug O'Gorman[1], Andrew R. Pepper[1], Peter Senior[1,2], Atul Humar[2], AM. James Shapiro[1,3,4].

Affiliation:

1. Clinical Islet Transplant Program, University of Alberta. Edmonton, Alberta, Canada
2. Department of Medicine. University of Alberta.
3. Department of Surgery. University of Alberta.
4. Corresponding author.

Running title: Microbial contamination in islet transplant.

Corresponding author:

A.M. James Shapiro, MD, PhD, FRCS (Eng), FRCSC, MSM
Professor of Surgery, Medicine and Surgical Oncology
Director Clinical Islet and Living Donor Liver Transplant Programs
Clinical Senior Scholar Alberta Innovates - Healthcare Solutions (AIHS)
University of Alberta.
2000 College Plaza, 8215-112th St, Edmonton AB T6G 2C8
Phone 1 (780) 407- 7330, Fax 1 (780) 407- 8259
amjs@islet.ca

4.1. - ABSTRACT

Several published studies have analyzed microbial contamination rates of islet products, ranging from 0 – 16%. However, few studies refer to potential clinical consequences for transplant recipients and possible impact on islet survival. The current study defines rates of microbiological contamination of islet products under good manufacturing practice conditions in 164 patients receiving 343 transplants at a single institution. Nineteen (5.5%) islet preparations showed positive microbial growth with a majority (79.4%) due to Gram-positive organisms. The most frequently identified microorganism was coagulase-negative *Staphylococcus* (9/19; 47.3%) followed by polymicrobial organisms (8/19; 42.1%). No patient developed signs of clinical infection, and there were no hepatic abscesses evident on imaging by ultrasound or magnetic resonance imaging (0/19, 0%), despite the use of potent T-depletional induction. Finally, we could not demonstrate any negative impact of microbiological contamination upon long-term islet graft survival. Microbiological contamination of the final islet preparation appears to have little or no effect on patients or on islet survival when appropriate antibiotics are given. However, preparation sterility should be guaranteed at all cost in order maximize patient safety and avoid potential complications in immunosuppressed patients.

4.2. - INTRODUCTION

Clinical islet transplantation is an accepted treatment modality to stabilize frequent hypoglycemia or severe glycemic lability in highly selected subjects with type 1 diabetes and poor glycemic control that cannot be stabilized by other means.(1, 2) Established final islet product release criteria must be met prior to clinical transplantation, and include adequate islet yield, purity, tissue volume, viability, negative Gram stain, and *post hoc* confirmation of microbiological sterility, an important consideration in the setting of immunodepletion and immunosuppression for transplant recipients (3).

Several studies have reported microbial contamination rates of islet products, ranging from 0% – 16% during pancreatic retrieval, in transport media, islet isolation and during islet culture (4-8). It is generally believed that the major source of bacterial contamination arises from the retrieved duodenal segment of small bowel attached to the pancreas. However, few studies have explored the potential clinical consequences for transplant recipients or the potential impact on islet survival. The objective of the study was to monitor the rate of microbiological contamination of islet products under current good manufacturing practice (cGMP) conditions at a large volume transplant center and the clinical consequences for patients, both in terms of infectious complications and graft function.

4.3. - MATERIALS AND METHODS

4.3.1. – Patients

Between March 1999 and July 2012, the clinical islet transplant program in Edmonton, AB, Canada, has carried out 358 islet transplants procedures in 171 subjects with type 1 diabetes mellitus under a series of evolving induction and maintenance immunosuppressive protocols. Patients received a median of two procedures (range, one to four). Seven subjects participating in an NIH trial (CIT-04) using belatacept (Nulojix®; Bristol-Myer Squibb, Devens, MA, USA) induction were excluded from the current analysis. Thus, the study population consisted of 164 subjects receiving 343 IT procedures, with a female:male ratio of 88:76 and a mean age of 46.3 years. All subjects underwent complete pre-transplant evaluation. Informed consent was obtained, and ethical approval for this study was covered under protocol 1120, approved by the Health Research Ethics Board at the University of Alberta, and by Health Canada under Clinical Trial Agreements NCT00014911, NCT00175253, NCT00175266, NCT00434811 and NCT00468403, as registered with ClinicalTrials.gov.

4.3.2. - Transplant procedures

Islets were prepared as previously described, using a modified Ricordi protocol (9-12). In brief, human cadaveric pancreata were recovered from deceased donors and transported to the cGMP-grade clinical islet isolation laboratory. Upon arrival, the pancreatic duct was cannulated and collagenase blend enzyme solution was perfused transductally (Serva Collagenase NB1,

Crescent Pharmaceuticals, Islandia NY) with Liberase HI, or more recently Mammalian Tissue-free (MTF) enzyme, (Roche Diagnostics Corp., Indianapolis, IN, USA) (13). The pancreas was enzymatically and mechanically dissociated in a Ricordi® Chamber and then purified on a refrigerated centrifuge (model Cobe 2991; Cobe BCT, Lakewood, CO, USA) with continuous density gradient separation with Ficoll™ (Sigma-Aldrich, St. Louis, MO) or, more recently Biocoll™ (Biochrom AG, Cederlane, Burlinton, ON, Canada) separating solution (14). The majority of the islet preparations were placed in culture (median 13.0 h; range 6.4–23.0 h) before infusion to facilitate timing of islet infusion or as part of the immunosuppressive protocol. Subjects then underwent percutaneous transhepatic portal access in the Radiology Department under local anesthesia and with fluoroscopic and ultrasonic guidance, and islets were infused under gravity pressure from a 100 ml of medium-containing intravenous islet bag (15). Portal pressure was monitored during and after infusion and afterwards the catheter tract was ablated to minimize the risk of bleeding.

4.3.3. - Microbiological testing

The majority of clinical islet preparations were placed in culture containing ciprofloxacin (Cipro®, Bayer A.G. Toronto, ON, Canada) at a concentration of 20 mg/ml. Samples for Gram stain and microbiological culture were taken immediately before transferring islets to the final container for transplant and evaluated at the Provincial Laboratory of Public Health at the University of Alberta Hospital. Both a negative Gram stain and endotoxin content less than 5 endotoxin units (EU)/kg of recipient's body weight are mandatory requirements prior to islet release for transplantation. Results of samples undergoing microbiological culture for aerobic

and anaerobic bacterial, fungal, mycoplasma and mycobacterial contamination were made available from 2 - 7 weeks post-transplant and did not constitute product release testing.

4.3.4. - Antibiotic coverage

All patients undergoing islet transplantation in Edmonton routinely receive prophylactic antibiotic treatment consisting of a single dose of cefazolin (Baxter Co. Missisauga, ON, Canada) 1g IV pre-procedure, or clindamycin (Sandoz Canada Inc., Boucherville, QC, Canada) 600mg IV if known allergy to cephalosporins or severe penicillin allergy.
In the rare case of contaminated islet preparation discovered after transplantation, consultations from transplant infectious diseases were made to design treatment strategy. Imaging studies (ultrasound, computed tomography, and magnetic resonance), as clinically indicated, was used to rule out development of intrahepatic abscess and directed antibiotic therapy was given according to microbiological culture results. In the absence of symptoms, normal liver imaging and absence of abscess, patients received a minimum of 7 days of broad-spectrum (culture-sensitive) antibiotic or antifungal treatment by peroral route if appropriate, or intravenous where peroral was judged to be inadequate.

4.3.5. - Immunosuppression protocols

Induction and maintenance immunosuppressive protocols have evolved in our program over time. Initially our practice was to induce with an IL-2 receptor monoclonal antibody (daclizumab [Zenapax®; Hoffman-La Roche Ltd., Missisauga, ON, Canada]) 2 mg/kg intravenously at

transplant and at 5 days post-transplant), combined with tacrolimus (Prograf®; Astellas Pharma Canada Inc. Markham, ON, Canada) for a target trough level of 3–6 ng/ml and sirolimus (Rapamune®; Pfizer Canada Inc., Kirkland, QC, Canada) for target trough levels of 12–15 ng/ml for the first 90 days and 8–10 ng/ml thereafter (the '*Edmonton Protocol*') (2, 12). Subsequently, basiliximab (Simulect®. Novartis Pharmaceuticals Canada Inc., Dorval, QC, Canada) (20mg intravenous on day 0 and 4) has been used in place of daclizumab, with the combination of tacrolimus (target trough level of 8 – 10 ng/ml) and mycophenolate mofetil (CellCept®; Hoffman-La Roche Ltd., Missisauga, ON, Canada) (up to 2g daily in divided dose as tolerated). Before 2003, daclizumab was given at a dose of 1 mg/kg every 2 weeks for five doses (10). Other protocols included the use of infliximab (10 mg/kg) given at the time of transplant, combined with daclizumab; alternative use of basiliximab (two doses of 20 mg), etanercept (embrel®; Amgen Canada Inc., Missisauga, ON, Canada) (50mg weekly) or most recently potent lymphocyte depletion protocols based on alemtuzumab (Mabcampath®; Genzyme Canada, Missisauga, ON, Canada) or Anti-thymocyte globulin (Rabbit) (Thymoglobulin®; Genzyme Canada Mississauga, ON, Canada).

4.3.6. - Management of patients receiving contaminated islet preparation

When a positive culture was obtained from the islet product, a personalized management was designed based on the patient's characteristics, the immunosuppression regimen, the transplant interval and the identity of the organism(s) isolated in culture. Routine abdominal ultrasound and abdominal CT or magnetic resonance imaging were also performed to rule out liver abscess when a positive culture was received and complementary antimicrobial treatment was ordered

depending on the particular microorganism growing in the culture media and its sensitivity profile. Further measures were also available upon clinical evidence of infection.

4.3.7. - Graft function

In addition to standing graft function determination based on insulin requirement, glycemic control, hemoglobin A1C, protection from hypoglycemia, and fasting C-peptide testing, more definitive stimulated C-peptide levels were obtained at the time of mixed meal tolerance testing scheduled at intervals post-transplant (3 monthly for one year, then 6 monthly thereafter). Loss of C-peptide production was defined by stimulated C-peptide levels below 0.2 nmol/L after mixed meal tolerance test or in the presence of a fasting glucose >15mmol/l.

4.3.8. - Statistical analysis

Results are expressed as means ± SE values or the median (25^{th} – 75^{th} percentile) as appropriate. Comparisons were made with a two-tailed Student's *t* test, paired or unpaired as appropriate. Graft survival analysis was performed using Kaplan-Meier with log-rank test to compare differences between groups. All statistical analyses were performed using SPSS version 12.0 (SPSS Inc., Chicago, IL, USA). Significance was considered when $P< 0.05$.

4.4. – RESULTS

After more than 358 procedures, no single islet preparation showed a positive gram stain or endotoxin level greater than 5 EU/kg. Of those 343 islet transplants included in this study, 19 (5.5%) showed positive microbial growth in the final islet preparation. This prevalence is well within the incidence previously reported by centers worldwide.(4, 5, 8, 16) Thus, two groups of patients are reported: those with contamination (n=18) (one patient received two contaminated islet preparations) and those without (n=146). Among the contaminated islet preparations, the most common isolates were gram-positive organisms (79.4%), followed by *Candida sp.* (11.7%) and Gram-negative organisms (8.8%) (**Table 4.1**). The most frequently identified microorganisms were coagulase-negative *Staphylococcus* (nine of 19 [47.3%]), followed by *Enterococcus* and *Candida albicans* (four of 19 [21%] for each*).* Eight islet preparations had polymicrobial contamination (42.1%). Other microorganisms also cultured in lesser frequency were *Pseudomonas* sp*., Streptococcal* sp., Aerobic spore-bearing bacilli and *Mycoplasma* sp. All patients in whom islet cultures were positive received appropriate antimicrobial prophylaxis usually of 1-2 weeks duration. No patient in our series experienced clinical features of infection related to the procedure regardless of the induction immunosuppression utilized and blood cultures sent in those patients resulted in no growth (**Table 4.2**).

We also analyzed the possible impact of contaminated preparations on short and long-term islet graft function assessed by various post-transplant tests over a mean follow up of 134.6 months. The analysis did not demonstrate any significant difference between the two groups in terms of measurable or stimulated C-peptide peak values at one moth post-transplant and onwards. Insulin-independence rates were similar among both study groups and finally, the mixed meal

Table 4.1 - Microbial species cultured from 19 contaminated human islet preparations

Pathogen	*Frequency*	*Percentage*
Staphylococcus sp.	9/19	47.3
Enterococcus sp.	4/19	21
C. albicans	4/19	21
Streptococcus sp.	3/19	15.8
Aerobic spore-bearing bacilli	3/19	15.8
Micrococcus sp.	1/19	5.26
Diphtheroid sp.	1/19	5.26
Rothia sp.	1/19	5.26
Mycoplasma sp.	1/19	5.26
Propionibacterium sp.	1/19	5.26
Ureaplasma urealyticum	1/19	5.26
Polymicrobial contamination	8/19	42.1

Table 4.2 Summary of 18 patients receiving islet transplant with microbial contamination in the final preparation. Asterisk marks patient No.8 who received contaminated preparations on two occasions.

Patient number	*Pathogen(s)*	*Clinical manifestations*	*Treatment*	*Most recent C-peptide*	*Imaging*	*Outcome*
1	*Staphylococcus* sp.	None	Cephalosporin course	Negative	Negative US, CT	No effect
2	Aerobic Gram-negative bacilli/*C. albicans*	None	Cephalosporin/ antifungal course	Positive	Negative US, CT	No effect
3	*Staphylococcus* sp.	None	Cephalosporin course	Positive	Negative US	No effect
4	*Bacillus* sp./*Streptococcus viridans*	None	Cephalosporin course	Positive	Negative US	No effect
5	*Staphylococcus* sp.	None	Cephalosporin course	Positive	Negative US	No effect
6	*Enterococcus* sp.	None	Vancomycin course	Negative	Negative US, CT	No effect
7	*Streptococcus*/*Micrococcus* sp.	None	Vancomycin course	Positive	Negative US, MR	No effect
8[a]	*Staphylococcus* sp. Aerobic spore-bearing bacilli	None	Cephalosporin course Negative	Negative Positive	Negative US Negative US, CT	No effect
9	*Rothia* sp./streptococci	None	Cephalosporin course	Positive	Negative US	No effect
10	*Mycoplasma*	None	Quinolone course	Negative	Negative US	No effect
11	*Staphylococcus* sp./ *Propionibacterium*	None	Cephalosporin course	Positive	Negative US	No effect
12	*C. albicans*/diphtheroids	None	Quinolone and antifungal course	Positive	Negative US, CT	No effect
13	*Enterococcus* sp./*C. albicans*	None	Linezolid and antifungal course	Positive	Negative US, CT	No effect
14	*C. albicans*	None	Fluconazole course	Positive	Negative US, CT	No effect
15	*Staphylococcus* sp.	None	Cephalosporin course	Negative	Negative US	No effect
16	*Staphylococcus* sp./ *Enterococcus* sp.	None	Linezolid course	Positive	Negative US, CT	No effect
17	Aerobic spore-bearing bacilli	None	Cephalosporin course	Positive	Negative US	No effect
18	*C. albicans*	None	Fluconazole course	Positive	Negative US, CT	No effect

[a]Patient 8 received contaminated preparations on two occasions.
CT, computed tomography; MR, magnetic resonance; US, ultrasound.

tolerance test (Ensure® [Abbot Nutrition Canada; Saint-Laurent, QC, Canada] test) and other metabolic monitoring tools (intravenous glucose tolerance test, oral glucose tolerance test and arginine stimulation test) also failed to demonstrate any differences in graft function after receiving non-contaminated/contaminated islet preparations. Moreover, we could not demonstrate any negative impact of microbiological contamination upon long-term islet graft survival, with loss of stimulated C-peptide over time (**Figure 4.1**). Mean C-peptide survival was 112.4 months in subjects with no contamination (n=146) vs 112.7 months in those receiving contaminated preparations (n=18), (Log-rank p=0.39).

4.5. – DISCUSSION

We herein report on the largest single center series of intraportal islet transplantation, with respect to risk of transplantation of microbiologically contaminated islet preparations in immunocompromised recipients. Importantly, despite a 5.5% risk of transplantation of a contaminated product, there were no clinical sequelae and no negative impact upon islet function in any of the 18 recipients. Clearly, despite the introduction of rigorous cGMP manufacturing conditions and strict protocols for handling of biological tissues, there is still a potential risk for transmission of microbiological contaminants (3-5, 8, 16). Since all of the materials, reagents and media are strictly quarantined and monitored, and full aseptic technique used throughout the processing, it seems most likely that the greatest source of microbiological contamination originates from the donor pancreas and in the retrieved segment of donor duodenum. Previous studies addressing contamination of the pancreas preservation solution (University of Wisconsin

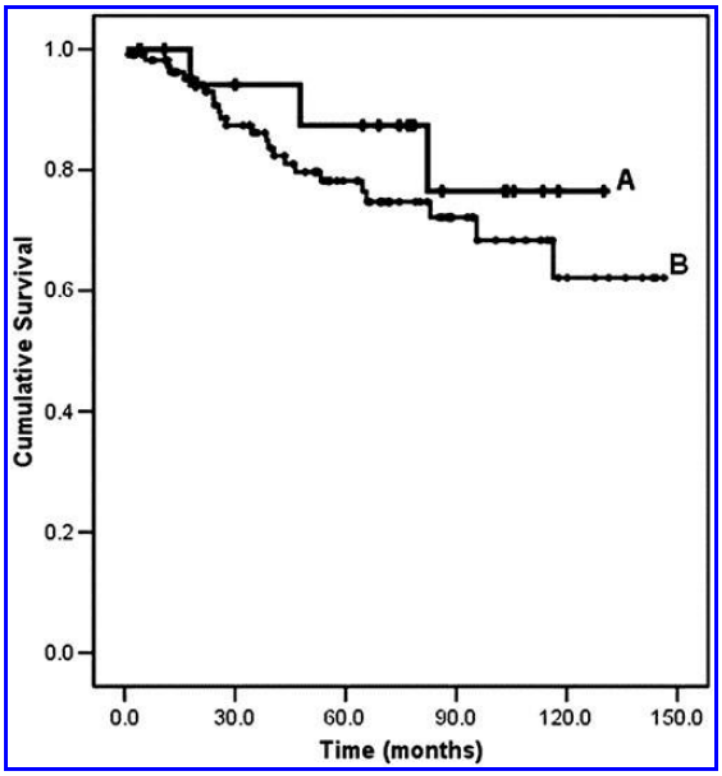


Figure 4.1 Kaplan Meier Graft survival curves comparing the maintenance of stimulated C-peptide levels in subjects who did or did not receive contaminated islet preparations. Mean C-peptide survival was 112.4 months in subjects with no contamination (**B**, n=115) vs 112.7 months in those receiving contaminated preparations (**A**, n=18) (Log-rank p=0.39).

solution or Histidine-Tryptophan-Ketoglutarate) both in whole pancreas and in islet isolation suggest that the donor duodenal segment provides the greatest source of contamination (4, 8). Our study reports a contamination rate of 5.5% with absolutely no influence of harvesting techniques or preservation solutions as previously reported by our center (8). In fact, previous studies on microbial contamination during islet isolation have suggested that a majority of microorganisms are washed, diluted or eliminated during pancreas processing. However, de novo contamination during the last stages of the process is still reported in various centers (4, 8). Previous reports on this topic make minimal reference to the consequences of patients receiving these contaminated islets in the presence of potent T-directed immunodepletion and immunosuppression regimes (121 of 358 [33.8%]). To our knowledge, previous reports have not addressed clinical risk, impact and potential negative effects upon islet engraftment and survival. In the current study, we looked for possible infectious complications in 18 subjects receiving positive-culture islet preparations. Our current series expands on previous findings, and clearly demonstrates exceedingly low risk of clinical sequelae provided the initial microbiological load is low (negative Gram stain and low endotoxin content) and appropriate antimicrobial prophylaxis is used. Furthermore, the intrahepatic site for islet delivery may provide an especially protective environment for a potentially contaminated islet product, due to the excellent intrahepatic blood flow, exposure to prophylactic and treatment directed antibiotics where indicated, and most likely due to the presence of large numbers of phagocytic Kupffer cells (17).

When looking into the possible long-term effects of this contamination we could not find any significant differences in islet survival between the two groups. This finding is similar to another study recently published by our group when analyzing the influence of cytomegalovirus infection

in clinical islet transplanted patients (18). In that analysis, we reported an association between the use of T-depletion immunosuppressant and the occurrence of cytomegalovirus transmission. The sterile technique during islet isolation continues to be of paramount importance in line with the continual improvement in islet transplantation safety. The use of cGMP conditions remains standard and an integral part of the safety of clinical grade islet manufacture. Despite these standards, microbiological contamination of islet products may still occur on rare occasions, but is only identified after the transplant procedure. Fortunately, it appears to have little or no effect on patient health or on islet survival. Nonetheless, utilization of sterile preparations should remain a priority in order to maximize patient safety and avoid potential complications in immunosuppressed patients. Microbial surveillance remains an important element in clinical islet transplantation, and where found the risks can be negligible provided appropriate directed antimicrobial therapies are given (4).

4.6. – REFERENCES

1. Ryan EA, Bigam D, Shapiro AM. Current indications for pancreas or islet transplant. Diabetes Obes Metab 2006;8(1):1-7.

2. Shapiro AM, Ricordi C, Hering BJ, Auchincloss H, Lindblad R, Robertson RP et al. International trial of the Edmonton protocol for islet transplantation. N Engl J Med 2006;355(13):1318-1330.

3. Taylor G, Kirkland T, Lakey J, Rajotte R, Warnock G. Bacteremia due to transplantation of contaminated cryopreserved pancreatic islets. Cell Transplant 1994;3.

4. Bucher PO, J.; Bosco, D.; Mathe, Z.; Toso,, C.; Buhler LHB, T.; Morel, P. Microbial surveillance during human pancreatic islet isolation. Transpl Int 2005;18:584 - 589.

5. Carroll PBR, C.; Fontes, P.; Rilo, H. R.; Phipps,, J.; Tzakis AGF, J. J.; Starzl, T. E. Microbiologic surveillance as part of human islet transplantation: Results of the first 26 patients. Transplant Proc 1992;24:2798–2799.

6. Lakey JRR, R. V.; Warnock, G. L. Microbial surveillance of human islet isolation, in vitro culture, and cryopreservation. Clin Invest Med 1995;18:168–176.

7. Scharp DWL, P. E.; McLear, M.; Longwith, J.; Olack, B. The bioburden of 590 consecutive human pancreata for islet transplant research. Transplant Proc 1992;24:974–975.

8. Kin T, Rosichuk S, Shapiro A, Lakey J. Detection of Microbial Contamination During Human Islet Isolation. Cell Transplant 2006;15:1–100.

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

10. 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.

11. Ryan EA, Lakey JR, Paty BW, Imes S, Korbutt GS, Kneteman NM et al. Successful islet transplantation: continued insulin reserve provides long-term glycemic control. Diabetes 2002;51(7):2148-2157.

12. 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.

13. O'Gorman D, Kin T, Imes S, Pawlick R, Senior P, Shapiro AM. Comparison of human islet isolation outcomes using a new mammalian tissue-free enzyme versus collagenase NB-1. Transplantation 2010;90(3):255-259.

14. Barbaro B, Salehi P, Wang Y, Qi M, Gangemi A, Kuechle J et al. Improved human pancreatic islet purification with the refined UIC-UB density gradient. Transplantation 2007;84(9):1200-1203.

15. Baidal DA, Froud T, Ferreira JV, Khan A, Alejandro R, Ricordi C. The bag method for islet cell infusion. Cell Transplant 2003;12(7):809-813.

16. Lloveras J, Farney AC, Sutherland DE, Wahoff D, Field J, Gores PF. Significance of contaminated islet preparations in clinical islet transplantation. Transplant Proc 1994;26(2):579-580.

17. Barshes NR, Wyllie S, Goss JA. Inflammation-mediated dysfunction and apoptosis in pancreatic islet transplantation: implications for intrahepatic grafts. J Leukoc Biol 2005;77(5):587-597.

18. Gala-Lopez BL, Senior PA, Koh A, Kashkoush SM, Kawahara T, Kin T et al. Late cytomegalovirus transmission and impact of T-depletion in clinical islet transplantation. Am J Transplant 2011;11(12):2708-2714.

CHAPTER 5.

CLINICAL SUBCUTANEOUS ISLET TRANSPLANTATION INTO A PRE-VASCULARIZED SUBCUTANEOUS DEVICE - EXPERIENCE IN THE FIRST THREE CASES

5. - Clinical Subcutaneous Islet Transplantation into a Pre-Vascularized Subcutaneous Device - Experience in the First Three Cases

American Journal of Transplantation

American Journal of Transplantation

Clinical Subcutaneous Islet Transplantation into a Pre-vascularized Subcutaneous Device - Experience in the First Three Cases.

Journal:	*American Journal of Transplantation*
Manuscript ID	AJT-B-16-00105
Wiley - Manuscript type:	B - Brief Communication
Date Submitted by the Author:	24-Jan-2016
Complete List of Authors:	Gala-Lopez, Boris; University of Alberta, Surgery Pepper, Andrew; University of Alberta, Surgery Senior, Peter; University of Alberta, Clinical Islet Transplant Program Dinyari, Parastoo; University of Alberta, Surgery Malcolm, Andrew; University of Alberta, Department of Medicine; University of Alberta, Department of Surgery Kin, Tatsuya; University of Alberta, Clinical Islet Laboratory; Pawlick, Rena; University of Alberta, Surgery Siroen, Delfina; Sernova Corp MacGillivary, Amanda; Sernova Corp Toleikis, Philip; Sernova Corp Shapiro, A.M.; University of Alberta, Surgery
Keywords - Scope:	clinical research / practice, health services and outcomes research
Keywords - Discipline:	islet transplantation
Keywords - Focus:	bioengineering, clinical trial, diabetes: type 1, islets of Langerhans

SCHOLARONE™
Manuscripts

Note: A version of this section has been submitted as A Brief Communication to the *Am J Transplant* May 2016.

BRIEF COMMUNICATION

Title: **Clinical Subcutaneous Islet Transplantation in a Prevascularized Subcutaneous Pouch – Preliminary Experience.**

Authors: Gala-Lopez, Boris L.[1]; Pepper, Andrew R.[1]; Dinyari, Parastoo[1]; Malcolm, Andrew J[1]; Kin, Tatsuya[1]; Pawlick, Rena L.[1] and Shapiro, A.M.James[1,2].

Affiliations:

1. Clinical Islet Transplant Program and Department of Surgery, University of Alberta
2. Department of Medicine, University of Alberta

Corresponding author contact information:

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

Canada Research Chair in Transplantation Surgery and Regenerative Medicine

Professor of Surgery, Medicine and Surgical Oncology

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

tel. (780) 407 7330 fax. (780) 407 8259 Email: amjs@islet.ca

Running title: Subcutaneous Clinical Islet Transplantation

5.1. – ABSTRACT

Despite advances in clinical islet transplantation, intraportal islet delivery is limited by engraftment, neovascularization, immune protection and functional survival. An alternative pre-vascularized, subcutaneous device could solve these issues and be relevant for future transplantation of insulin-producing stem cells. We herein report a first-in-human trial with a newly developed pre-vascularized subcutaneously placed pouch as an innovative approach for human islet implantation. Three longstanding type-1 diabetes subjects underwent subcutaneous implantation of therapeutic and sentinel pouches. After a median delay of 53 days (range 22 – 130), inner rods were removed and voids filled with purified human islets. In this preliminary experience, the primary endpoint of safety was met, and surviving, vascularized human islets were visualized on histological examination after pouch explantation by 6 weeks post-transplant. Islets retained macro-structure of beta and alpha cells in all cases, and demonstrated neovascularization. The early secondary endpoint of efficacy was not met, as no functional islet engraftment was detectible despite transplantation of a substantial islet mass in each case. Early peak C-peptide at 24 hours followed by absence subsequently suggested early engraftment failure. This pre-vascularized subcutaneous device offers potential for human islet or stem cell engraftment in a superficial retrievable site, but requires further refinement.

5.2. - INTRODUCTION

Clinical islet transplantation has advanced beyond 'proof-of-concept' demonstrating that cellular replacement therapy can effectively treat type 1 diabetes mellitus (T1DM) (1). Currently, islet transplantation into the portal 'black-box' of the liver has clear limitations for engraftment, functional survival, immune protection, monitoring and imaging of grafts, and many subjects require more than one islet infusion to achieve and maintain protection from hypoglycemia or insulin independence (2, 3). The elements of any future widespread cell-based approach to restore beta-cell mass through transplantation beyond cadaveric donation will require a vast supply of compatible and safe insulin-producing cells. If these cells are derived from embryonic or adult stem cell lines, the potential for unregulated growth, teratoma or malignant transformation, will likely dictate a need for graft retrievability, at least in the early phase safety trials (4). The hepatic portal system is therefore likely unsuitable for infusion of stem cell-derived therapies, as a major hepatectomy or liver transplantation would be required if graft retrieval is needed.

Numerous studies have explored alternative suitable sites for islet engraftment (5). Empirically, the intraportal site is used routinely for clinical islet transplantation, and is currently the only site that has consistently provided protection from hypoglycemia and insulin-independence. Intramuscular (6-8) and bone marrow (9) implantation have generated interest, but no patients have achieved insulin independence with such an approach to date. The subcutaneous site for surrogate beta-cell implantation remains attractive, but has previously failed to offer an adequate milieu for vascularity, oxygen, hormonal and metabolite exchange (10-12). Furthermore,

placement of non-encapsulated islets or stem cell derived insulin producing cells within the unmodified subcutaneous space has met with limited success (13).

Over the past six years, Sernova Corp. (London, ON) developed and refined a proprietary, scalable, implantable polymer chambered medical device (Cell Pouch™) designed for human cellular replacement therapies (14). A scaled down Cell Pouch™ prototype for small animal testing demonstrated long-term insulin independence in a marginal mass islet transplant model (15, 16). Initial results suggested the device could provide a critical, unmet need in development of the subcutaneous space for islet, and especially for alternate stem cell derived therapeutic Cell Transplant. The pouch is contract-manufactured from medical-grade materials, under ISO13485, US FDA Quality System Regulations (QSR) 21 CFR 820 standards, and sterilized according to ANSI/AAMI/ISO 11135-1: 2007. The device previously demonstrated a favorable safety profile in multiple animal models and met ISO10993 biocompatibility studies. This human-scaled device is approximately 60mm x 60mm and is placed in the deep subcutaneous space, in a minimally-invasive surgical procedure (**Figure 5.1**).

Based on safety and efficacy validation in small and large animal islet transplant models (isograft, autograft and allograft), the University of Alberta's Clinical Islet Transplantation Program initiated a pilot phase I/II clinical trial to evaluate safety and efficacy of this device in a planned recruitment of 20 subjects with type I diabetes (Clinical Trials.gov NCT01652911). This study was authorized by the Health Research Ethics Board of the University of Alberta (Protocol number PRO00028097), Data and Safety Monitoring Board and by Health Canada. Therapeutic cells are regulated by the Biologic and Genetic Therapies Directorate and the Cell Pouch™ is regulated by the Therapeutic Products Directorate - Medical Devices Bureau of Health Canada. This paper presents our preliminary experience in the first three enrolled

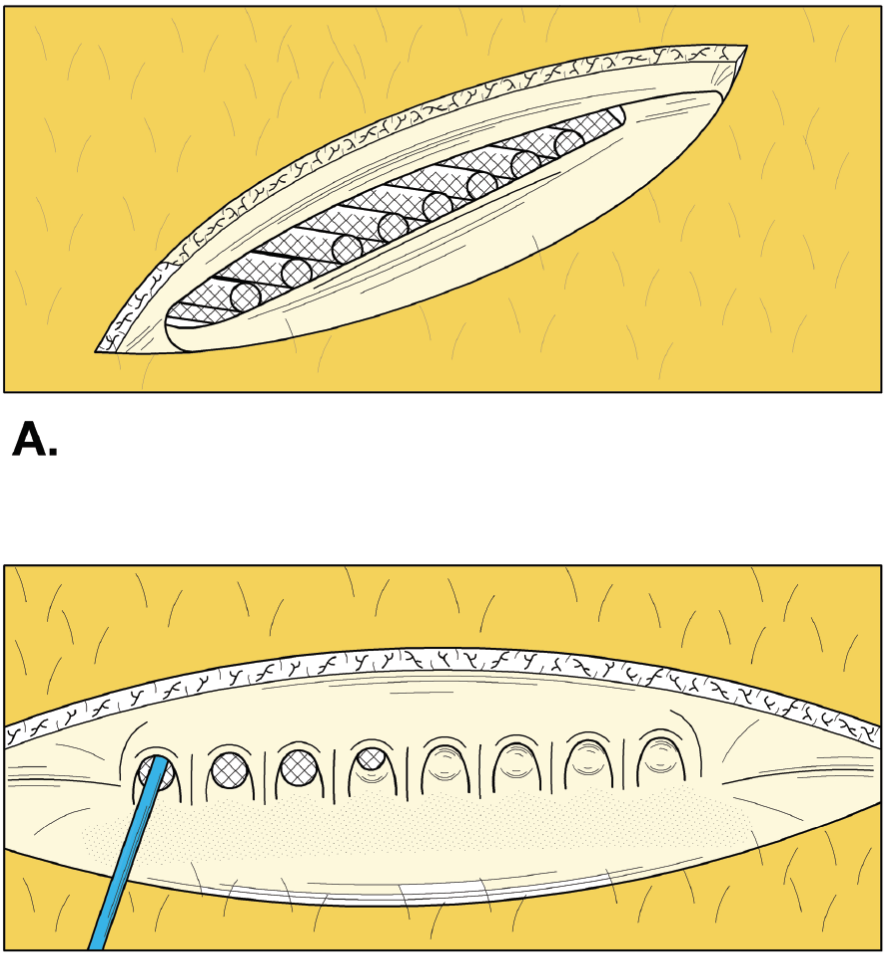


Figure 5.1 . Illustration of the Sernova Cell Pouch™. (**A**) Plugs in situ within the Cell Pouch™. (**B**) Illustration of islet infusion within the chambers of the Cell Pouch™.

subjects and the lessons learned from a first-in-human assessment of the Cell Pouch™ using human islets.

5.3. - CASE 1

A 60-year-old male subject with longstanding T1DM of 35 years was listed for clinical islet transplantation based on frequent recurrent hypoglycemia and glycemic lability (Clark score of 5/7, Lability Index 530, Hypo score of 2,704) (17). Baseline characteristics and glycemic control are shown in **Table 5.1**. At the time of device implantation, 1g cefazolin (Ancef, SmithKline Beecham, Mississauga, ON.) was administered intravenously, and implantation was accomplished under local anesthesia. Two 10-plug pouches were implanted in the abdominal wall through 2 limited transverse incisions. The devices were positioned lying flat in the deep subcutaneous space. A third small 2-plug pouch, designed as a sentinel, was placed in the volar forearm.

Approximately 13 days post-implant this subject experienced a minor wound seroma, which was aspirated percutaneously. The cultured seroma fluid was initially sterile, but 7 days later ongoing wound discharge was culture positive for *propionibacterium acnes*, a likely skin contaminant. The seroma resolved completely on clinical inspection, and confirmed by superficial ultrasound interrogation 28 days later.

On day 53 post-implant, and under general anesthesia, the devices were accessed, the inner rods removed, and 448,612 islet equivalents (IEQ) distributed evenly across all channels (**Table 5.1 and Figure 5.1**). Immunosuppression consisted of our local standard alemtuzumab (MabCampath, Genzyme Corp.) 30 mg intravenously induction, etanercept (Enbrel; Amgen

Table 5.1 Summarized demographic, glycemic and transplant characteristics of three subjects receiving subcutaneous Cell Pouch™ islet transplantation. Islet channel density was estimated by dividing the total islet mass infused (in IEQ) by the total number of therapeutic channels used. Subject 3 received a combination of two islet preparations; **a**. Right-sided therapeutic pouch islet density for subject 3 (first donor); **b.** Left-sided therapeutic pouch islet density for subject 3 (second simultaneous donor); **c**. Only sentinel devices were explanted in Subject 3. Therapeutic devices remain in place.

	Subject 1	Subject 2	Subject 3
Age (years)	60	45	29
Weight (Kg)/BMI	79.3/23.8	83.5/27.4	67.3/26
Basal insulin requirement (U/Kg/day)	0.45	0.37	0.40
Basal HbA1C (%)	7.7	8.3	7.8
Time from implant to transplant (days)	53	22	130
Total islet mass (IEQ)	448,612	506,844	368,696 [a] 794,615 [b]
Islet mass per body weight (IEQ/Kg)	5,657	6,070	5,478 [a] 11,807 [b]
Islet purity (%)	90	75	40 [a] 57.5 [b]
Islet viability (%)	93.5	84	92 [a] 84 [b]
Packed cell volume (mL)	1.5	3.0	3.0 [a] 3.0 [b]
Number of sentinel devices implanted	1	1	2
Number of therapeutic devices implanted	2	2	4
Number of therapeutic devices transplanted	2	1	4
Islet density (therapeutic channel) (IEQ/channel)	22,430	50,684	46,087 [a] 99,327 [b]
Time from transplant to explant (days)	30	14	40 [c]

Canada Inc., Mississauga, ON.) 50 mg IV on day 0, and 25 mg subcutaneously on days 3, 7 and 10 post-transplant, and anakinra (Kineret, Amgen Canada Inc., Mississauga, On.) 100 mg subcutaneously on day 0 and daily for 7 days. Maintenance twice daily tacrolimus (Prograf, Astellas Pharma Canada Inc., Markham, ON.) was adjusted to provide target trough levels of 10 – 12 µg/L, together with mycophenolate mofetil (CellCept®, Hoffmann-La Roche Ltd., Mississauga, ON.) up to 2 g per day in divided dose, as tolerated. Cephalexin (Keflex, Eli Lilly Canada Inc. Toronto, ON.) was given at a dose of 500 mg 4 times daily, orally for 10 days.

The early post-transplant period was uneventful. In this subject, systemic evidence of graft function was not observed at early time points (negative C-peptide on day 7, 14 and 21, with no change of insulin requirement). On day 14 post-transplant, a sterile local wound discharge was observed from the right and left abdominal incision sites, and cultures were positive for anaerobic non-spore forming Gram-positive bacilli, a possible skin contaminant. The patient was prescribed cephalexin 500 mg q6h for 14 days and by post-transplant day 23 the discharge was resolving.

On post-transplant day 30, accommodating patient request, all devices were explanted under general anesthesia. The integrity of the pouches was confirmed, and appeared to be vascularized and integrated with surrounding tissues. Tissue fluid from each of the incisions was cultured and demonstrated no bacterial or fungal growth.

Histology and immunohistochemistry of the excised devices confirmed the devices to be well-integrated with neovascularization (positive staining for von Willebrand factor) and intact, viable islets were present in limited sections with β-cells stained positive for insulin (**Figure 5.2A,B**). α-cells, staining positive for glucagon, and δ-cells staining positive for somatostatin were also observed (images not shown).

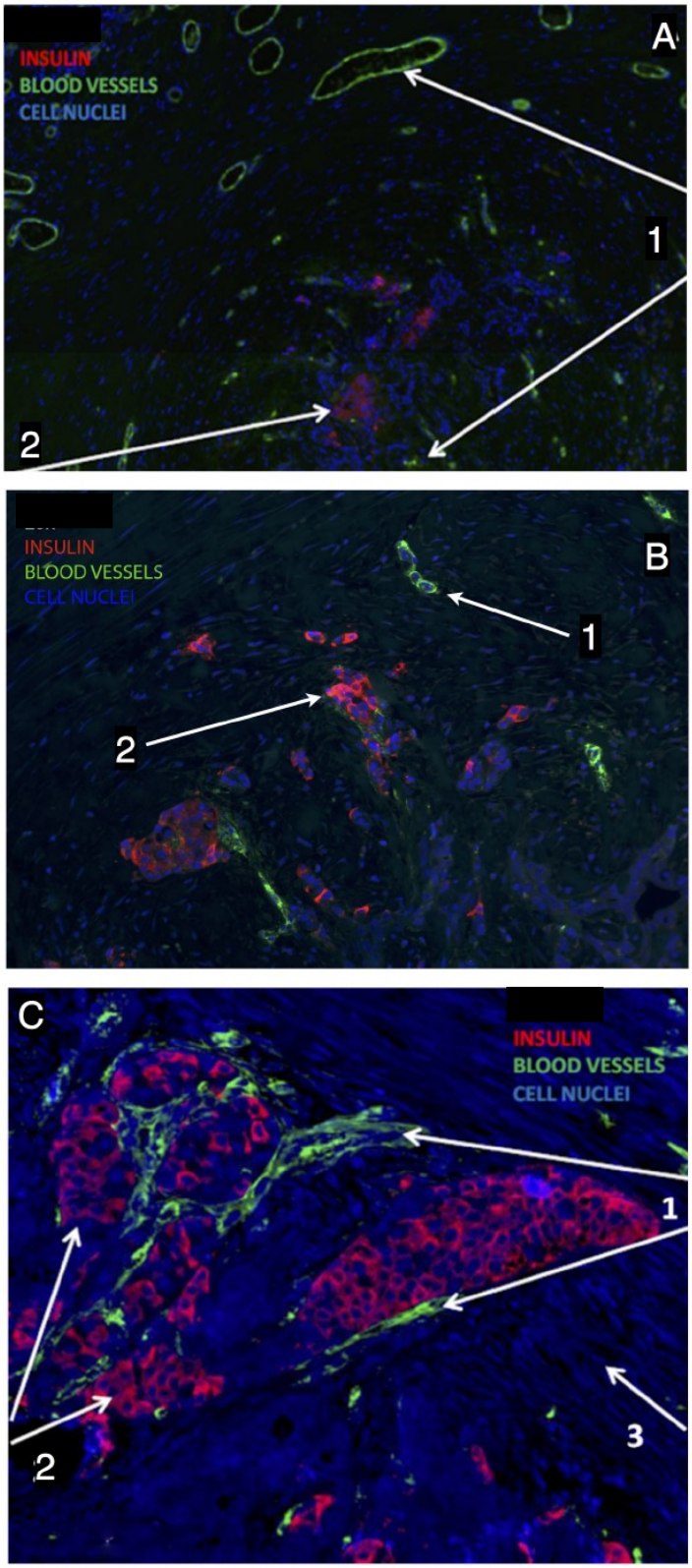

A
INSULIN
BLOOD VESSELS
CELL NUCLEI
1
2
B
INSULIN
BLOOD VESSELS
CELL NUCLEI
1
2
C
INSULIN
BLOOD VESSELS
CELL NUCLEI
1
2
3

Figure 5.2 Histology showing surviving islets surrounded new blood vessels in areas of the therapeutic devices explanted from Subjects 1 and 2 (paraffin embedded, 5 micron sections). White arrows (**1**) highlight strong von Willebrand Factor (vWF) staining in green; White arrows (**2**) demonstrate insulin staining (red) and cell nuclei (**3**) staining blue with 4',6-diamidino-2-phenylindole (DAPI). **2A**. Selected section of therapeutic Cell Pouch™ explanted at day 30 post-transplant from Subject 1, at 100x magnification to show overview of surrounding large vessels in the graft perimeter together with intra-islet micro vessels; **2B**. Similar section as 2A, but magnified to 200x; **2C**.Selected section of therapeutic Cell Pouch™ explanted at day 14 post-transplant from Subject 2, at 200x magnification.

Where islets were identified, their presence was low volume and the distribution was patchy. No evidence of acute cellular rejection or autoimmune infiltrates was seen, without foreign body reaction and no macrophage or monocytic infiltration.

5.4. - CASE 2

A 58-year-old-female was enrolled with T1DM of 45 years duration (Clark score 7, Lability Index 592, Hypo score 671 (**Table 5.1**). The Cell Pouch™ implants were carried out under local anesthesia similar to Case 1 (two 10-plug pouches placed in the lower abdominal wall and one 2-plug sentinel pouch in the volar left forearm).

There were no local wound complications after device implantation. On day 22 post-implant, under local anesthesia, 506,844 IEQ islets were infused evenly within the left-sided 10-plug pouch and sentinel (**Table 5.1**). Since the preparation was very pure, the remaining right-sided device was left *in situ*. Similar immunosuppression was given, and cephalexin prescribed (500mg 4 times daily orally for 10 days).

The patient did well initially, but subsequently developed superficial cellulitis of the left forearm at day 10 post-transplant, which responded to intravenous ceftriaxone (Sandoz Pharmaceuticals, Sandoz, Boucherville, QC) 2 g daily.

Again, upon patient request, all devices were explanted on day 14 post-transplant. In this patient, no detectable C-peptide was evident and no meaningful reduction in insulin requirement was detected at this day 10 early time point. She did not reach the protocol defined 3-month post-transplant first efficacy assessment, nor was she offered a second transplant.

Staphylococcus aureus was isolated from the left upper quadrant abdominal site on delayed cultures. Immunohistochemistry of the explanted devices showed extensive vessel ingrowth by von Willebrand factor staining, and viable islets were identified with patchy distribution, with β-cells staining positive for insulin (**Figure 5.2B**), α-cells positive for glucagon, and δ-cells positive for somatostatin (images not shown). There was no evidence of acute cellular rejection or autoimmune infiltrates, foreign body reaction and no macrophage or monocytic infiltration.

5.5. - CASE 3

Based on our preliminary experience above, we minimized the use of electrocautery during implantation, prolonged prophylactic oral antibiotics, and extended the period from device to cell implantation to 4 months to ensure mature device incorporation. A 29-year-old female with 25 years of T1DM was then enrolled (Clark score 4, Lability Index 610, Hypo Score 501) (**Table 5.1**). In this case we doubled the initial device implant numbers, placing four 8-plug pouches in the deep subcutaneous space of the lower abdominal wall, with two 2-plug sentinel devices placed laterally (**Figure 5.3**). The rationale for the increased device number and configuration was to limit exposure to repeated surgeries, and to potentially accommodate simultaneous transplantation of two clinical islet preparations from separate donors. At 3 weeks post implantation, a large sterile fluctuant seroma developed, extending bilaterally across the dependent lower abdominal wall. There was no pain or discomfort, and no evidence of infection, cellulitis or discharge, and we chose to allow the seroma to resolve fully and spontaneously before proceeding with islet implantation. Complete resolution was confirmed subsequently by superficial ultrasound examination at 3 months.

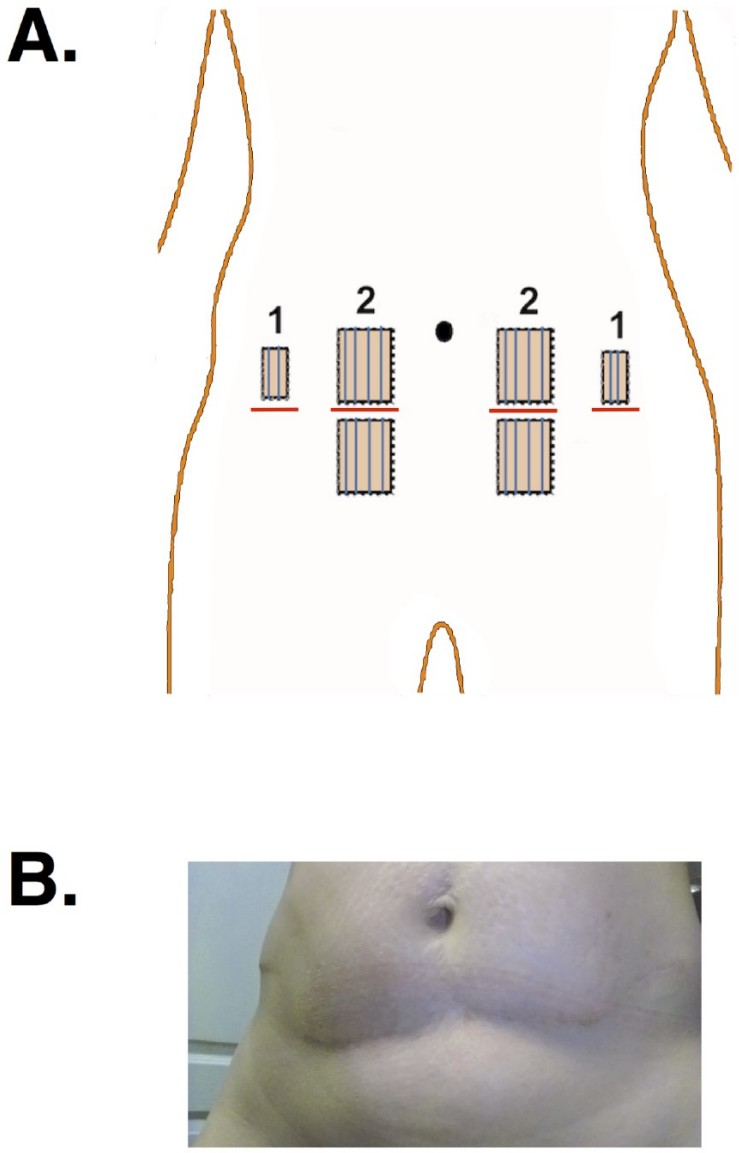


Figure 5.3 (**A**) Illustrative diagram showing surgical sites for implantation of Cell Pouch™ devices in subject three. 1. Site of the lateral sentinel pouches placed above the iliac crests. 2. A total of four therapeutic pouches placed in pairs above and below each transverse incision on the lower anterior right and left abdominal wall. Note: Cell Pouches™ are not scaled to size. (**B**) 2-year post-transplant photograph of the anterior abdominal wall of subject 3.

This subject was transplanted 130 days after pouch implantation, when extensive device incorporation and vascularization was observed. Two donor islet preparations become available simultaneously with cumulative islet mass of 1,294,900 IEQ and these were distributed evenly across all four major pouches, with a small fraction allocated to the two laterally placed sentinel devices. Approximate comparison of islet density per plug channel is outlined in **Table 5.1**. Post-transplant immunosuppression was similar to the previous cases, but antibiotic prophylaxis was extended to 7 days. The transplant procedure concluded without complications.

The two sentinel pouches were excised as per protocol, for histological assessment 6 weeks after transplantation. Histologic and immunohistochemical analysis of the sentinel pouches confirmed neo-angiogenesis and patchy islet fragments present within the device, staining positively for insulin and glucagon (**Figure 5.4A-C**) and no evidence of immune cell infiltration by CD3 staining (**Figure 5.5**). The examination also identified that exocrine tissue (as identified by pancreatic amylase and CK-19 staining) was abundant to a variable degree (**Figure 5.6**).

5.6. - SERIAL C-PEPTIDE MONITORING AND SUBSEQUENT COURSE

All three subjects experienced acute, short-lived peaks in serum C-peptide levels within the initial 24h post-transplant, most evident in subject 3 that received the largest islet mass (**Figure 5.7A,B**). All three subjects remained C-peptide negative after post-transplant day 2.

The three subjects made a complete recovery without long-term sequelae, and the second and third subject were relisted for 'standard-of-care' intraportal islet transplantation outside of this research protocol. Subject 1 chose not to participate in further islet transplants, remained C-peptide negative, and has not been followed actively (**Figure 5.8A,B**).

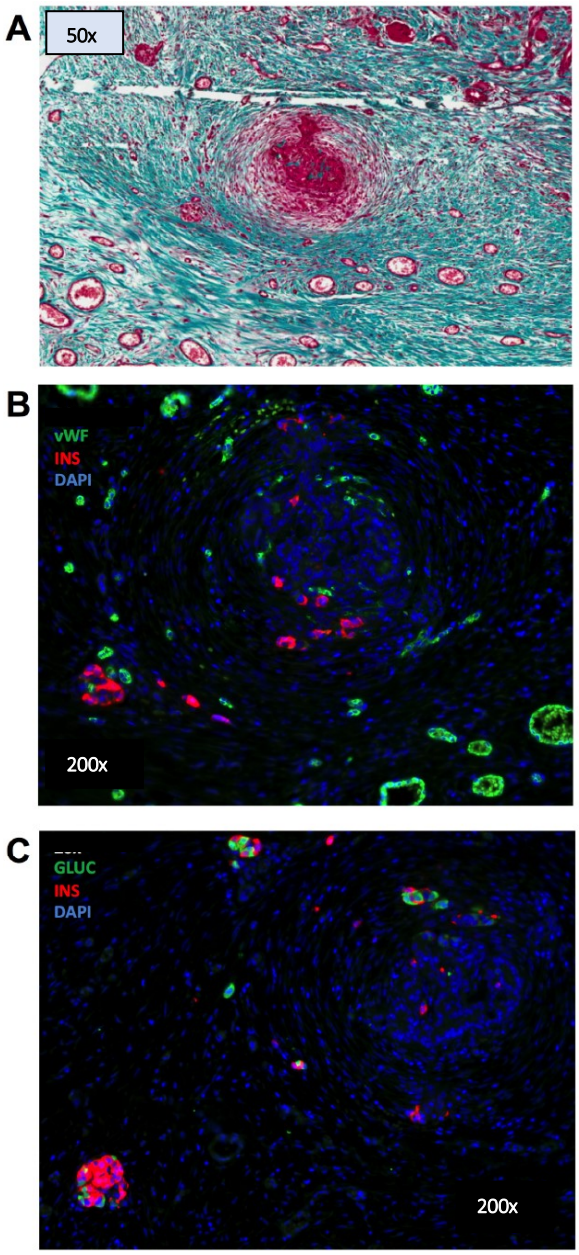


Figure 5.4 Selected histology and immunohistochemistry from the sentinel devices explanted at 30 days post-transplant in subject 3, showing surviving islets surrounded by tissue and new blood vessels within the device. **A.** Masson's trichrome stain at 50x magnification showing location of the transplanted cells within the channel lumen; **B.** Positive immunofluorescence staining for insulin/vWF/DAPI at 200x magnification (insulin red (INS), von Willebrand Factor green (vWF), 4',6-diamidino-2-phenylindole blue; **C.** Glucagon green (GLUC), INS (red) and DAPI (blue) at 200x magnification. /DAPI antibodies.

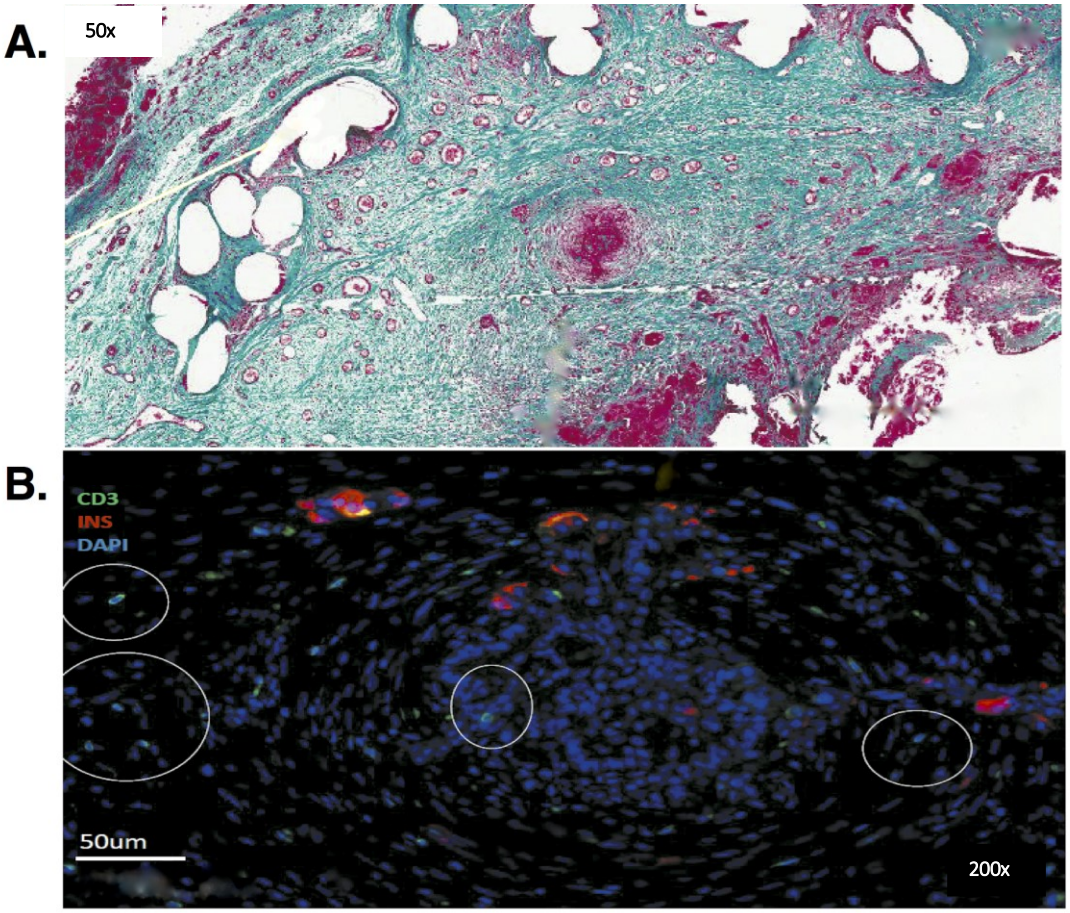


Figure 5.5 A. Masson's trichrome stain of a selected histology image of a Cell Pouch™ explanted after day 30 post-transplant from Subject 3, showing outer device tines (large white voids in upper portion of slide) with a portion of an islet graft seen centrally (50x magnification); **B.** Immunofluorescence staining for CD3 (green); insulin (INS, red) and DAPI (blue), demonstrating no evident T-cell infiltrate (200x magnification). White circles highlight occasional CD3 positive cells.

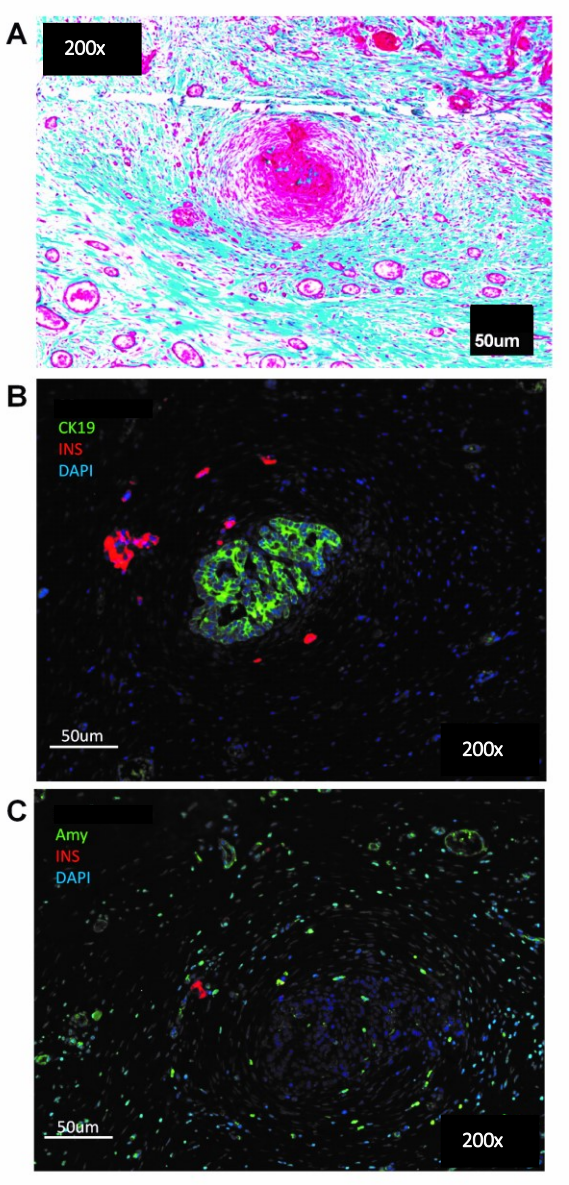


Figure 5.6 Evidence for survival of pancreatic exocrine tissue and ductal elements (CK19 positive) within the sentinel devices explanted from Subject 3. **A.** Masson's trichrome stain of a selected histology image of a Cell Pouch™ explanted after day 30 post-transplant from Subject 3 (200x magnification); **B.** Selected section stained by immunofluorescence for CK-19 (green), insulin (INS, red) and DAPI (blue) (200x magnification); **C.** Immunostaining for Amylase (Amy (green), insulin (INS, red) and DAPI (blue) (200x magnification).

Subject 2 received a first intraportal islet transplant 11 months after the subcutaneous procedure and promptly attained positive C-peptide status with marked reduction in exogenous insulin requirement and correction of HbA1C. A second intraportal islet infusion was given at 16 months, which rendered her insulin free (**Figure 5.8C,D**). Subject 3 was the only patient in the study to reach the 3-month efficacy assessment. Evidence of graft function was not observed at any time point up to and beyond 4 months post-transplant in this subject, as determined by serial measurement of mixed meal stimulated C-peptide (Ensure^{TM}), decrease in insulin requirement, or protection from hypoglycemia. While a decrement in HbA1C was noted, in the absence of C-peptide this likely reflects more optimized glycemic and insulin monitoring rather than graft function *per se* (**Figure 5.8E,F**).

Subject 3 underwent two subsequent intraportal islet infusions on day 131 and 202 after the subcutaneous intervention, and promptly achieved attained C-peptide status, correction of HbA1C and periods of insulin independence (**Figure 5.8E, F**).

All subjects remained non-sensitized with negative panel reactive antibody (PRA) following device transplantation, and there were no donor-specific antibodies identified directed against any of the subcutaneous donors.

Finally, other than minor superficial scars, no major aesthetic concerns were apparent as a consequence of the pouch implantation, cell transplants or sentinel retrievals. Indeed, subject 3 still has four 8-plug pouches *in situ* at 24 months post-surgery with no safety sequelae (**Figure 5.3B**).

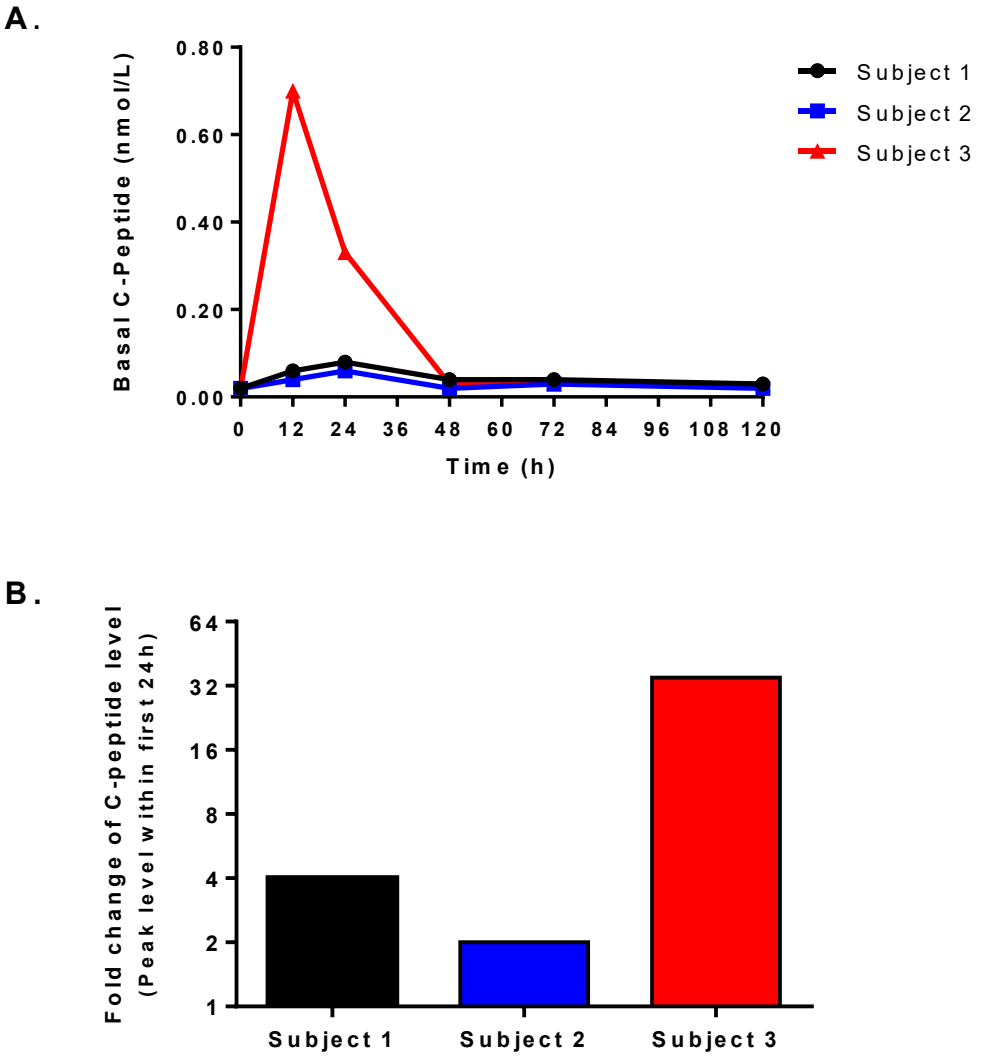


Figure 5.7 Early post-transplant basal levels of C-peptide. **A.** Post-transplant levels of C-peptide for all three subjects showing peak increase within the first 24h, most substantial in Subject 3 who received a larger transplant dose; **B.** Corresponding fold-change in basal C-peptide levels for all subjects seen within the initial 24h post-transplant.

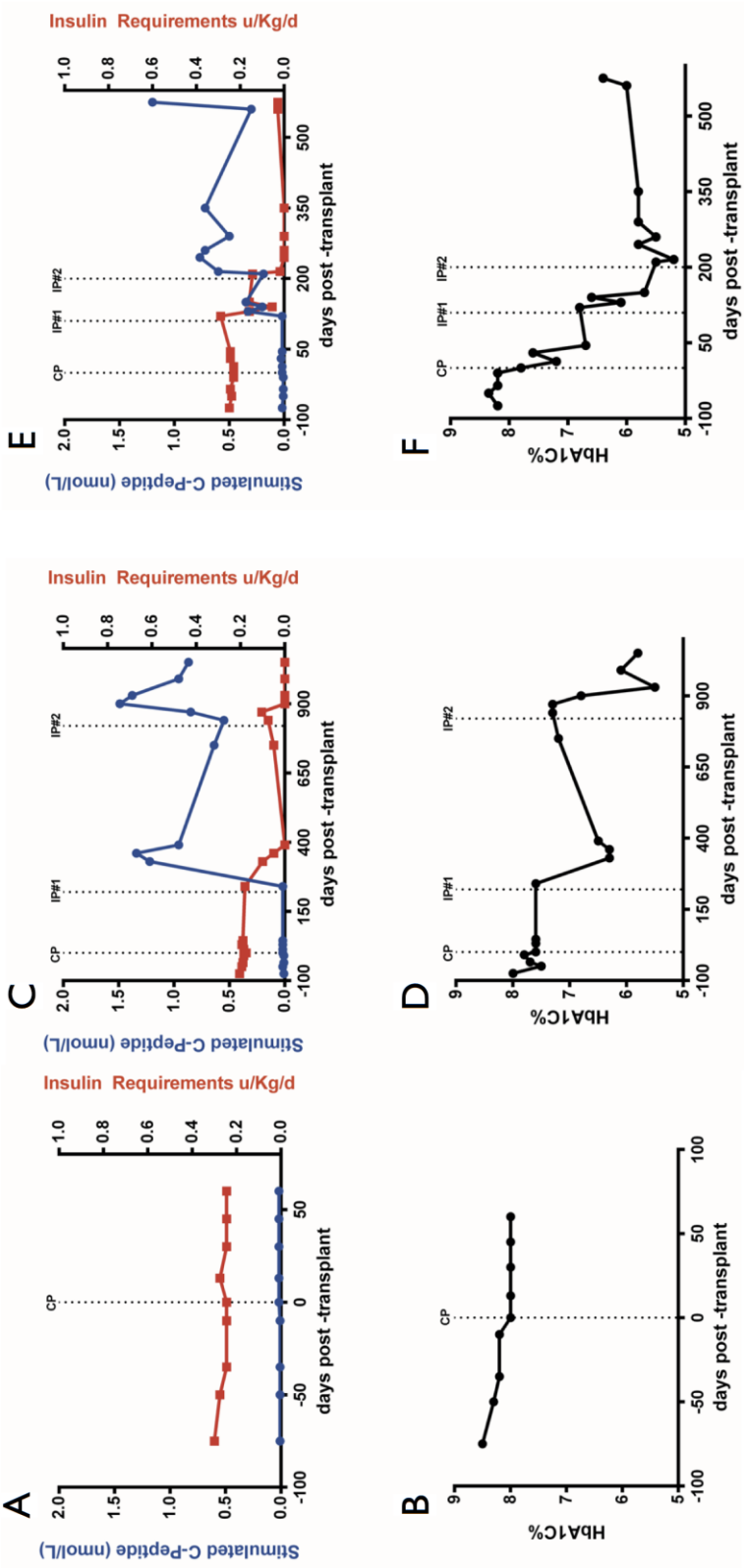

A
Stimulated C-Peptide (nmol/L)
2.0
1.5
1.0
0.5
0.0
-100
-50
0
50
CP
days post -transplant
B
HbA1C%
9
8
7
6
5
-100
-50
0
50
100
CP
days post -transplant
C
Insulin Requirements u/Kg/d
1.0
0.8
0.6
0.4
0.2
0.0
CP
IP#1
IP#2
-100
150
400
650
900
days post -transplant
Stimulated C-Peptide (nmol/L)
D
HbA1C%
CP
IP#1
IP#2
-100
150
400
650
900
days post -transplant
E
Insulin Requirements u/Kg/d
CP
IP#1
IP#2
-100
50
200
350
500
days post -transplant
Stimulated C-Peptide (nmol/L)
F
HbA1C%
CP
IP#1
IP#2
-100
50
200
350
500
days post -transplant

Figure 5.8 Pre and post-transplant function of the 3 study subjects as determined by stimulated C-peptide response to a mixed meal challenge (Ensure™), daily insulin requirement and glycated hemoglobin over time. There was no detectible function of islets contained within any of the Cell Pouches™ in any of three subjects, but Subjects 2 and 3 subsequently received two intraportal islet infusions and both became promptly C-peptide positive, insulin independent for a period, and corrected hemoglobin A1C (HbA1C). **A, B**. Data from Subject 1; **C, D.** Data from Subject 2; **E, F.** Data from Subject 3. CP: Cell Pouch™; IP#1: first intraportal transplant; IP#2: second intraportal transplant. HbA1C: hemoglobin A1C.

5.7. – DISCUSSION

We herein report our initial first-in-human experience with a pre-vascularized subcutaneous islet transplant device, Sernova's Cell Pouch™, in a single center phase I/II study. The results clearly show the device and surgical approach to be relatively safe. Minor wound complications occurred in all 3 cases, including cellulitis and seroma, and either resolved spontaneously over time (third subject) or following device explantation (first and second subject). An important observation was the clear histologic identification of human islets staining positively for insulin, glucagon and somatostatin within the pouch, with incorporated neovascular ingrowth in all cases at different time points. Although the human islet distribution was patchy and found only in limited sections, where present, cells were surrounded by viable stroma and demonstrated no features of immune rejection or infection.

While the device materials have been shown by the manufacturer to be biocompatible in small and large animal models, the occurrence of seroma in human subjects was not predicted in previous studies, perhaps suggesting attenuated foreign-body reaction responses in human subjects with longstanding diabetes (18). Based on our preliminary observations especially in subject 3, we would advocate delaying implantation of viable cellular material until the devices have become fully incorporated.

The lack of demonstrable graft functionality in this preliminary experience, an important secondary endpoint, is disappointing. We have closed enrollment of further subjects based on this finding. In our own preclinical studies with a sentinel-sized Cell Pouch^{TM}, it took approximately 20 days with full islet mass, and 40 days with a marginal mass to fully reverse diabetes in mice (16). However a steady improvement in glycemic control was observed within

the initial 5-10 days of transplantation in these mice (16). These findings contrast with our routine clinical experience with intraportal islet transplantation in diabetic patients, where measurable C-peptide function, marked decrease in insulin requirement or insulin independence with stabilization of hypoglycemic events occur within days of transplantation (**Figure 5.8**). Due to pre-emptive device explantation within 15 and 30 days in the first 2 cases, insulin independence was not expected, but a decrement in insulin requirement and the presence of detectable C-peptide should have occurred. Further developmental work will be required to determine the functional utility of this approach. Subject 3 maintained therapeutic Cell Pouches™ containing a substantial islet mass for 131 days before intraportal islet transplantation intervened (**Figure 5.8**). The presence of measurable C-peptide in all 3 subjects within the initial 24 hours, and the high peak observed in subject 3 (**Figure 5.7**), is consistent with substantial acute islet demise during the initial engraftment period, with passive insulin and C-peptide release.

The contrast between lack of detectable function and histologic survival suggests, especially in the light of the acute C-peptide release at 24 hours, that the vast majority of the transplanted islets failed to survive the engraftment process. The patchy finding of occasional islets is consistent. The study was not designed to assess precise, longitudinal, histological quantification of islet cell survival, but there was clearly a marked discrepancy between the number transplanted and number surviving. The optimal islet density per device channel still remains to be defined for human islets in patients, and it seems likely that in three cases we may have overwhelmed the capacity of the Cell Pouch to provide oxygen and nutrient exchange (**Table 5.1**).

The presence of contaminating exocrine and ductal components may be an important differentiating factor between Cell Pouch™ studies in mice and patients. The human islet preparations selected for Subjects 1 and 2 were of high purity. The third subject received a larger islet mass distributed across almost twice as many channels, but also contained more substantial exocrine pancreatic tissue (**Table 5.1**). While such a preparation is generally accommodated within the intraportal space, this may have further contributed to cellular hypoxia and demise within the limited confines of the device. Of interest, Subject 3 was found to have surviving exocrine tissue in the sentinel Cell Pouch™ (**Figure 5.6**), in contrast to intraportal islet transplantation where exocrine tissue is not routinely found (19).

The pre-vascularized subcutaneous Sernova Cell Pouch™ approach offers a potentially retrievable site for human islet implantation, and future application of embryonic or adult stem cell-derived expanded beta cells in diabetes. Based on our limited preliminary experience, and acknowledging technical challenges in our early learning curve, the current device and surgical techniques likely require further modification to optimize accommodation of functional cells. Prevascularization of the subcutaneous site through a variety of approaches may transform a non-favorable site for therapeutic cellular engraftment, thereby broadening future potential cell transplant approaches in regenerative medicine.

5.8. – REFERENCES

1. 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.

2. Shapiro AM. State of the art of clinical islet transplantation and novel protocols of immunosuppression. Curr Diab Rep 2011;11(5):345-354.

3. Eich T, Eriksson O, Lundgren T. Visualization of early engraftment in clinical islet transplantation by positron-emission tomography. N Engl J Med 2007;356(26):2754-2755.

4. Blum B, Benvenisty N. The Tumorigenicity of Human Embryonic Stem Cells. In: George FVW, George K, (eds). Adv Cancer Res 2008: 133-158.

5. 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.

6. Juang JH, Hsu BR, Kuo CH. Islet transplantation at subcutaneous and intramuscular sites. Transplant Proc 2005;37(8):3479-3481.

7. Rafael E, Tibell A, Rydén M, Lundgren T, Sävendahl L, Borgström B et al. Intramuscular Autotransplantation of Pancreatic Islets in a 7-Year-Old Child: A 2-Year Follow-Up. Am J Transplant 2008;8(2):458-462.

8. Sakata N, Aoki T, Yoshimatsu G, Tsuchiya H, Hata T, Katayose Y et al. Strategy for clinical setting in intramuscular and subcutaneous islet transplantation. Diabetes Metab Res Rev 2013.

9. Maffi P, Balzano G, Ponzoni M, Nano R, Sordi V, Melzi R et al. Autologous Pancreatic Islet Transplantation in Human Bone Marrow. Diabetes 2013;62(10):3523-3531.

10. Juang JH, Bonner-Weir S, Ogawa Y, Vacanti JP, Weir GC. Outcome of subcutaneous islet transplantation improved by polymer device. Transplantation 1996;61(11):1557-1561.

11. Kawakami Y, Iwata H, Gu YJ, Miyamoto M, Murakami Y, Balamurugan AN et al. Successful subcutaneous pancreatic islet transplantation using an angiogenic growth factor-releasing device. Pancreas 2001;23(4):375-381.

12. Kawakami Y, Iwata H, Gu Y, Miyamoto M, Murakami Y, Yamasaki T et al. Modified subcutaneous tissue with neovascularization is useful as the site for pancreatic islet transplantation. Cell transplant 2000;9(5):729-732.

13. Sakata N, Sumi S, Yoshimatsu G, Goto M, Egawa S, Unno M. Encapsulated islets transplantation: Past, present and future. World J Gastrointest Pathophysiol 2012;3(1):19-26.

14. Ford O. Sernova gets approval to start Cell Pouch™ clinical trial in Canada. Medical Device Daily;16(90).

15. Kriz J, Vilk G, Mazzuca DM, Toleikis PM, Foster PJ, White DJ. A novel technique for the transplantation of pancreatic islets within a vascularized device into the greater omentum to achieve insulin independence. Am J Surg 2012;203(6):793-797.

16. Pepper AR, Pawlick R, Gala-Lopez B, MacGillivary A, Mazzuca DM, White DJ et al. Diabetes Is Reversed in a Murine Model by Marginal Mass Syngeneic Islet Transplantation Using a Subcutaneous Cell Pouch Device. Transplantation 2015;99(11):2294-2300.

17. Ryan EA, Shandro T, Green K, Paty BW, Senior PA, Bigam D et al. Assessment of the severity of hypoglycemia and glycemic lability in type 1 diabetic subjects undergoing islet transplantation. Diabetes 2004;53(4):955-962.

18. Socarras TO, Vasconcelos AC, Campos PP, Pereira NB, Souza JP, Andrade SP. Foreign body response to subcutaneous implants in diabetic rats. PloS One 2014;9(11):e110945.

19. Toso C, Isse K, Demetris AJ, Dinyari P, Koh A, Imes S et al. Histologic graft assessment after clinical islet transplantation. Transplantation 2009;88(11):1286-1293.

CHAPTER 6.

A PREVASCULARIZED SUBCUTANEOUS DEVICE-LESS SITE FOR ISLET AND CELLULAR TRANSPLANTATION

6. - A prevascularized subcutaneous device-less site for islet and cellular transplantation

nature biotechnology

ARTICLES

nature biotechnology

A prevascularized subcutaneous device-less site for islet and cellular transplantation

Andrew R Pepper[1], Boris Gala-Lopez[1], Rena Pawlick[1], Shaheed Merani[1], Tatsuya Kin[1,2] & A M James Shapiro[1,2]

Transplantation of donor-derived islets into the liver is a successful cellular replacement therapy for individuals with diabetes. However, the hepatic vasculature is not an optimal transplant site for several reasons, including graft attrition and the inability to retrieve or image the islets. Here we describe islet transplantation into a prevascularized, subcutaneous site created by temporary placement of a medically approved vascular access catheter. In mice with streptozotocin (STZ)-induced diabetes, transplantation of ~500 syngeneic islets into the resulting 'device-less' space reversed diabetes in 91% of mice and maintained normoglycemia for >100 days. The approach was also effective in mice with pre-existing diabetes, in another mouse strain that mounts a more vigorous inflammatory response, and across an allogeneic barrier. These results demonstrate that transient priming of a subcutaneous site supports diabetes-reversing islet transplantation in mouse models without the need for a permanent cell-encapsulation device.

© 2015 Nature America, Inc. All rights reserved.

Cellular transplantation is an attractive and growing treatment strategy for a variety of disease processes, including diabetes, Parkinson's disease, myocardial ischemia, stroke, metabolic liver disease and hemophilia. A prototypic example of cellular replacement therapy is intrahepatic transplantation of donor-derived pancreatic islets of Langerhans in individuals with type 1 diabetes mellitus who have unstable glucose control. The 'Edmonton protocol' for administering this therapy achieved high rates of insulin independence[1]. Initial long-term analysis indicated that insulin independence was not durable, as most recipients eventually returned to requiring moderate amounts of insulin, although they remained protected from recurrent hypoglycemia[2]. Recent results from six islet centers suggest marked improvement in durable graft function, with insulin independence now seen in more than half of recipients at five years after transplantation[3]. However, the procedure often results in acute or gradual graft attrition, and carries risks of bleeding, thrombosis and localized steatosis. Moreover, intrahepatic transplantation does not permit imaging or retrieval of donor islets. The ability to retrieve the graft is especially important for current efforts to replace donor-derived islets with cells produced from human pluripotent stem cells, which may have unwanted effects. These considerations suggest that the liver is not the optimal site for islet transplantation[4,5].

Islets isolated for transplantation have lost their natural vascularized and specialized extracellular matrix[6,7] and, for successful engraftment, must receive nutritional and physical support from the host through the formation of new blood vessels around and within the graft. The density of newly formed vessels after transplantation is much lower than that in native islets[8,9], irrespective of whether islets are delivered to the human liver, kidney or spleen[9]. Research on the development of alternative transplant sites[9–11] (**Supplementary Tables 1** and **2**) has suggested that an optimal site for islet transplantation should (i) have an adequate tissue volume capacity, (ii) be in close proximity to vascular networks, ensuring a sufficient oxygen supply to the graft before revascularization, (iii) allow for dynamic communication between the graft and the systemic circulation in a physiologically relevant manner, (iv) facilitate minimally invasive methods to transplant, biopsy and retrieve the graft, and (v) elicit minimal inflammation to reduce immunogenicity and promote long-term graft survival[11]. In theory, subcutaneous transplantation should be superior to portal vein infusion as it provides ready access to the graft and the possibility of monitoring function through imaging[12–14]. However, islet transplantation into an unmodified subcutaneous site has never reversed diabetes in animals or humans as the microenvironment is inhospitable to cell survival owing to poor oxygen tension and inadequate vascularization[15]. Stimulation of angiogenesis is critical to successful subcutaneous islet transplantation[9,11,14,16]. Oxygen generators, polymers, meshes, encapsulation devices, matrices, growth factors (including fibroblast growth factor, hepatocyte growth factor and vascular endothelial growth factor) and co-transplantation of mesenchymal stem cells have all been explored with variable success (**Supplementary Table 1**).

Strategies for subcutaneous transplantation that rely on biomaterials often fail because of the foreign-body and inflammatory reaction—a complex, dynamic process that can persist for the lifetime of the implant[17]. Physical contact of the implant with host blood, lymph, exudate or other fluids triggers an instant inflammatory response that leads to spontaneous adsorption to the biomaterial of host blood proteins, including albumin, fibrinogen, complement, fibronectin and γ-globulin[17–19]. Host cells responsible for wound healing encounter this layer and release cytokines, chemokines, reactive oxygen species and other enzyme products that recruit tissue-resident macrophages and undifferentiated monocytes to the wound site[17–19]. As macrophages

[1]Clinical Islet Transplant Program, Alberta Diabetes Institute, University of Alberta, Edmonton, Alberta, Canada. [2]Department of Surgery, University of Alberta, Edmonton, Alberta, Canada. Correspondence should be addressed to: A.M.J.S. (amjs@islet.ca).

Received 6 October 2014; accepted 12 March 2015; published online 20 April 2015; doi:10.1038/nbt.3211

Note: A version of this section was published as an Original Paper in *Nature Biotechnol* 2015 May; 33(5): 518 - 23.

ORIGINAL PAPER

A Prevascularized Subcutaneous Device-Less Site for Islet and Cellular Transplantation

Andrew R. Pepper,[1] Boris Gala-Lopez,[1] Rena Pawlick,[1] Shaheed Merani,[1] Tatsuya Kin, [1, 2] A. M. James Shapiro[1, 2]

[1] *Clinical Islet Transplant Program, Alberta Diabetes Institute, University of Alberta, Edmonton, AB, Canada*

[2] *Department of Surgery, University of Alberta, Edmonton, AB, Canada*

Correspondence to: A.M. James Shapiro, **MD, PhD, Professor, Director of Clinical** Islet **and** Living Donor Liver Transplant Programs, Clinical Islet Transplant Program, University of Alberta. 2000 College Plaza, 8215-112th St, Edmonton T6G 2C8, Alberta, Canada. amjs@islet.ca

Telephone: +1-780-4077330, **Fax:** +1-780-4078259

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.

6.1. – ABSTRACT

Transplantation of donor-derived islets into the liver is a successful cellular replacement therapy for individuals with diabetes. However, the hepatic vasculature is not an optimal transplant site for several reasons, including graft attrition and the inability to retrieve or image the islets. Here we describe islet transplantation into a prevascularized, subcutaneous site created by temporary placement of a medically approved vascular access catheter. In mice with streptozotocin (STZ)-induced diabetes, transplantation of ~500 syngeneic islets into the resulting 'device-less' space reversed diabetes in 91% of mice and maintained normoglycemia for >100 days. The approach was also effective in mice with pre-existing diabetes, in another mouse strain that mounts a more vigorous inflammatory response, and across an allogeneic barrier. These results demonstrate that transient priming of a subcutaneous site supports diabetes-reversing islet transplantation in mouse models without the need for a permanent cell-encapsulation device.

6.2. – INTRODUCTION

Cellular transplantation is an attractive and growing treatment strategy for a variety of disease processes, including diabetes, Parkinson's disease, myocardial ischemia, stroke, metabolic liver disease and hemophilia. A prototypic example of cellular replacement therapy is intrahepatic transplantation of donor-derived pancreatic islets of Langerhans in individuals with type 1 diabetes mellitus who have unstable glucose control. The 'Edmonton protocol' for administering this therapy achieved high rates of insulin independence (1). Initial long-term analysis indicated that insulin independence was not durable, as most patients eventually returned to requiring moderate amounts of insulin, although they remained protected from recurrent hypoglycemia (2). Recent results from six islet centers suggest marked improvement in durable graft function, with insulin independence now seen in more than half of recipients at five years after transplantation (3). In addition to the possible occurrence of acute or gradual graft attrition, the procedure carries risks of bleeding and thrombosis, and of localized steatosis. These considerations suggest that the liver may not be the optimal site for transplanting insulin-secreting cells (4, 5). For successful intrahepatic transplantation, islets must receive nutritional and physical support from the hepatic vascular space, as they are devoid of their natural vascularized and specialized extracellular matrix (6, 7). To regain proper islet function, new blood vessels must form around and within the graft, however, the density of newly formed vessels is much lower than in native islets (8, 9), irrespective of whether islets are transplanted into the human liver, the kidney or the spleen (9). Moreover, intrahepatic transplantation does not permit graft retrieval, an important requirement when transplanting stem-cell derived insulin-secreting cell populations, which could lead to unwanted effects such as teratoma formation.

For these reasons, and as new, alternative stem cell derived insulin-secreting cells become available, substantial research efforts have been dedicated to the pursuit of alternative transplant sites as retrievability becomes an imperative requirement, (9-11) (**Tables 6.1 and 6.2**).

It has been suggested that an optimal site should: 1) have an adequate tissue volume capacity, 2) be in close proximity to vascular networks, ensuring a sufficient oxygen supply to the graft before revascularization, 3) allow for dynamic communication between the graft and the systemic circulation in a physiologically relevant manner, 4) facilitate minimally invasive means to transplant, biopsy and retrieve the graft, and 5) elicit minimal inflammation to reduce immunogenicity and promote long-term graft survival (11). In theory, subcutaneous transplantation should be superior to portal vein infusion as it provides ready access to the graft and the possibility of monitoring function through imaging (12-14). However, transplantation of islets into an unmodified subcutaneous site has universally failed to reverse diabetes in animal models or in humans owing to poor oxygen tension and inadequate vascularization (15). Stimulation of angiogenesis is critical to successful subcutaneous islet transplantation (9, 11, 14, 16). Oxygen generators, polymers, meshes, encapsulation devices, matrices, growth factors (including fibroblast growth factor, hepatocyte growth factor and vascular endothelial growth factor) and co-transplantation of mesenchymal stem cells have all been explored with variable success (**Table 6.1**).

Strategies for subcutaneous transplantation that rely on biomaterials often fail because of the foreign-body and inflammatory reaction, a complex, dynamic process that can persist for the lifetime of the implant (17). Physical contact of the implant with host blood, lymph, exudate or other fluids triggers an instant inflammatory response that leads to spontaneous adsorption to the biomaterial of host blood proteins, including albumin, fibrinogen, complement, fibronectin and

γ-globulin (17-19). Host cells responsible for wound healing encounter this layer and release cytokines, chemokines, reactive oxygen species and other enzyme products that recruit tissue-resident macrophages and undifferentiated monocytes to the wound site (17-19).

As macrophages engage the biomaterial, they form foreign-body giant cells. These cells secrete signaling molecules (e.g., IL-1, IL-6, IL-10, IL-12, TNF-α, TGF-β), attracting fibroblasts, which secrete collagen during cellular proliferation and neovascularization (17, 20). It has been theorized that in the early stages of the foreign body reaction, cellular infiltrates are confronted with a reduced oxygen tension environment, stimulating macrophages to activate hypoxia-inducible factors and in turn induces the expression of pro-angiogenic factors including vascular endothelial growth factors and platelet-derived growth factors driving the neovascularization process (21).

Table 6.1 Translation of subcutaneous islet transplantation therapies. Summary of experimental approaches used to optimize the subcutaneous space for experimental transplantation.

Author	Year	Transplant Model	Intervention	Reference
Juang JH	1996	Mouse	Prevascularization by implantation of polymer	(1)
Tatarkiewicz K	1999	Mouse	Subcutaneous transplantation of macroencapsulated islets	(2)
Kawakami Y	2000	Rat	Prevascularization by basic fibroblast growth factor	(3)
Wang W	2002	Nude Mouse (Xenotransplant)	Bioartificial pancreas in a prevascularized subcutaneous site	(4)
Yang Z	2002	NOD Mouse	Islet xenografts in the Theracyte device	(5)
Bharat A	2005	Mouse (Xenotransplant)	Use of Matrigel	(6)
Pileggi	2006	Rat	Prevascularization by implantation of polytetrafluoroethylene	(7)
Sörenby AK	2008	Mouse	Pre-implantation of an immunoprotective device to lower the curative islet dose	(8)
Perez-Basterrechea M	2009	Rat	Islets containing Plasma-fibroblast gel	(9)
Dufrane D	2010	Cynomologous Monkey (Xenotransplant)	Alginate macroencapsulated pig islets in primates	(10)
Ludwig B	2010	Porcine	Immunoprotective device with oxygen-refuelling macrochamber, 'βAir'	(11)
Kim J	2012	Mouse	Fibrin / islet composites as a scaffold	(12)
Ludwig B	2012	Rats	Immunoprotective device with enhanced oxygen supply and growth hormone release agonist	(13)
Vériter S	2013	Primates	Vascularized bioartificial pancreas bioby co-encapsulation of pig islets and mesenchymal stem cells in primates	(14)
Ludwig B	2013	Human Allograft	Immune-isolating device with enhanced oxygen supply	(15)

Table 6.2 Islet transplantation sites. Summary comparison of advantages and disadvantages of alternative sites for islet implantation.

Transplant Site	Advantage	Disadvantage	Evidence
Intra-Portal/Portal Vein	• Surgical accessible • Minimally invasive • Physiological glyco-insular response (portal insulin delivery) • Safety established clinically • Only site to reliably provide insulin independence in human subjects treated with islet transplants to date	• Early graft loss due to instant blood mediated inflammatory response (IBMIR) • Immune exposure • Local transplant induced hypoxia • Risk of hemorrhage and thrombosis • Risk to host organ (liver) • Limited ability to biopsy islets • Inability to retrieve • Limited imaging capability • Limited transplant volume capacity	(16-20)
Renal Subcapsular	• Well established efficacy pre-clinically (but only in mice/rats) • Highly vascularized • Avoidance of IBMIR • Robust evidence in rodent models	• Surgically invasive • Systemic (non-portal), non-physiologic glyco-insular response • Hypoxia • Immune exposure • Limited ability to biopsy or retrieve • Limited transplant volume capacity • Cannot be translated due to differences in renal capsule between rodents and human	(21-24)
Omentum/Intraperitoneal	• Physiological glyco-insular response (portal insulin delivery) • Vascularized • Immune privileged (only when islets encapsulated) • Large transplant volume capacity • Surgically accessible with minimal access approach	• Limited ability to biopsy and retrieve • Limited ability to image • Moderately invasive • Provokes inflammatory response	(25-28)
Pancreas	• Native highly vascular • Physiological glyco-insular response • High oxygen tension	• Surgically invasive • Provokes inflammatory reaction • Risk to host organ • Pancreatic fistula, necrosis of high risk to recipient • Limited ability to biopsy and retrieve • Limited ability to image • Limited transplant volume capacity • Local autoimmunity	(29-31)
Spleen	• Highly vascular • Physiological glyco- insular Response • Good evidence in canine studies	• Exposure to IBMIR • Risk of hemorrhage, thrombosis – patient deaths described • Limited ability to biopsy and retrieve • Limited transplant volume capacity • Immune exposure	(32-34)
Gastric Submucosa	• Physiological glyco-insular response • Avoidance of IMBIR • Accessible by gastroscopy • Minimally invasive • Large transplant volume capacity • High oxygen tension • Capacity to biopsy	• Surgical risk (gastric perforation) • Limited ability to retrieve transplant	(35-37)
Bone Marrow	• Highly vascularized	• High immune exposure • Invasive • Non-physiological glyco-insular response • Limited ability to biopsy and retrieve • Limited ability to image • Limited transplant volume capacity	(38, 39)
Brain	• Highly vascularized • Immune privileged	• Invasive • Risk of cerebral ischemia • Non-physiological glyco-insular response • Inability to biopsy and retrieve • Limited transplant volume capacity	(40-42)
Testis	• Immune privileged	• Non-physiological glyco-insular response • Invasive • Limited ability to biopsy and retrieve • Risk of host organ complication • Limited transplant volume capacity • Therapy only translatable to the male population	(43, 44)

Table 6.2 Islet transplantation sites. Continued

Transplant Site	Advantage	Disadvantage	Evidence
Thymus	• Immune privileged • Efficacy demonstrated in pre-clinical models • Surgically accessible • Possibility of immunological reset to auto and allo-islet antigens • Avoidance of IMBIR	• Requirement of large quantities of islets • Non-physiological glyco-insular response • Risk of host organ complications • Limited transplant volume capacity • Thymic involution after the neonatal period	(45-48)
Anterior Chamber of Eye	• Immune privileged • Efficacy demonstrated in non-human primates • Avoidance of IMBIR • Ability to image	• Non-physiological glyco-insular response • Risk of host organ complication (blindness) • Limited transplant capacity • Pre-existing retinopathy in diabetic population	(49)
Intramuscular	• Non-invasive transplantation • Ability to retrieve graft • High oxygen tension • Limited efficacy in clinical autotransplantation • Non-invasive imaging capacity	• Non-physiological glyco-insular response • Limited transplant volume capacity • Susceptibility to excessive fibrosis	(50-54)
Subcutaneous	• Non-invasive transplantation • Ability to retrieve graft • Ability to biopsy • Non-invasive imaging capacity • Large transplant volume capacity • Proximity to blood supply	• Non-physiological glyco-insular response • Low oxygen tension • Hypoxic environment • Requirement for prevascularization (e.g. devices, exogenous oxygen supply, over-expression of angiogenesis growth factors-VEGF, MSC) • Chronic inflammatory response with implanted devices • Free islets fail to function post-transplant	(2, 4, 7, 13-15, 55-58)
Subcutaneous 'Device-less'	• Non-invasive transplantation • Accessibility to biopsy or graft retrieval • Non-invasive imaging capability • Large transplant volume capacity • Proximity to blood supply • Avoidance of IMBIR and chronic inflammatory response • Avoidance of exogenous oxygen supply and growth factors	• Non-physiological glyco-insular response • Delayed engraftment period	(59)

Within several weeks, a dense collagenous fibrotic capsule forms around the implant, isolating it from the host (17, 22). The fibrotic capsule hinders metabolic exchange, cell signaling, healing, tissue-device integration, and increases the risk of bacterial infection (17, 22).

Here we report a subcutaneous device-less (DL) transplant technique that enables successful transplantation of mouse or human islets in mice. The approach was designed to harness the innate foreign-body response in a controlled manner to induce local neovascularization favorable to islet survival and function. A hollow nylon catheter is implanted subcutaneously, inducing a foreign-body response, and withdrawn after one month. Removal promptly extinguishes the foreign-body response, leaving a transplant space lined with neovessels **(Figure 6.1 and Figure 6.2)**. Transplantation of islets into this space facilitates reversal of diabetes without the need for a permanent device or exogenous growth factors.

6.3. – METHODS

6.3.1. - Creation of the DL subcutaneous transplant site

A common ability of all cellular transplant based devices or transplant strategies is the formation of a vascularized scaffold to house the transplanted tissue (11). We aimed to harness the natural foreign-body response elicited by medically approved vascular catheters, as a means to develop a DL transplant site. This technique transforms the tissue under the skin from a hypoxic and avascular space, into a densely vascularized cellular graft-supporting matrix.

Three to six weeks prior to islet transplant, 2 cm segments of a 5-French (Fr.) textured nylon radiopaque angiographic catheter (Torcon NB® Advantage Beacon® tip Cook Medical, Indiana,

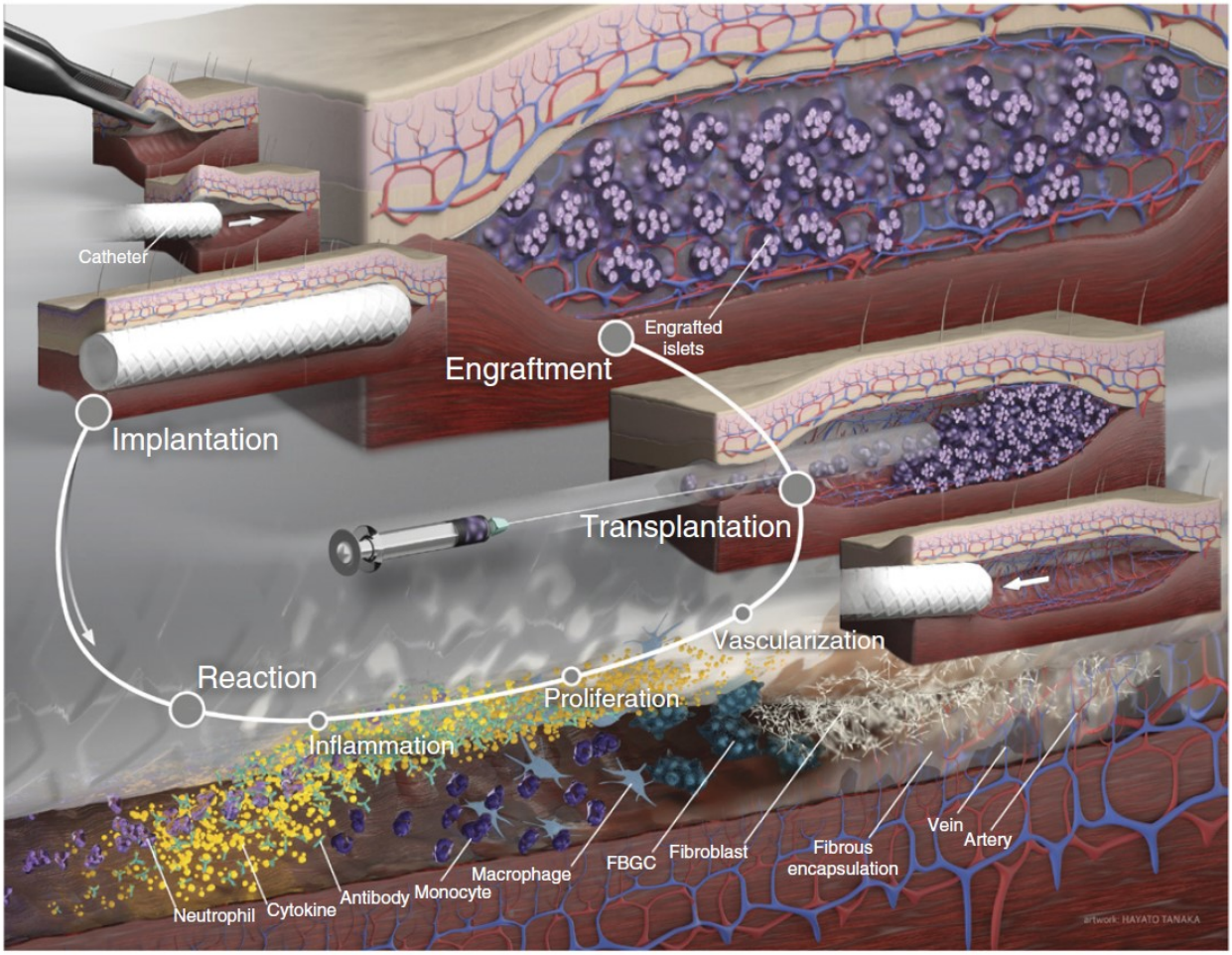


Figure 6.1 Design and characteristics of the subcutaneous DL cellular transplant site. Artwork by Hayato Tanaka.

USA) or a 6.5-French smooth silicone catheter (Cook Medical, Indiana, USA) were implanted subcutaneously into the lower left quadrant of 20-25 gram male BALB/c or C57BL/6 mice (Jackson Laboratories, Canada) for mouse syngeneic islet transplantation, (**Figure 7.2A**) or B6.129S7-Rag1^{tm1Mom} immunodeficient mice for human islet transplantation. A 4 mm lateral transverse incision was made caudal to the rib cage allowing for a small pocket to be created inferior to the incision line using blunt dissection. An adequate void (1 cm by 3 cm) was created. The catheter segment was implanted into the space such that the catheter laid parallel to the midline. The incision was closed with a surgical staple (Autoclip®, Becton Dickinson, Sparks, MD) (**Figure 6.2B**). Once implanted, the catheter became adherent with blood proteins, leading to the formation of densely vascularized tissue, which exhibited a minimally visible profile (**Figure 6.2C - 6.2F**). At the time of transplant, removal of the catheter revealed a vascularized lumen allowing for cellular transplant infusion (**Figure 6.1 and Figure 6.3A**).

6.3.2. - Proinflammatory cytokine and chemokines measurements

A one-centimeter segment from either source catheter material was placed subcutaneously into the left lower quadrant of 20-25 gram male BALB/c mice. Implanted catheters with surrounding skin and muscle tissue margins were explanted 24 hours, 1 week and 2 weeks post-implantation. Similarly, tissue dissections were retrieved from the abdomen of non-implanted mice, serving as background cytokine and chemokine control specimens. The respective catheter segments were carefully removed from the surrounding tissue, yielding a hollow void encompassed by a vascularized matrix. Tissue samples were immediately placed in pre-weighed microcentrifuge tubes. The tissue weights were recorded, then subsequently flash frozen with liquid nitrogen and

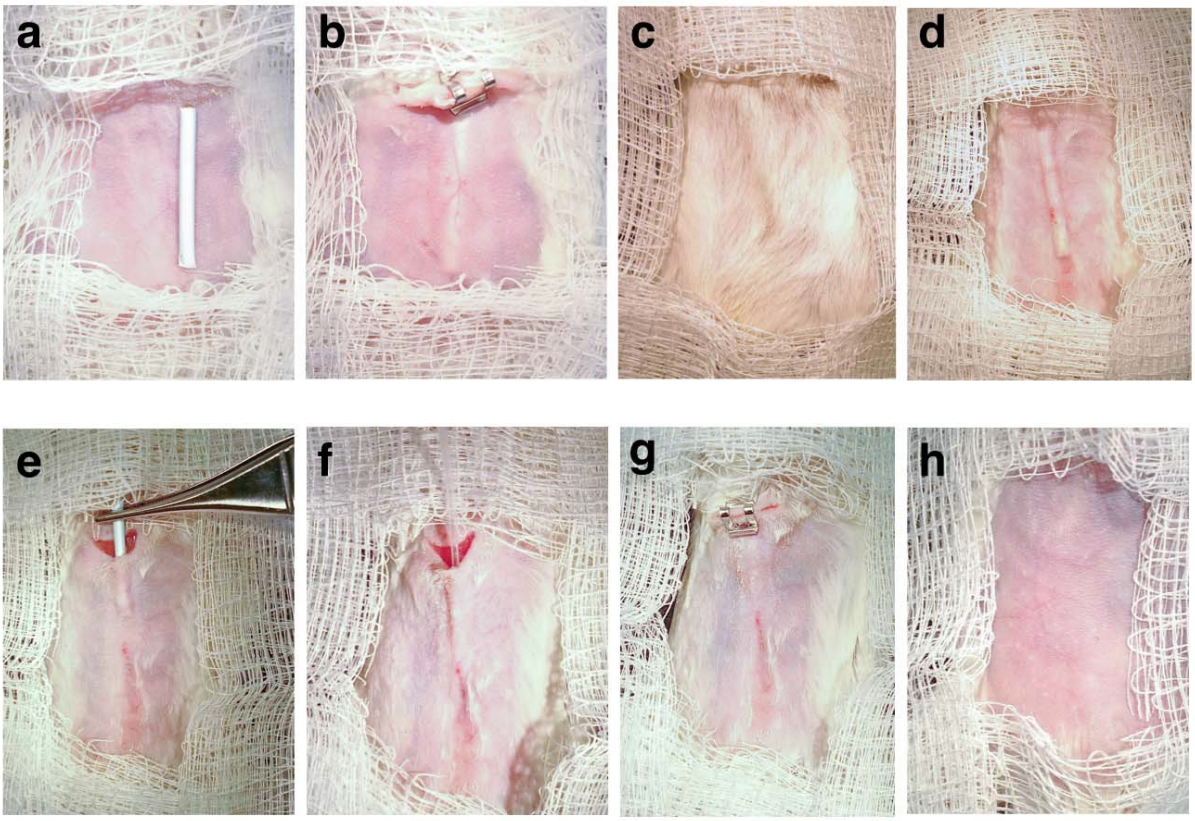


Figure 6.2 Overview of the subcutaneous, 'device-less' transplant approach. To create the 'device-less' transplant site, a 5-French textured nylon radiopaque angiographic catheter (Torcon NB® Advantage Beacon® tip Cook Medical, Indiana, USA) is: (**a**) Implanted beneath the skin; (**b,c,d**) Left for a period of 3-6 weeks; (**e**) Removed; (**f**) Subsequent to the implant period, the angiocatheter is removed (**e**) creating a vascularized void where the islet transplant is infused; (**f**) Islets are then infused via PE50 tubing (Instech Laboratories, Boston USA); (**g**) Incision site closed with a single surgical clip; and (**h**) The islet graft exhibited no visible profile post-transplant up to 100 days post-transplant.

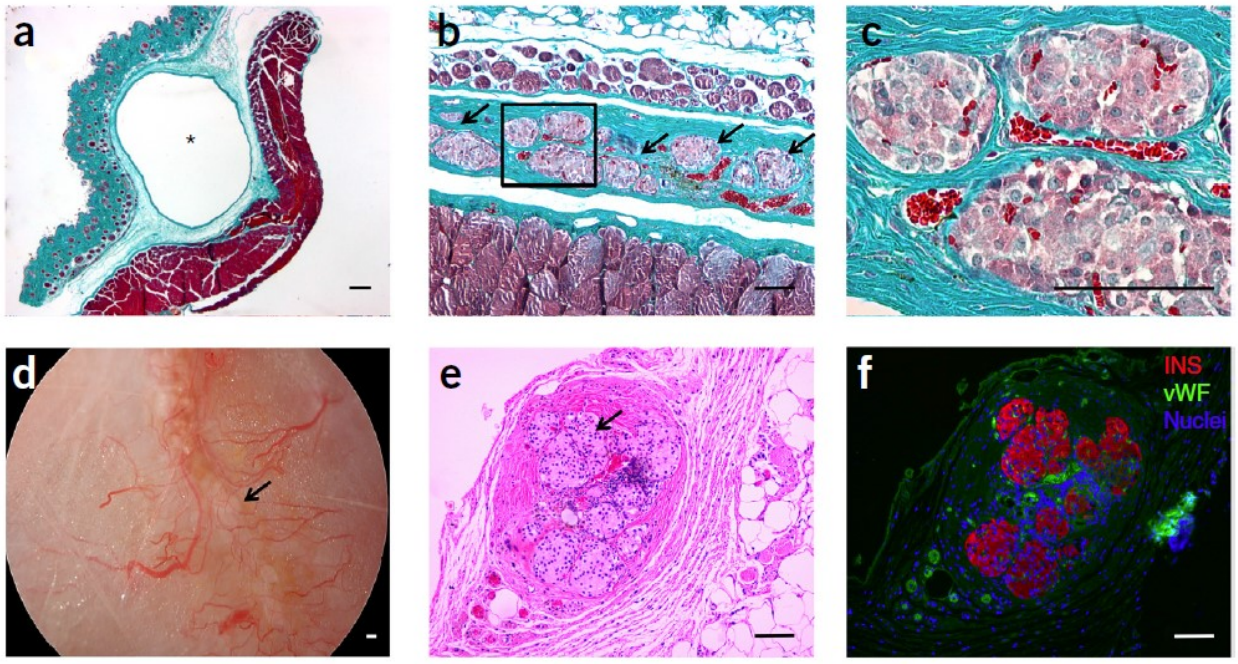


Figure 6.3 Histological analysis of islets transplanted long-term into the DL space. (**a**) Masson's trichrome staining of the cross-section of the DL site after removal of a catheter that had been implanted for 4 weeks. Islets were infused into the resulting lumen (*). Collagen (blue), smooth muscle and erythrocytes (red) at 20×. (**b**,**c**) Masson's trichrome staining at 100× (**b**) and 400× (**c**) of an islet graft in the DL site at >100 days after transplant, surrounded by collagen and blood vessels. Arrows indicate engrafted islets in a vascularized collagen scaffold. (**d**) Macroscopic image of the neovascularization penetrating the length of an islet graft in the DL site, >100 days after transplant. Arrow indicates islets within vascularized graft. (**e**) Hematoxylin and eosin staining of an islet graft cross-section, 100 days after transplant at 100×. Arrow indicates islets with the DL transplant site. (**f**) Fluorescent staining of the same cross-section stained for insulin (red), blood vessels (green) and nuclei (blue) at 100×. Scale bars, 100 μm.

stored at -80°C prior to conducting the cytokine and chemokine proinflammatory analysis. Once all tissue samples from respective implantation period were collected and frozen, 1 mL of lysis buffer (0.15 M NaCl, 1 mM Tris-HCl, 0.1% SDS, 0.1% Triton X-100, 20 mM Sodium deoxycholate, 5 mM EDTA) per 200 mg of tissue was added to the tissue containing microcentrifuge tube. Each tissue sample was homogenized (PowerGen, Fisher Scientific, Ontario, Canada) on ice for 30 sec x 2 replications. Samples were then sonicated (VirSonic, VirTis, NY, USA) with 10 quick pulses while on ice. Lysed tissue samples were centrifuged at 18,300 g (RCF) for 10 min at 4°C to remove cellular debris. The resulting supernatant was collected and placed in a microcentrifuge tube containing 10 μL of a protease inhibitor cocktail (Sigma-Aldrich Canada Co., Oakville, ON, Canada) per 1 mL of lysate (1:100). Peri-implant cytokine and chemokine (IL-1β, IL-12p70, IFN-γ, IL-6, KC/GRO, IL-10 and TNF-α) measurements were conducted using a Multi-Spot Mouse ProInflammatory 7-Plex Ultra-Sensitive kit (Meso Scale Discovery®, Gaithersburg, MD, USA) requiring 25 μL of lysate/replicate and analyzed on a SECTOR Imager (Meso Scale Discovery®, Gaithersburg, MD, USA).

6.3.3. - Mouse pancreatectomy and islet isolation

Pancreatic islets were isolated from 8 to 12 week old male BALB/c or C57BL/6 mice. Animals were housed under conventional conditions having access to food and water *ad libitum*. The care for all mice within the study was in accordance with the guidelines approved by the Canadian Council on Animal Care. Before pancreatectomy, the common bile duct was cannulated with a 27 G needle and the pancreas distended with 0.125 mg/mL cold Liberase TL Research Grade

enzyme (Roche Diagnostics, Laval, QC, Canada) in Hanks' balanced salt solution (Sigma-Aldrich Canada Co., Oakville, ON, Canada). Islets were isolated by digesting the pancreata in a 50 mL tube placed in a 37°C water bath for 14 minutes with gentle shaking. Subsequent to digestion, islets were purified on histopaque-density gradients (1.108, 1.083 and 1.069 g/mL, Sigma-Aldrich Canada Co., Oakville, ON, Canada).

6.3.4. - Human islet isolation

Pancreata from multi-organ deceased donors were procured post-aortic cross-clamp and infused with preservation solutions. Consent for islet isolation was obtained in all cases. Islets from two separate human islet preparations were isolated implementing a modified Ricordi technique (23, 24). In short, the pancreas was distended with collagenase blend solution and digested in a Ricordi® Chamber. When islets were adequately dissociated from surrounding acinar tissue, the pancreatic digest was collected. Islets were purified using a continuous density gradient on a cell processor centrifuge (Model 2991, Cobe, Lakewood, Co, USA). All human islet preparations were processed with intent for clinical transplantation, and were only made available for research when the islet yield fell below that of the minimal mass required for clinical transplantation. Permission for these studies was granted by the Health Research Ethics Board of the University of Alberta, Edmonton, Alberta Canada. Human islets were cultured overnight in CMRL-1066 media supplemented with insulin selenium-transferrin and insulin-like growth factor-1 at 22°C before transfer to the laboratory for experimentation.

6.3.5. - Diabetes induction and islet transplantation

Three to five days before transplantation, implanted mice were rendered diabetic through administration of an intraperitoneal injection of STZ at 185 mg/kg in acetate phosphate buffer, pH 4.5 (Sigma-Aldrich Canada Co., Oakville, ON, Canada). Animals were considered diabetic when their blood glucose levels exceeded a pre-established value of 15 mmol/L (350 mg/dL) for two consecutive daily readings. At the time of transplantation, ~500 mouse islets ± 10% with purity of 90% ± 5% or 2000 human islet equivalents (IEQ) were aspirated into polyethylene (PE-50) tubing using a micro-syringe, and centrifuged into a pellet suitable for transplantation. Islet preparations were distributed randomly to all three transplant recipient groups: Device-less, kidney capsule or subcutaneous alone. DL recipient mice were maintained under anesthesia with inhalant isoflurane, and placed in a supine position. A field surrounding the implanted catheter was prepared by shaving and surface disinfected with soap scrub, povidone-iodine (Betadine, Purdue Pharma, Oakville, ON, Canada) and isopropyl alcohol. Cranial to the superior edge of the implanted catheter, a small (4mm) incision was made to gain access to the catheter. The tissue matrix surrounding the superior margin of the catheter was dissected to withdraw and remove the catheter (**Figure 6.1 and Figure 6.2E**). The PE-50 tubing and islet preparation was delivered within the vascularized lumen, and islets expelled into the void using a micro-syringe (**Figure 6.1, Figure 6.3 and Figure 6.3F**). The incision was closed with a surgical staple (Autoclip®, Becton Dickinson, Sparks, MD) (**Figure 6.2G**). Prior to recovery, recipients received a 0.1 mg/kg subcutaneous bolus of buprenorphine. Control animals were rendered diabetic and transplanted with ~500 mouse islets/recipient as described above, however the islets were infused into the subcutaneous space alone (no prevascularization or catheter implant). In

addition, a subset of diabetic animals was transplanted with ~500 mouse islets/recipient under the kidney capsule (KC), the standard site for rodent islet transplantation. For all experiments, islets were pooled, batched and transplanted in random allocation to either the DL or KC sites. To facilitate the KC transplants, a left lateral paralumbar subcostal incision was made and the left kidney was delivered into the wound. The renal capsule was incised and space was made under the capsule to allow transplantation of the islets using PE-50 tubing (Instech Laboratories, Boston USA). The subcostal incision was closed in two layers. The efficacy of mouse islets transplanted into the DL site to reverse diabetes was compared to the engraftment efficacy of islets transplanted in the unmodified subcutaneous site or under the renal subcapsule.

6.3.6. - Evaluation of islet graft function

Islet graft function was assessed through non-fasting blood glucose measurements, using a portable glucometer (OneTouch Ultra 2, LifeScan, Canada) three times per week following islet transplantation, in all groups transplanted. Reversal of diabetes was defined as two consecutive readings <11.1 mmol/L (in accordance with the American Diabetes Association) and maintained until study completion.

In addition, glucose tolerance tests were conducted at 60 or 100 days post-transplant, as a means to further assess metabolic capacity in response to a glucose bolus, mimicking postprandial stimulus. Animals were fasted overnight prior to receiving an intraperitoneal glucose bolus (3 g/kg). Blood glucose levels were monitored at 0, 15, 30, 60, 90 and 120 minutes post injection, allowing for area under the curve (AUC-blood glucose) to be calculated and analyzed between transplant groups.

6.3.7. - Long-term islet graft retrieval

To confirm graft dependent euglycemia, and to eliminate residual or regenerative native pancreatic beta cell function, animals with functional grafts had their islet transplants explanted either by nephrectomy or subcutaneous graft excision. Renal sub-capsular islet transplant recipients were placed under anesthesia, and the graft-bearing kidney exposed. A LT200 Ligaclip (Johnson & Johnson, Inc., Ville St-Laurent, QC, CA) was used to occlude the renal vessels and the ureter at the pedicle. The left kidney was dissected and the explanted graft preserved for immunohistochemistry in 10% formalin. Likewise, the subcutaneous islet grafts within the DL transplanted animals, which exhibited no visible profile post-transplant (**Figure 6.2H**), were excised with a margin of surrounding abdominal skin containing the islet graft. Following islet-graft removal non-fasting blood glucose measurements were monitored for the subsequent 7 days to observe a return to hyperglycemia, confirming post-transplant graft function.

6.3.8. - Histological assessment

Immunofluorescence was used to identify endothelial cells for assessment of vascularization using anti-von Willebrand factor (vWF) antibody and anti-insulin and anti-glucagon antibodies to identify the presence of pancreatic β-cells and α-cells, respectively. Briefly, following deparaffinization and antigen heat retrieval, the graft sections were washed with phosphate buffered saline supplemented (PBS) 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), rabbit anti-pig von

Willebrand factor (Dako A0082) diluted 1:400 (PBS with 1% goat Serum) and or rabbit anti-glucagon (Abcam) diluted 1:200 (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), and goat anti-rabbit (Vector Fl-1000) diluted 1:500 (PBS with 1% goat serum) for 30 minutes 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. Vascular density was quantified by ImageJ software (National Institute of Health, Bethesda MD), and reported as the percentage of the islet graft staining positive for von Willebrand staining. In addition, to assess the incorporation of vascularized collagen tissue into surrounding the DL islet-grafts, representative sections were stained with hematoxylin/eosin and Masson's trichrome.

6.3.9. - Statistical analysis

Non-fasting blood glucose and proinflammatory data are represented as the mean ± standard error of mean (s.e.m.). Sample size calculations were based on reversal of glycemia rates in control mice with islets placed subcutaneously (0%) vs. a projected estimate of 60% engraftment in the DL site (Sample size n=20 or more per group; alpha 5%, power 100%). 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). Newman-Keuls 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. $P<0.05$ was considered significant.

6.4. – RESULTS

6.4.1. - Design and testing of the subcutaneous DL site

Initial studies summarized in **Table 6.3** describe how we chose the biomaterial used in the present study. Briefly, we compared a range of hydrophilic and hydrophobic catheters of different diameters for their ability to create a space that supported transplantation of syngeneic BALB/c mouse islets. Reversal of diabetes rates and intraperitoneal glucose tolerance tests (IPGTT) were measured **(Figure 6.4 and 6.5).** Of the catheters tested, the 5-French (Fr.) nylon catheter induced the most optimal, thin supporting collagen matrix (**Figure 6.3A**) and led to extensive neovascularization of transplanted islets (**Figure 6.3B – 6.3F**). We also compared implant periods of 2-4 weeks. We selected a 4-week period, as vascularized collagen space appeared to be more favorable histologically for neovascularization (data not shown). These results suggested that a time-limited induction of the foreign-body response converts the subcutaneous space into a favorable site for islet engraftment, although direct causality was not proven.

We observed extensive differences in tissue cytokine expression, in BALB/c mice, represented as mean pg/g tissue ± standard error of mean (s.e.m.), in response to the nylon catheter compared to the silicone catheter and unmodified subcutaneous controls (**Figure 6.6A – 6.6R**). Both nylon and silicone elicited strong interleukin (IL-) IL-1β, IL-10 and keratinocyte growth factor

(KC/GRO) responses, but the responses to nylon were much faster (24 hours vs 1 week). IL-6 expression was elevated within 24 hours, and remained mildly elevated during the 2 week experiment in both groups. In contrast, tumor necrosis factor α (TNF-α) expression became elevated in both groups by one week post implant. IFN-γ was analyzed but was not detected in any samples.

These data suggest that the temporary presence of the nylon catheter induces strong cytokine and chemokine responses that contribute to host inflammatory cell recruitment (e.g. neutrophils, macrophages, fibroblasts) and neovascularization. We do not, however, have causative data demonstrating that the degree of inflammatory marker response relates directly to the degree of diabetes reversal.

We compared the efficacy of the subcutaneous space generated by various biomaterials supporting islet engraftment, in BALB/c recipients (**Table 6.3**). The nylon and silicone based-catheters appeared to be most effective and were studies in more depth. Glycemic control was monitored over time, and intraperitoneal glucose tolerance testing (IPGTT) was conducted at 60 days. In kidney capsule (KC) controls, by 3 weeks 95% of mice (19/20) became euglycemic and remained so for >60 days (**Figure 6.4**).

In controls using unmodified subcutaneous transplantation (SubQ), no mice (0 of 10) showed diabetes reversal. In the silicone-DL group, 35% of the mice (6/17) became normoglycemic by 3 weeks, and by Day 60 65% (11/17) were normoglycemic. In contrast, in the nylon-DL group, 57% of the mice (12/21) became normoglycemic by 3 weeks, and by Day 60 91% (19/21) were normoglycemic, similar to the KC control group (**Figure 6.4**).

Table 6.3 Rationale and optimization for biomaterial selection in creation of prevascularized subcutaneous 'device-less' sites used in the present manuscript. Modified from Pepper AR, et al. Nature Biotech 2015; 35(5):518-23.

Generic name	Biomaterial composition	Critical surface tension of biomaterial (dynes/cm)	Water contact angle (°)	Outer diameter	Average transplant dose (islet/recipient)	Reversal of diabetes (%)	No of recipients
T-Tube	Butyl rubber	27	112.1	8.0 Fr.	1,350	0	5
I.V. Catheter	FEP	19.1	108.5	4.0 Fr.	500	50.0	4
Long-term indwelling catheter	Silicone*	20.1	107.2	5.0 Fr.	500	33.3	12
Radiopaque angiocatheter	Nylon**	43.9	62.6	5.0 Fr.	500	91.3	23

Abbreviations: FEP – Fluorinated Ethylene Propylene; Fr. – French; I.V. – Intravenous; *Polydimethylsiloxane; **Nylon 6 (Aramid™).

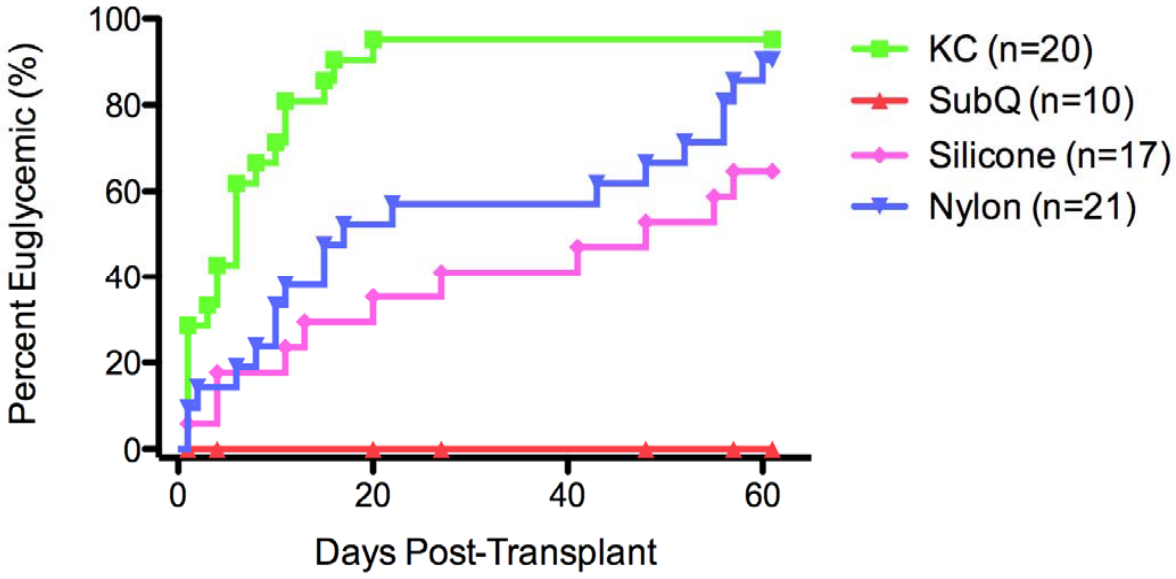
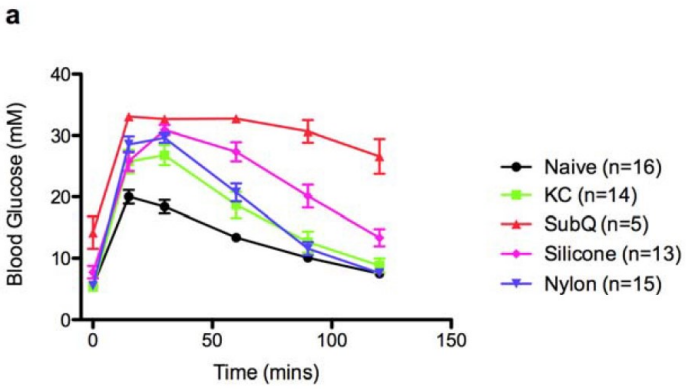


Figure 6.4 Rate of diabetes reversal, defined as percent euglycemic, in mouse recipients of syngeneic BALB/c islet grafts. Glycemic control, measured by twice weekly non-fasting blood glucose levels, was monitored for 60 days post-islet transplant in chemically induced (STZ) diabetic mice. Reversal of diabetes was defined as a maintained non-fasting blood glucose level of <11.1 mM. Recipients received 500 BALC/c islets. Islet transplant groups: Kidney Capsule (KC – green, n=20), subcutaneous alone (SubQ –red, n=10), 'device-less' silicone (Silicone – purple, n=17) and 'device-less' nylon (Nylon – blue, n=21). Data points represent blood glucose mean ± s.e.m. Islets transplanted were from 10 separate isolations (n=20 pancreata per isolation).


a
Blood Glucose (mM)
40
30
20
10
0
0
50
100
150
Time (mins)
Naive (n=16)
KC (n=14)
SubQ (n=5)
Silicone (n=13)
Nylon (n=15)

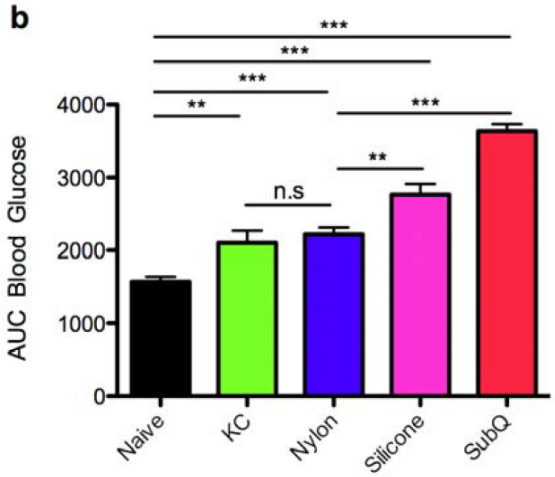

b
***
***
***
**
***
**
n.s
AUC Blood Glucose
4000
3000
2000
1000
0
Naive
KC
Nylon
Silicone
SubQ

Figure 6.5 Intraperitoneal glucose tolerance tests in syngeneic BALB/c islet recipients. Intraperitoneal glucose tolerance tests in syngeneic BALB/c islet recipients under the Kidney Capsule (KC) or subcutaneous 'device-less' (DL) site, at 60 days post-transplant. (**a**) Blood glucose post-dextrose bolus (**b**) area under the curve (AUC) analysis did not differ between the KC (green, n=14) and nylon-DL (blue, n=15) recipients (p NS, one-way Anova-Newman-keuls post-hoc). Nylon-DL profiles were significantly improved compared to silicone-DL (pink, n=13), (**p<0.01 one-way Anova-Newman-keuls post-hoc). Islets transplanted beneath the skin without prevascularization, (SubQ – red, n=5), demonstrated diabetic profiles (***p<0.001) compared with Nylon-DL). Naïve were normal, non-diabetic control BALB/c mice (black, n=16), and showed most optimal glycemic profiles (**p<0.01 and ***p<0.001) compared with Nylon-DL and KC respectively. Blood glucose was measured at 0, 15, 30, 60, 90 and 120 minutes. Data points represent blood glucose mean ± s.e.m. Islets transplanted were from 10 separate isolations (n=20 pancreata per isolation).

IPGTT showed that mice in the KC (n=14) and nylon DL (n=15) groups rapidly returned to normoglycemia (**Figure 6.5A**). There was no difference as measured by mean area under the curve (AUC) ± s.e.m. (AUC KC: 2101 ± 168 mmol/L/120min vs. nylon DL: 2219 ± 93 mmol/L/120min, $p>0.05$, ANOVA, **Figure 6.5B**). Silicone-DL mice (n=13) were more glucose intolerant than nylon-DL mice (AUC silicone-DL: 2764 ± 149 mmol/L/120 min vs. nylon-DL, $p<0.01$, ANOVA, **Figure 6.5B**), and all mice in the unmodified subcutaneous group (n=5) had diabetic profiles (AUC SubQ 3635 ± 95 mmol/L/120 min, compared to nylon-DL, $p<0.001$, ANOVA, **Figure 6.5B**). These data demonstrate that the choice of biomaterial affects the rates of islet engraftment *in vivo* and further support the superiority of nylon over silicone.

6.4.2. - Long-term function and vascularization of DL islet grafts

We followed graft function for >100 days to measure long-term performance. Grafts were subsequently retrieved to confirm prompt reversion to hyperglycemia. Recipient BALB/c mice were rendered diabetic with STZ and ~500 syngeneic mouse islets were transplanted in the DL space, under the KC, or in the unmodified subcutaneous space. In addition, ~2000 human islet equivalents (IEQ) were transplanted in the DL space in diabetic $Rag^{-/-}$ mice to test compatibility of the DL approach with clinical-grade human islets. Syngeneic islets transplanted under the KC reversed diabetes in 100% of recipients (10/10) (**Figure 6.7Aa**) within 11.5 ± 2.9 days (**Figure 6.7B**). Transplants in the unmodified subcutaneous space did not reverse diabetes (0%; 0 of 10) at any time point (**Figure 6.7A and 6.7B**). Transplants in the DL space reversed diabetes in 91.3% of the mice (21/23) (**Figure 6.7A**), with marked improvement compared with the unmodified subcutaneous group

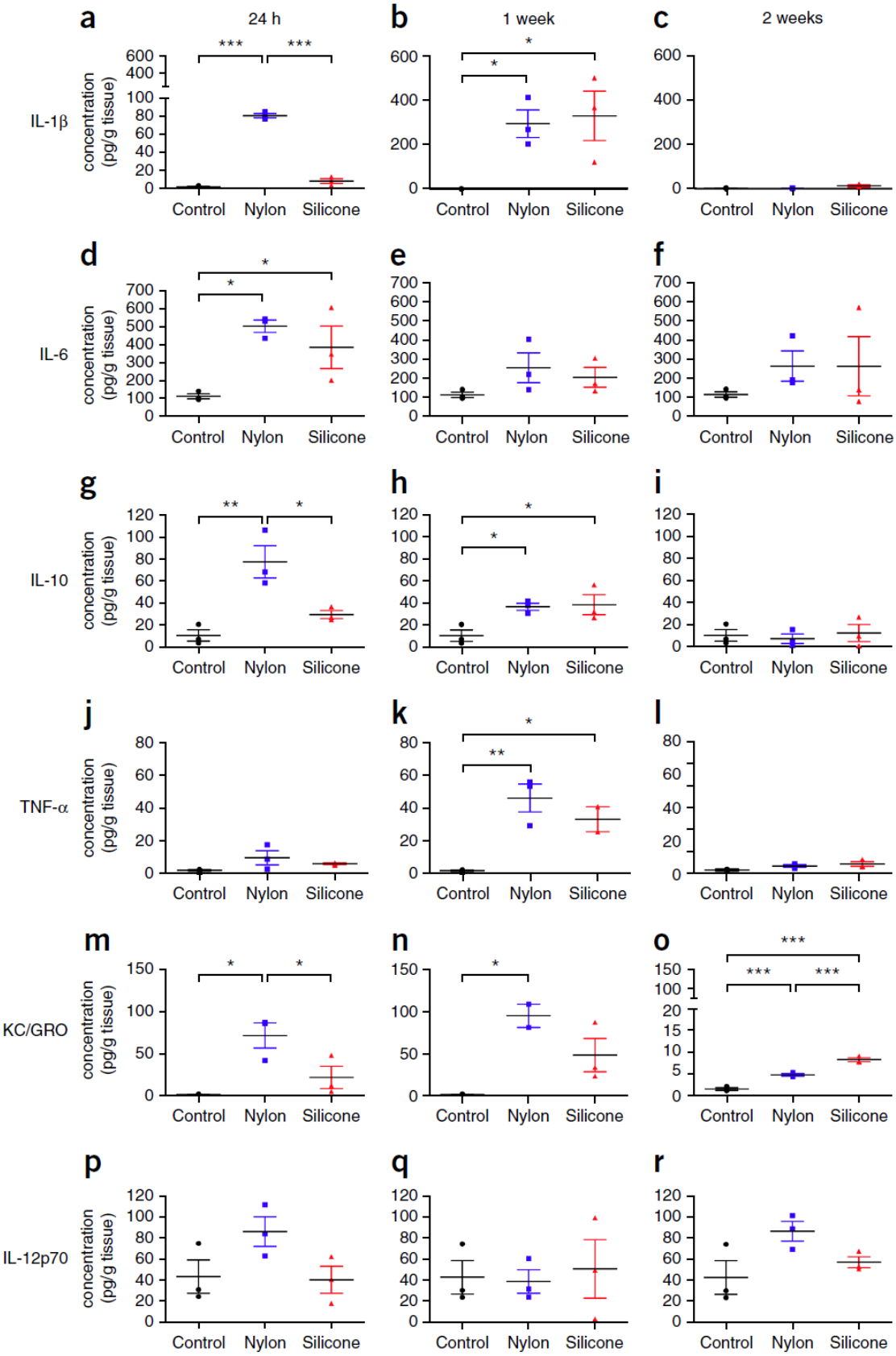

a
24 h
b
1 week
c
2 weeks
IL-1β
IL-6
IL-10
TNF-α
KC/GRO
IL-12p70
concentration (pg/g tissue)
Control
Nylon
Silicone
d
e
f
g
h
i
j
k
l
m
n
o
p
q
r

Figure 6.6 The proinflammatory response elicited by angiocatheters composed of nylon (blue) or silicone (red) when implanted subcutaneously for 24 h, 1 week and 2 weeks. (**a**–**r**) The peri-implant concentrations of IL-1β (**a**–**c**), IL-6 (**d**–**f**), IL-10 (**g**–**i**), TNF-α (**j**–**l**), KC/GRO (**m**–**o**) and IL-12p70 (**p**–**r**). Data points represent mean ± s.e.m. for pg/g tissue, **P* < 0.05, ***P* < 0.01, ****P* < 0.001 (*n* = 3/time point). One-way ANOVA was calculated with Newman-Keuls *post-hoc* testing for multiple comparisons between controls and individual biomaterials tested. *Y* Axis labels for all graphs indicated in left margin.

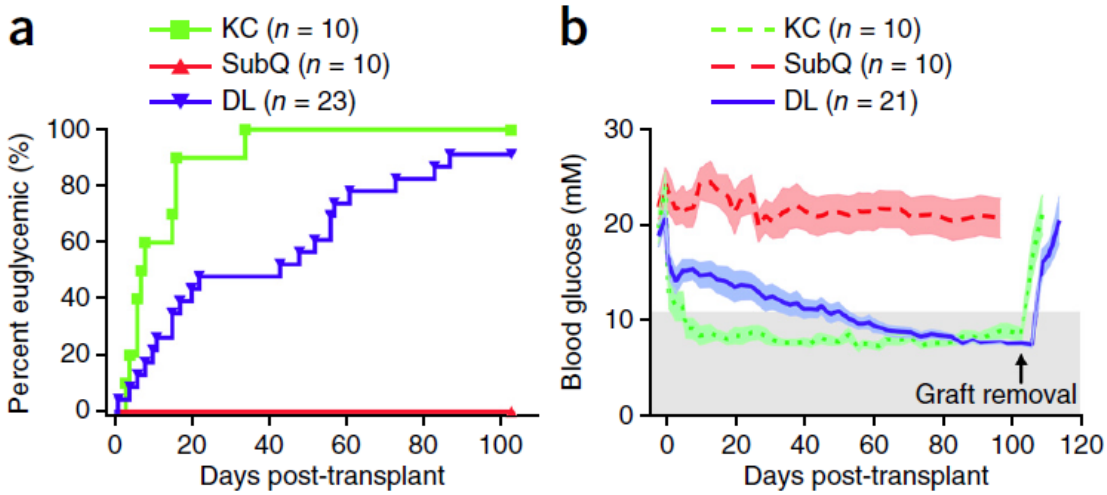


Figure 6.7 Long-term function of syngeneic islet grafts transplanted into the DL space. (**a**) The proportion of animals that achieved euglycemia was similar in KC recipients (n = 10) and DL space recipients (n = 21) 100 days after transplant, with KC recipients reversing diabetes earlier (P = 0.001, log-rank, Mantel-cox test). (**b**) Nonfasting blood glucose measurements showed that both KC and DL space recipients maintained normoglycemia until the graft was retrieved (arrow), at which point they reverted to their pre-transplant hyperglycemic state. Islets transplanted in the unmodified subcutaneous space did not provide glycemic control (SubQ-red, n = 10). Shaded area represents a nonfasting physiological range (<11.1 mM). Data points represent blood glucose mean ± s.e.m. Islets transplanted were from ten separate islet isolations (n = 20 pancreata per isolation).

($p<0.0001$, log-rank). Engraftment in the DL space was delayed, with mean diabetes reversal at 35.4 ± 6.0 days post-transplant ($p<0.05$, t-test), compared to KC engraftment (**Figure 6.7B**). After 100 days, we conducted IPGTT (**Figure 6.8A**). Both KC (n=10) and DL (n=15) groups had well-preserved glucose clearance profiles that were not significantly different. AUCs ± s.e.m. for glucose clearance (**Figure 6.8B**) were similar (KC: 1771 ± 212 mmol/L/120min vs. DL: 2095 ± 138 mmol/L/120min, $p>0.05$, ANOVA) (**Figure 6.8B**). By contrast, glucose profiles in the unmodified subcutaneous group were significantly worse (AUC 3635 ±95 mmol/L/120min, $p<0.001$, ANOVA) (**Figure 6.8A and 6.8B**). Normal, non-diabetic, non-transplanted BALB/c mice demonstrated the best glycemic profiles (AUC naïve: 1375 ± 62 mmol/L/120min, vs. DL recipients, $p<0.01$, ANOVA) (**Figure 6.8B**). The glycemic profiles of $Rag^{-/-}$ diabetic mice that received human islets in the DL space were similar to those of BALB/c mice that received syngeneic islets (**Figure 6.9A**). To confirm graft-dependent euglycemia, we explanted the grafts and in all cases saw prompt return of diabetes (**Figure 6.7B and 6.9A**). Histological analysis of explanted grafts from the DL site revealed islets enveloped in a vascularized collagen scaffold between skin and musculature (**Figure 6.3B-6.3F and 6.9B-6.9G**). Extensive vascular networks were visible macroscopically and penetrated the islet tract created by the nylon catheter (**Figure 6.3D**). Of note, capillary networks were localized to the DL area, whereas outside of the tract margins, planes were relatively avascular. Grafts in the DL space stained positive for insulin, glucagon and for the presence of endothelial cells in new intra-islet microvessels (**Figure 6.3E-6.3F and 6.9B-6.9G**). In contrast, islets transplanted into the unmodified subcutaneous space underwent extensive necrosis and a destructive inflammatory response, resulting in graft failure (**Figure 6.10A and 6.10B**).

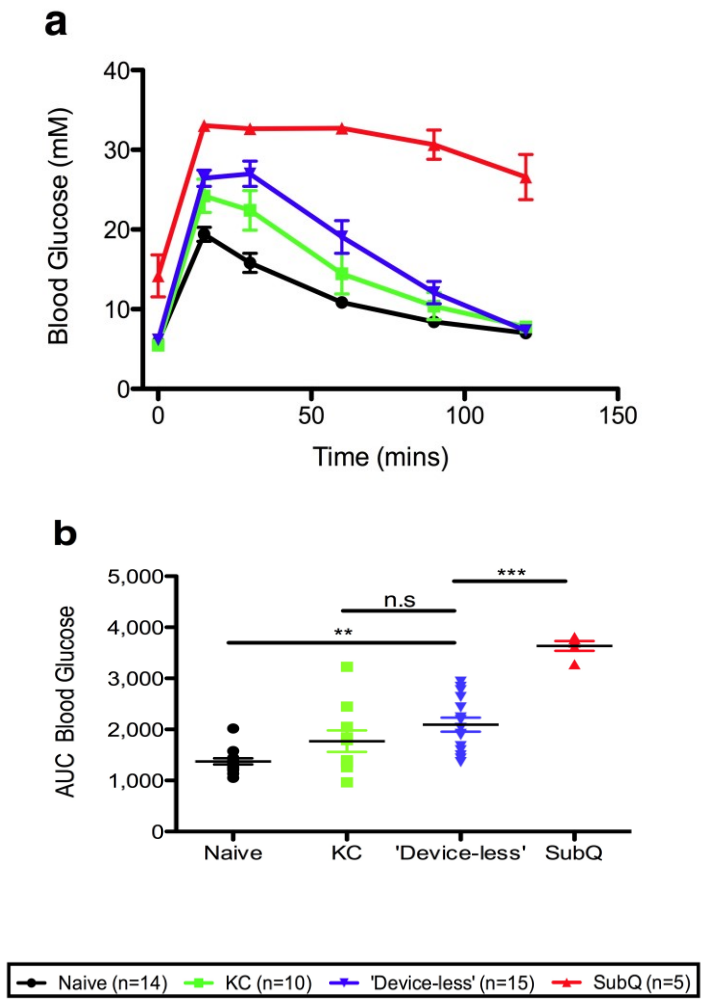


Figure 6.8 IPGTTs of syngeneic mouse islets transplanted under the KC or into the DL site, 100 days after transplant. (**a**,**b**) Blood glucose after dextrose bolus (**a**) AUC analysis (**b**) did not differ between the KC (n = 10) and DL space (n = 15) recipients ($P > 0.05$, one-way ANOVA with Newman-Keuls *post-hoc* testing for multiple comparison between transplant groups). Naive represents nondiabetic, nontransplanted BALB/c mice (black, n = 14), which were more tolerant to the metabolic test than the DL space recipients (**$P < 0.01$, $P > 0.05$, one-way ANOVA with Newman-Keuls *post-hoc* testing for multiple comparison to naive controls). Animals receiving islets in the unmodified SubQ (n = 5), were intolerant to the glucose challenge compared to DL space recipients (***$P <$ 0.001 one-way ANOVA with Newman-Keuls *post-hoc* testing for multiple comparison between transplant groups). Mice were administered 3 g/kg 50% dextrose i.p. Blood glucose measurements were monitored at t = 0, 15, 30, 60, 90 and 120 min. Data points represent blood glucose mean ± s.e.m. Islets transplanted were from ten separate islet isolations (n = 20 pancreata per isolation). n.s., not significant.

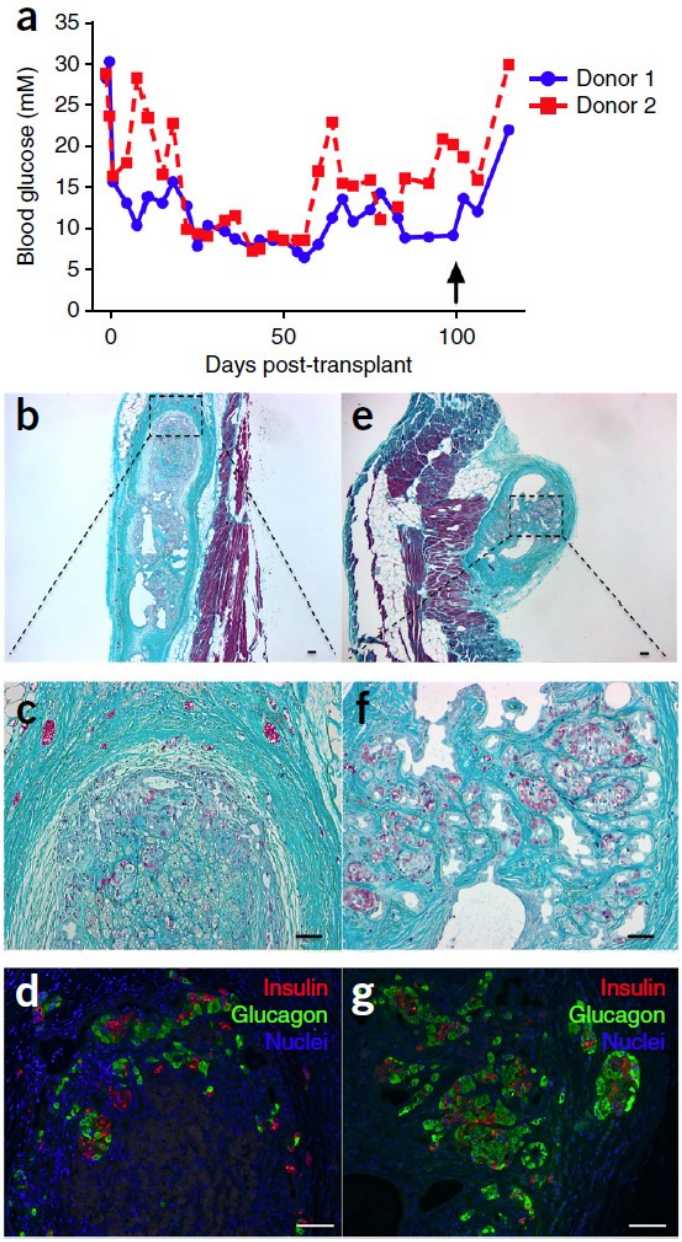


Figure 6.9 Long-term function of human islets transplanted into the DL space. (**a**) Nonfasting blood glucose measurements showed maintenance of normoglycemia until the time of graft retrieval (arrow), at which point recipients reverted to the pre-transplant hyperglycemic state (n = 2 human donors). (**b**–**f**) 20× (**b**,**e**) and 200× (**c**,**f**) Masson's trichrome staining of a long-term (>100 days) human islet graft in the DL site, surrounded with collagen and blood vessels. (**d**,**g**) Fluorescent staining of the same cross-section staining for insulin, glucagon and nuclei at 200×. Scale bars, 100 μm.

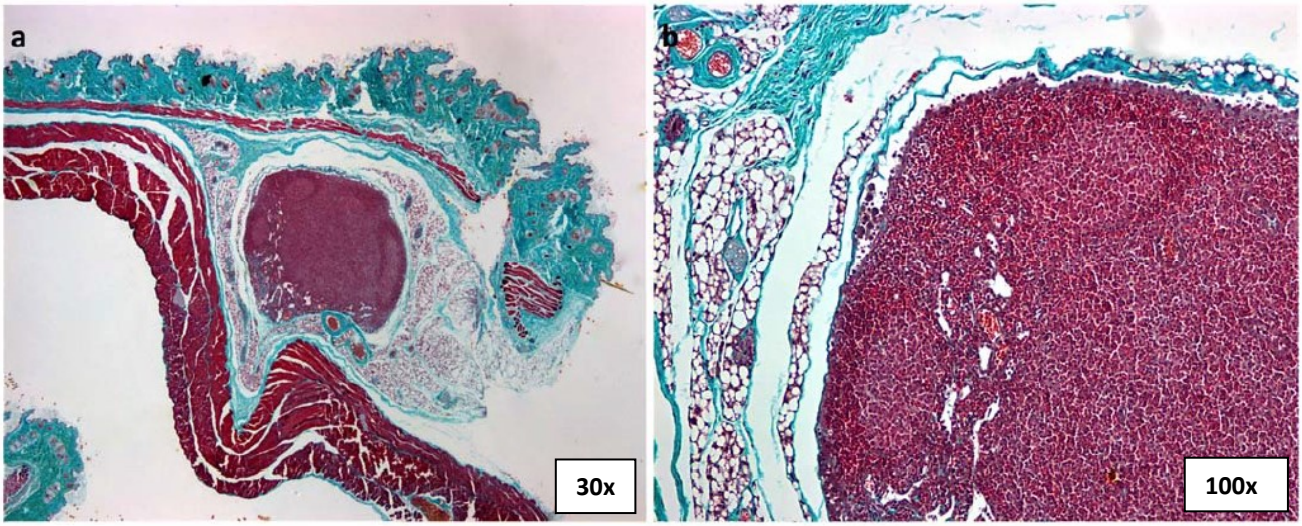


Figure 6.10 Immunohistochemistry of representative syngeneic (BALB/c) islet grafts transplanted beneath the skin without prevascularization. Immunohistochemistry of representative syngeneic (BALB/c) islet grafts transplanted beneath the skin without prevascularization, at 40 days post-transplant. Mason trichrome staining of cross-section of a subcutaneous islet graft (**a**) at 30x and (**b**) 100x magnification. Without prevascularization, islet necrosis and inflammatory destructive response ensued, resulting in graft loss.

We compared vascular density in DL and unmodified subcutaneous islet grafts at 100 days post-transplant (**Figure 6.11A-6.11C**). DL grafts showed a marked increase in neovascularization as measured by the percentage of the graft staining positive for von Willebrand (vWF) ± s.e.m. (2.30 ± 0.38% of the entire graft vWF+ in DL grafts vs. 0.56 ± 0.07% vWF+ in SubQ controls, $p<0.01$, t-test).

6.4.3. - Efficacy of DL transplantation in additional mouse models

To confirm that the potential of the prevascularized DL space is not unique to BALB/c mice, we studied C57BL/6 mice, a strain that, unlike BALB/c mice, has a vigorous foreign-body response. Transplantation of ~500 syngeneic islets in the DL space reversed diabetes more quickly in C57BL/6 mice than in BALB/c mice, as calculated by mean days post-transplant ± s.e.m. (11.3 ± 3.1 days vs. 35.5 ± 6.1 days, $p<0.05$, t-test) (**Figure 6.12**). Proportional rates of diabetes reversal were similar in the two strains by Day 50 post-transplant (C57BL/6: 75% (9/12) vs. BALB/c: 62% (13/21), $p>0.05$, t-test).

As diabetes may be associated with impaired wound healing, we studied mice with pre-existing diabetes. BALB/c and C57BL/6 mice were rendered diabetic with STZ one week before placement of the DL catheter and compared with similar mice that were not diabetic at the time of catheter placement. All mice were maintained for four weeks before catheter withdrawal and transplantation of syngeneic islets in the DL space. The pre-diabetic state did not inhibit diabetes reversal, as similar rates were observed in the two groups by Day 50 (78% (7/9) pre-diabetic versus 62% (13/21) non-diabetic at time of catheter placement ($p>0.05$, t-test)) (**Figure 6.13**).

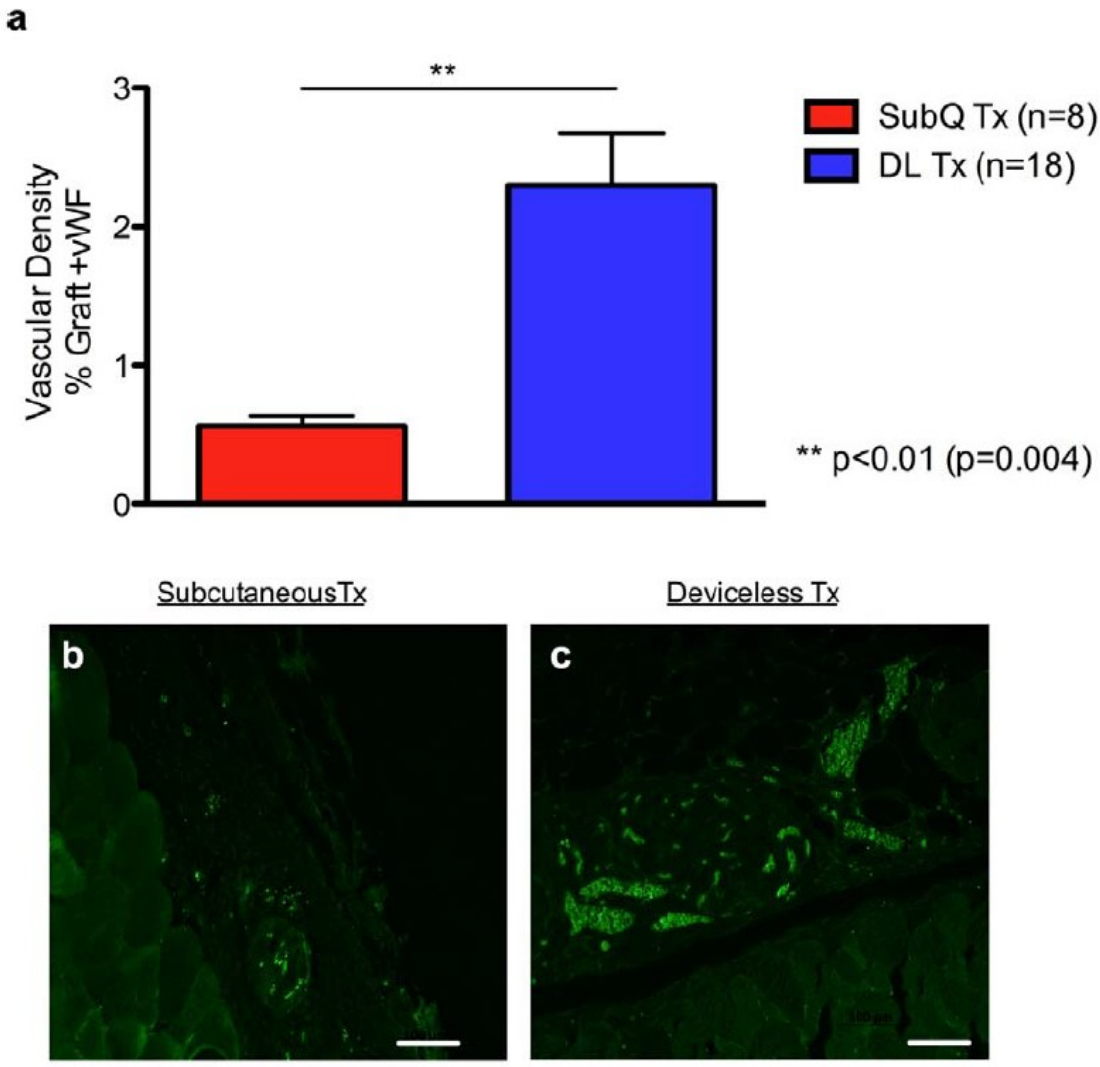


Figure 6.11 Vascular density of islet grafts post-transplantation. (**a**) Islets transplanted into the unmodified subcutaneous space (red: SubQ Tx, n=8) had markedly less graft neovascularization compared to islets transplanted into the prevascularized DL site (blue: DL Tx, n=18) ($p<0.01$, unpaired t-test). Vascular density was quantified by measuring percentage of islet grafts staining positive for the vascular wall marker, von Willebrand (vWF) (green), using ImageJ software (ImageJ, National Institutes of Health, Bethesda MD). Representative images of vWF positive staining within (**b**) subcutaneous and (**c**) DL islet grafts. Scale bar represents 100μm. Values represent mean percentage of graft staining positive for vWF ± s.e.m.

To determine whether the DL transplant technique is efficacious across an alloimmune barrier, we transplanted ~500 BALB/c islets into the DL space of C57BL/6 recipients, rendered diabetic post-DL site formation. Rejection occurred within 7 days in mice that did not receive immunosuppression, whereas tacrolimus therapy (0.5 mg/kg for 28 days) led to prolonged allograft survival (**Figure 6.14**). These results show that the DL space is not immunoprotective but can still provide an effective microenvironment that supports allogeneic islet survival, despite the presence of calcineurin inhibition (**Figure 6.14**).

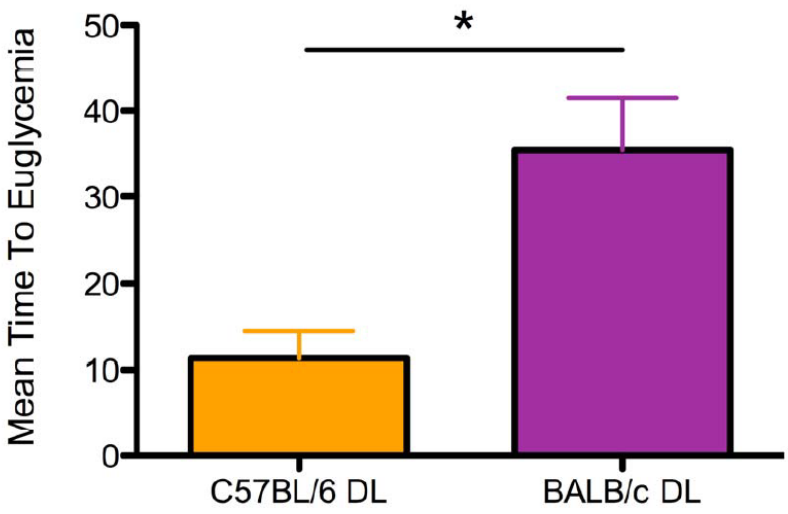


Figure 6.12 Time to normoglycemia in C57BL/6 vs. BALB/c mouse strains. Time to normoglycemia in C57BL/6 (n=12) vs. BALB/c (n=21) mouse strains, using the device-less (DL) subcutaneous approach. Glycemic control was measured three times per week with non-fasting glucose levels, and reversal of diabetes defined as glucose <11.1mM. C57BL/6 mice reversed diabetes at a more rapid rate than BALB/c mice (11.3 ± 3.1 vs. 35.5 ± 6.1 days, $p<0.05$, unpaired t-test). Data points represent mean days post-transplant ± s.e.m. C57BL/6 transplants were conducted from 5 separate isolations. BALB/c transplants were conducted from 10 separate islet isolations (n=20 pancreata per isolation).

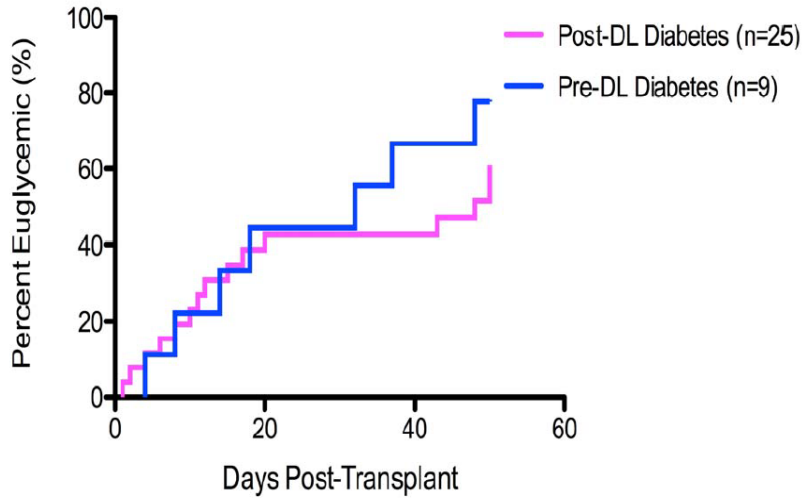


Figure 6.13 Impact of pre-existing diabetes before placement of the device-less (DL) catheter upon subsequent islet engraftment. Mice were rendered diabetic 7 days ahead of DL catheter placement, and remained diabetic for a further 4 weeks before transplantation of 500 syngeneic islets. Glycemic control, measured by three times per week, was monitored for 50 days post-islet transplant in chemically induced streptozotocin (STZ) diabetic mice. Reversal of diabetes was defined as glucose <11.1 mM. No significant difference was found between pre-existing diabetic state (n=9) vs. post DL catheter placement diabetic state (n=25), upon subsequent islet engraftment (p NS, log-rank, Kaplan-Meier). Islets were transplanted from 15 separate islet isolations (n=20 pancreata per isolation).

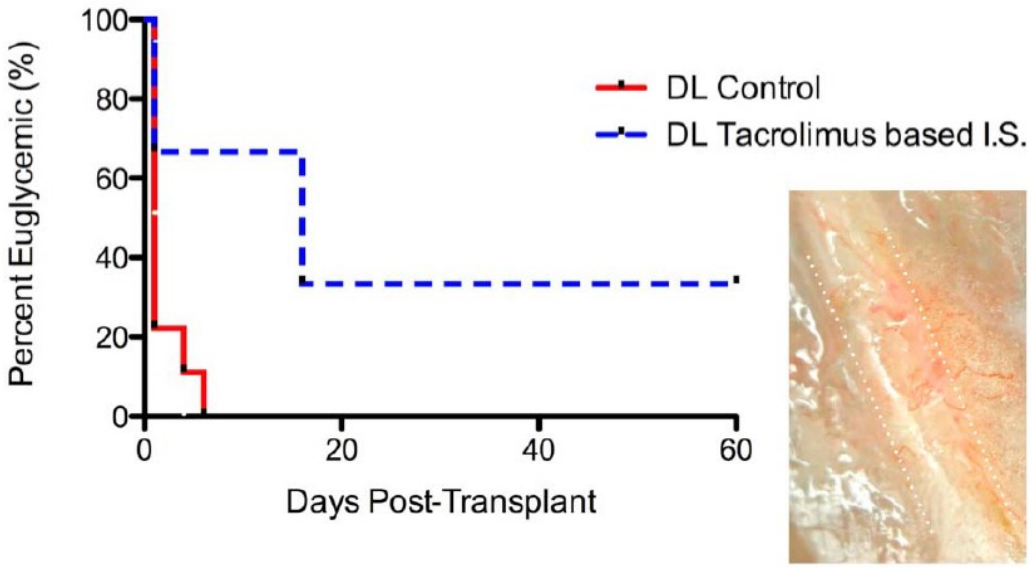


Figure 6.14 Impact of an allogeneic barrier upon diabetes reversal using the device-less (DL) subcutaneous site. 500 BALB/c islets were transplanted within the DL space of streptozotocin-diabetic C57BL/6 mice, in the presence or absence of immunosuppression (I.S.). Control mice (n=9) initially reversed diabetes, but then rapidly rejected allogeneic islet grafts. By contrast, with tacrolimus-based immunosuppression (0.5mg/kg/day for 28 days, n=3, subcutaneously via Alzet® mini-osmotic pump, (Alzet Cupertino, CA), rejection was delayed, and a proportion of grafts continued to function > 60 days. Hyperglycemia occurred promptly upon graft explantation. Kidney subcapsular allogeneic grafts (data not shown) rejected in a similar time-course. Insert depicts a representative islet allograft showing robust neovascularization, of similar response to that found in syngeneic grafts. Dashed lines indicate margins of prevascularized tract.

6.5. - DISCUSSION

Our results indicate that a controlled foreign-body response can be used to generate a prevascularized subcutaneous site that supports islet engraftment. Withdrawal of an implanted catheter after one month terminates the foreign-body reaction at a stage where a rich vascularized collagen network has formed but before permanent fibrosis and mature scar formation occur. An advantage of the DL approach is that it avoids the need for a permanent encapsulation device, which often generates an avascular fibrotic granular capsule(17, 19, 22, 25-27) and a chronic inflammatory response that contribute to graft failure. We showed that the neovascularization response and successful islet engraftment occurred in different mouse strains, in the presence of pre-existing STZ-induced diabetes, and with islet allografts. Notably, human islets transplanted into immunodeficient $Rag^{-/-}$ mice reversed diabetes to the limited extent tested. As $Rag^{-/-}$ mice have intact innate immunity, the foreign-body neovascularization response was preserved.

To discover conditions for generating a favorable DL site, we compared inflammatory responses to a selection of catheter materials with different surface properties and diameters and tested implant periods of 2-4 weeks. We chose catheter materials that are in routine clinical practice so that off-label clinical application would be relatively straightforward. Hydrophilic nylon induced stronger pro-inflammatory responses compared to silicone, which translated into more effective diabetes reversal. Four-week implantation of a 5-Fr. nylon catheter was used in subsequent studies. We were concerned that longer catheter implantation times could promote an excess collagen scar response but did not test these. Although the tempo of the inflammatory response and therefore the period of catheter indwell may vary between species, our observations are consistent with previous studies of subcutaneous devices in rodents (28). We did not test pro-

inflammatory markers in tissue immediately before or after islet transplantation, but measured these at 24 hours, 1 and 2 weeks after catheter implantation. Clearly, biomaterials could be engineered to further improve neovascularization responses in a DL subcutaneous site while minimizing fibrotic capsule formation. For example, zwitterionic polymers hinder the foreign-body response and increase vessel density around an implanted biosensor (22, 29).

Withdrawal of the catheter fragment after prevascularization could potentially impose mechanical or other stress responses at the time of islet implantation. We did not observe tract adhesion, bleeding or apparent tissue trauma after catheter withdrawal, immediately before islet transplantation.

We further acknowledge that the ~500 mouse islets used here represents a non-marginal graft. Had we transplanted fewer islets, we may have uncovered differences in engraftment efficiency between the KC and DL sites. Although the KC site has a track record of efficacy in mouse islet transplantation, it has not routinely afforded insulin independence in large animals or humans. KC transplantation of fetal pig islets in human type 1 diabetic patients undergoing kidney transplantation led to persistent urinary C-peptide excretion for >300 days (30), but the relevance of this approach to clinical cellular transplantation remains to be determined.

After intraportal transplantation, islets are initially avascular, denervated and isolated from contact with endogenous cells or extracellular matrix, resulting in delayed engraftment (31). Neovascularization initiates within the first two weeks and remodels extensively over months (32-34). Several previous studies have shown that islet neovessels are chimeric, consisting of both donor and host cells (35). Although we did not address this question here, we expect a similar chimeric vascular ingrowth/outgrowth response in the DL site.

The DL approach may be useful when transplanting islet cells derived from stem cells as the graft could be easily retrieved in the event of a local complication such as a teratoma, malignant transformation or unchecked hormone release. Islet progenitor cells may be more tolerant of hypoxia than are islets, and may induce local neovascularization during maturation. The DL site could provide a favorable environment and facilitate engraftment of both islets and insulin producing stem cells, especially in cases where intraportal delivery is contraindicated. Indeed, we have demonstrated herein return to the diabetic state in all cases promptly following excision of the subcutaneous implant tract, indicating graft depend euglycemia and a safe return to a pre-transplant state. Transplantation into the DL site also opens up the possibility of real-time, non-invasive imaging, including extra-hepatic labeling or techniques such as photoacoustic ultrasound(36), to monitor graft survival and immunological response. This is a clear limitation of clinical intraportal islet transplantation as practiced currently.

The relevance of our results to clinical transplantation remains to be determined. In BALB/c mice, which do not mount a strong foreign-body response, islet engraftment in the DL space was delayed compared with the KC site, but in C57BL/6 mice, which have a stronger foreign-body response, the time to normoglycemia was similar to that of the KC controls. By contrast, permanently implanted devices have traditionally failed in C57BL/6 mice but have worked well in alternative strains. We also showed that the DL site could support islet engraftment across a strong allogeneic barrier in mice, but only when tacrolimus immunosuppression is given to avert rejection. Thus, the DL site is not immunoprivileged. Notably, tacrolimus immunosuppression facilitated long-term islet engraftment despite the known diabetogenic side effects of this calcineurin inhibitor. Additionally, the DL site offers the possibility of co-transplanting immunoregulatory cells (e.g., mesenchymal stem cells or regulatory T cell cells) in a manner that

is not currently possible with a dispersed intrahepatic islet graft. Our results indicate that a stronger early inflammatory response actually favors rather than hinders neovascularization and islet engraftment in our model.

We further showed that a pre-existing diabetic state does not interfere with diabetes reversal rates, at least in mice. We cannot infer that a neovascularization response will occur to a similar degree in humans with longstanding diabetes, but it is of note that neovascularization remains a hallmark response to ischemia in end-stage secondary complications of clinical diabetes (37) (38, 39). Although we do not anticipate major limitations in neovascularization responses in humans with longstanding diabetes, this remains to be tested in first-in-human studies planned at the University of Alberta.

6.6. – REFERENCES

1. 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.

2. 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.

3. Shapiro AM, Ricordi C. islet Cell Transplant - Indications, Risks and Long-Term Outcomes. In: Kirk AD, Knechtle SJ, Larsen CP, Madsen JC, Pearson TC, Webber SA, (eds). Textbook of Organ Transplantation: Wiley-Blackwell, 2014: 1-1904.

4. Harlan DM, Kenyon NS, Korsgren O, Roep BO. Current advances and travails in islet transplantation. Diabetes 2009;58(10):2175-2184.

5. 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.

6. Ricordi C, Strom TB. Clinical islet transplantation: advances and immunological challenges. Nat Rev Immunol 2004;4(4):259-268.

7. 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.

8. Brissova M, Powers AC. Revascularization of transplanted islets: can it be improved? Diabetes 2008;57(9):2269-2271.

9. 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.

10. Merani S, Toso C, Emamaullee J, Shapiro AM. Optimal implantation site for pancreatic islet transplantation. Br J Surg 2008;95(12):1449-1461.

11. Veriter S, Gianello P, Dufrane D. Bioengineered sites for islet Cell Transplant. Curr Diab Rep 2013;13(5):745-755.

12. Nishimura R, Goto M, Sekiguchi S, Fujimori K, Ushiyama A, Satomi S. Assessment for revascularization of transplanted pancreatic islets at subcutaneous site in mice with a highly sensitive imaging system. Transplant Proc 2011;43(9):3239-3240.

13. Saito T, Ohashi K, Utoh R, Shimizu H, Ise K, Suzuki H et al. Reversal of diabetes by the creation of neo-islet tissues into a subcutaneous site using islet cell sheets. Transplantation 2011;92(11):1231-1236.

14. Sakata N, Aoki T, Yoshimatsu G, Tsuchiya H, Hata T, Katayose Y et al. Strategy for clinical setting in intramuscular and subcutaneous islet transplantation. Diabetes Metab Res Rev 2013.

15. Simeonovic CJ, Dhall DP, Wilson JD, Lafferty KJ. A comparative study of transplant sites for endocrine tissue transplantation in the pig. Aust J Exp Biol Med Sci 1986;64 (Pt 1):37-41.

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

17. Anderson JM, Rodriguez A, Chang DT. Foreign body reaction to biomaterials. Semin Immunol 2008;20(2):86-100.

18. Anderson JM. Biological response to materials. Annu Rev Mater Res 2001;31:81-110.

19. Kenneth Ward W. A review of the foreign-body response to subcutaneously-implanted devices: the role of macrophages and cytokines in biofouling and fibrosis. J Diabetes Sci Technol 2008;2(5):768-777.

20. Fujiwara N, Kobayashi K. Macrophages in inflammation. Curr Drug Targets Inflamm Allergy 2005;4(3):281-286.

21. van Amerongen MJ, Molema G, Plantinga J, Moorlag H, van Luyn MJ. Neovascularization and vascular markers in a foreign body reaction to subcutaneously implanted degradable biomaterial in mice. Angiogenesis 2002;5(3):173-180.

22. Grainger DW. All charged up about implanted biomaterials. Nat Biotechnol 2013;31(6):507-509.

23. 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.

24. Ricordi C, Lacy PE, Scharp DW. Automated islet isolation from human pancreas. Diabetes 1989;38 Suppl 1:140-142.

25. Sharkawy AA, Klitzman B, Truskey GA, Reichert WM. Engineering the tissue which encapsulates subcutaneous implants. I. Diffusion properties. J Biomed Mater Res 1997;37(3):401-412.

26. Sharkawy AA, Klitzman B, Truskey GA, Reichert WM. Engineering the tissue which encapsulates subcutaneous implants. III. Effective tissue response times. J Biomed Mater Res 1998;40(4):598-605.

27. Sharkawy AA, Klitzman B, Truskey GA, Reichert WM. Engineering the tissue which encapsulates subcutaneous implants. II. Plasma-tissue exchange properties. J Biomed Mater Res 1998;40(4):586-597.

28. Pileggi A, Molano RD, Ricordi C, Zahr E, Collins J, Valdes R et al. Reversal of diabetes by pancreatic islet transplantation into a subcutaneous, neovascularized device. Transplantation 2006;81(9):1318-1324.

29. Zhang L, Cao Z, Bai T, Carr L, Ella-Menye JR, Irvin C et al. Zwitterionic hydrogels implanted in mice resist the foreign-body reaction. Nat Biotechnol 2013;31(6):553-556.

30. Groth CG, Korsgren O, Tibell A, Tollemar J, Moller E, Bolinder J et al. Transplantation of porcine fetal pancreas to diabetic patients. Lancet 1994;344(8934):1402-1404.

31. Olsson R, Olerud J, Pettersson U, Carlsson PO. Increased numbers of low-oxygenated pancreatic islets after intraportal islet transplantation. Diabetes 2011;60(9):2350-2353.

32. Hellerstrom C, Andersson A, Korsgren O, Jansson L, Sandler S. Aspects of pancreatic islet transplantation in diabetes mellitus. Baillieres Clin Gastroenterol 1989;3(4):851-863.

33. Korsgren O, Lundgren T, Felldin M, Foss A, Isaksson B, Permert J et al. Optimising islet engraftment is critical for successful clinical islet transplantation. Diabetologia 2008;51(2):227-232.

34. Pileggi A, Ricordi C, Alessiani M, Inverardi L. Factors influencing Islet of Langerhans graft function and monitoring. Clin Chim Acta 2001;310(1):3-16.

35. Nyqvist D, Kohler M, Wahlstedt H, Berggren PO. Donor islet endothelial cells participate in formation of functional vessels within pancreatic islet grafts. Diabetes 2005;54(8):2287-2293.

36. Zhang HF, Maslov K, Stoica G, Wang LV. Functional photoacoustic microscopy for high-resolution and noninvasive in vivo imaging. Nat Biotechnol 2006;24(7):848-851.

37. Costa PZ, Soares R. Neovascularization in diabetes and its complications. Unraveling the angiogenic paradox. Life Sci 2013;92(22):1037-1045.

38. Tamarat R, Silvestre JS, Le Ricousse-Roussanne S, Barateau V, Lecomte-Raclet L, Clergue M et al. Impairment in ischemia-induced neovascularization in diabetes: bone marrow mononuclear cell dysfunction and therapeutic potential of placenta growth factor treatment. The Am J Pathol 2004;164(2):457-466.

39. Brekken RA, Overholser JP, Stastny VA, Waltenberger J, Minna JD, Thorpe PE. Selective inhibition of vascular endothelial growth factor (VEGF) receptor 2 (KDR/Flk-1) activity by a monoclonal anti-VEGF antibody blocks tumor growth in mice. Cancer Res 2000;60(18):5117-5124.

CHAPTER 7.

A NOVEL PRE-VASCULARIZED SUBCUTANEOUS SITE SAFELY ACCOMMODATES STEM CELL DERIVED THERAPIES FOR TREATING DIABETES

7. - A novel pre-vascularized subcutaneous site safely accommodates stem cell derived therapies for treating diabetes

Stem Cell & Transplantation Biology

http://elynsgroup.com
Copyright: © 2016 Gala-Lopez B, et al
Research Article | Journal of Stem Cell and Transplantation Biology | Open Access

A Novel Pre-Vascularized Subcutaneous Site Safely Accommodates Stem Cell Derived Therapies for Treating Diabetes

Gala-Lopez B[1,3], Pepper AR[1,3], Pawlick RL[1], Bruni A[1,3], Abualhassan N[1,3], Kin T[1,2], Keller G[4,5], Nostro MC[4,6,7], and Shapiro AMJ[1,2,3*]

[1]*Alberta Diabetes Institute, University of Alberta, Edmonton, Alberta, Canada*
[2]*Clinical Islet Transplant Program, University of Alberta, Edmonton, Alberta, Canada*
[3]*Canadian Transplant Research Program (CNTRP)*
[4]*McEwen Centre for Regenerative Medicine, Toronto, Ontario, Canada*
[5]*Princess Margaret Cancer Centre, University of Toronto, Toronto, Ontario, Canada*
[6]*Toronto General Research Institute (TGRI), Toronto, Ontario, Canada*
[7]*Department of Physiology, University of Toronto, Toronto, Ontario, Canada*

Received Date: March 23, 2016, **Accepted Date:** June 07, 2016, **Published Date:** June 13, 2016.

**Corresponding authors: A.M. James Shapiro, Clinical Senior Scholar Alberta Innovates - Healthcare Solutions (AIHS), Edmonton, Alberta, Canada, Tel: 780-407-7330; Fax: 780-407-8259; Email: amjs@islet.ca*

Abstract

Islet transplantation has become an important treatment modality for Type 1 Diabetes Mellitus (T1DM); nonetheless, the procedure may be limited by donor availability. An alternative has been the increasing use of cellular therapies derived from human Embryonic Stem Cells (hESC), showing very promising results in maturation, yield and ultimately, in insulin secretion in response to adequate stimuli. We recently developed a new technique for cellular transplantation under the skin. This manuscript evaluates the capabilities of the pre-vascularized Device-Less (DL) site to allow transplantation of Pancreatic Endoderm (PE) cells differentiated from hESC to treat diabetes mellitus. Fifty immunodeficient mice, n = 25 diabetic and n = 25 non-diabetic, were transplanted with PE cells. Animals were followed for 22 weeks and grafts were retrieved to evaluate engraftment and subsequent maturation. Diabetic mice showed slightly better engraftment (48% vs. 36%, $p = 0.19$) and secreted higher concentration of human C-peptide upon glucose stimulation (0.32 ± 0.15 ng/mL vs. 0.13 ± 0.09 ng/mL, $p = 0.30$), although differences were not significant. This maturation was not sufficient to successfully reverse diabetes. Monomorphic cystic changes were detected in 12% and 8%, respectively (diabetics vs. non-diabetics, $p = 0.32$) and all grafts seemed to be adequately contained by the surrounding collagen wall within the DL space. Our findings support the capabilities of the DL site to host PE cells and allow safe maturation as a new strategy to treat diabetes.

Keywords: Islet Transplantation; Embryonic Stem Cells; Cell Engraftment; Cell Maturation

Abbreviations

IT: Islet Transplantation; T1DM: Type 1 Diabetes Mellitus; hESC: Human Embryonic Stem Cells; DL: Device-Less; PE: Pancreatic Endoderm; DL: Device-Less; H&E: Hematoxylin and Eosin; STZ: Streptozotocin; SEM: Standard Error of the Mean.

Introduction

The recent advances in immunotherapy have allowed Islet Transplantation (IT) to become a mainstay treatment for Type 1 Diabetes Mellitus (T1DM). Today, the procedure is safer and long-term graft survival is comparable to that of pancreas transplant alone, with a reduced risk for complications [1,2]. Nonetheless, the IT procedure is limited by donor availability and usage. Significant variability is associated with this treatment modality and many factors may affect the successful utilization of a donated pancreas. In fact, the entire donation-transplant process depends upon many variables related to the donor clinical characteristics, the type of donation (living, brain death, cardiac death, etc.), the outcomes of islet isolation, and recipient characteristics. As a consequence, the process is not always efficient and like other transplant types, the demand may surpass the available donation pool.

An alternative to IT may be to use renewable sources for insulin secretion from proliferative stem cell populations. In particular, research using insulin-producing cells derived from human embryonic stem cells (hESC) has shown very promising results in maturation yield and ultimately, in insulin secretion in response to adequate stimuli [3-6]. The focus is now on optimizing the existing differentiation protocols to allow for a successful and stable diabetes reversal. However, finding the most efficient transplant site remains a dilemma given the infusion volume needed at the time of transplant and the potential need for graft retrieval in the event of tumor formation [7,8]. These reasons are a deterrent to use the conventional intra portal route for this transplantation modality.

Our group recently described a novel pre-vascularized Device-Less (DL) technique for cell transplantation in the subcutaneous space [9]. This approach was successful in reversing diabetes with mouse and human islets and is currently being used for other cell therapies. We herein describe the use of the DL technique to safely allow engraftment and maturation of Pancreatic Endoderm (PE) cells derived from a hESC line in an experimental xeno-transplant model of diabetes.

Materials and Methods

Human Embryonic Stem Cells-derived Pancreatic Endoderm

Pancreatic Endoderm (PE) cells derived from a human embryonic cell line were kindly provided by Drs. M.C. Nostro and G. Keller at the McEwen Centre for Regenerative Medicine in Toronto. Their differentiation protocol uses a combination of cytokines and small molecules to simulate pancreatic development and produces multipotent pancreatic progenitor cells with the potential to differentiate into all pancreatic lineages [10,11]. At the time of transplant, cells were harvested and shipped overnight to Edmonton for immediate implantation.

Transplantation of PE cells

Immunodeficient 8-12 week B6.129S7-Rag1^{tm1Mom} mice (Jackson Laboratory, Bar Harbor, ME, USA) were used for all experiments. Animals (n = 50) were housed under conventional conditions with access to food and water *ad libitum* and their care was in accordance with guidelines approved by the Canadian Council on Animal Care.

The DL space was created as previously reported by inserting a nylon catheter subcutaneously in the left lower abdomen and left for five weeks before transplant [9].

Note: A version of this section was published as an Original Paper in *J Stem Trans Bio* 2016; 2(1):107.

RESEARCH ARTICLE

TITLE: A novel pre-vascularized subcutaneous site safely accommodates stem cell derived therapies for treating diabetes

AUTHORS: Boris L. Gala-Lopez[1,3], Andrew R. Pepper[1,3], Rena L. Pawlick[1], Antonio Bruni[1,3], Nasser Abualhassan[1,3], Tatsuya Kin[1,2], Gordon Keller[4,5], M. Cristina Nostro[4,6,7*], A.M. James Shapiro[1,2,3*]

AFFILIATIONS:

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

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

[3]Canadian Transplant Research Program (CNTRP)

[4]McEwen Centre for Regenerative Medicine, Toronto, Ontario, Canada

[5]Princess Margaret Cancer Centre, University of Toronto, Toronto, Ontario, Canada

[6]Toronto General Research Institute (TGRI), Toronto, Ontario, Canada

[7]Department of Physiology, University of Toronto, Toronto, Ontario, Canada

[*]Denotes senior author

CORRESPONDING AUTHOR:

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

Fellow of the Royal Society of Canada

Canada Research Chair in Transplant Surgery and Regenerative Medicine

Professor of Surgery, Medicine and Surgical Oncology

AHS Director Clinical Islet and Living Donor Liver Transplant Programs

Clinical Senior Scholar Alberta Innovates - Healthcare Solutions (AIHS)

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

tel. (780) 407 7330 fax. (780) 407 8259 Email: amjs@islet.ca

7.1. - ABSTRACT

Islet transplantation has become an important treatment modality for type 1 diabetes mellitus; nonetheless, the procedure may be limited by donor availability. An alternative has been the increasing use of cellular therapies derived from human embryonic stem cells (hESC), showing very promising results in maturation, yield and ultimately, in insulin secretion in response to adequate stimuli. We recently developed a new technique for cellular transplantation under the skin. This manuscript evaluates the capabilities of the pre-vascularized Device-Less (DL) site to allow transplantation of pancreatic endoderm (PE) cells differentiated from hESC to treat diabetes mellitus. Fifty immunodeficient mice, n=25 diabetic and n=25 non-diabetic, were transplanted with PE cells. Animals were followed for 22 weeks and grafts were retrieved to evaluate engraftment and subsequent maturation. Diabetic mice showed slightly better engraftment (48% vs. 36%, p=0.19) and secreted higher concentration of human C-peptide upon glucose stimulation (0.32 ± 0.15 ng/mL vs. 0.13 ± 0.09 ng/mL, p=0.30), although differences were not significant. This maturation was not sufficient to successfully reverse diabetes. Monomorphic cystic changes were detected in 12% and 8%, respectively (diabetics vs. non-diabetics, p=0.32) and all grafts seemed to be adequately contained by the surrounding collagen wall within the DL space. Our findings support the capabilities of the DL site to host PE cells and allow safe maturation as a new strategy to treat diabetes.

7.2. – INTRODUCTION

The recent advances in immunotherapy have allowed islet transplantation (IT) to become a mainstay treatment for type 1 diabetes mellitus (T1DM). Today, the procedure is safer and long-term graft survival is comparable to that of pancreas transplant alone, with a reduced risk for complications (1, 2). Nonetheless, the IT procedure is limited by donor availability and usage. This restriction certainly poses a significant variability to this treatment modality, given the many factors that may affect the successful utilization of a donated pancreas. In fact, the entire donation-transplant process depends upon many variables related to the donor clinical characteristics, the type of donation (living, brain death, cardiac death, etc.), the outcomes of islet isolation, and recipient characteristics. As a consequence, the process is not always efficient and like other transplant types, the demand may surpass the available donation pool.

An alternative to IT may be to use renewable sources for insulin secretion from proliferative stem cell populations. In particular, research using insulin-producing cells derived from human embryonic stem cells (hESC) has shown very promising results in maturation yield and ultimately, in insulin secretion in response to adequate stimuli (3-6). The focus is now on optimizing the existing differentiation protocols to allow for a successful and stable diabetes reversal. However, finding the most efficient transplant site remains a dilemma given the infusion volume needed at the time of transplant and the possibility of retrieving the graft upon a potential tumor transformation (7, 8). These reasons are a deterrent to use the conventional intraportal route for this transplantation modality.

Our group recently described a novel pre-vascularized Device-Less (DL) technique for Cell Transplant in the subcutaneous space (9). This approach was successful in reversing diabetes with mouse and human islets and is currently being used for other cell therapies.
We herein describe the use of the DL technique to safely allow engraftment and maturation of pancreatic endoderm (PE) cells derived from a hESC line in an experimental xenotransplant model of diabetes.

7.3. - MATERIALS AND METHODS

7.3.1. - Human Embryonic Stem Cells-derived Pancreatic Endoderm

Pancreatic Endoderm (PE) cells derived from a human embryonic cell line were provided by Drs. M.C. Nostro and G. Keller at the McEwen Centre for Regenerative Medicine in Toronto. Their differentiation protocol uses a combination of cytokines and small molecules to simulate pancreatic development and produces multipotent pancreatic progenitor cells with the potential to differentiate into all pancreatic lineages (10, 11). At the time of transplant, cells were harvested and shipped overnight to Edmonton for immediate implantation.

7.3.2. - Transplantation of PE cells

Immunodeficient 8-12 week B6.129S7-Rag1^{tm1Mom} mice (Jackson Laboratory, Bar Harbor, ME, USA) were used for all experiments. Animals (n=50) were housed under conventional conditions

with access to food and water *ad libitum* and their care was in accordance with guidelines approved by the Canadian Council on Animal Care.

The DL space was created as previously reported by inserting a nylon catheter subcutaneously in the left lower abdomen and left for 5 weeks before transplant (9).

Diabetes was chemically induced by intraperitoneally injecting 180mg/kg of streptozotocin (STZ; Sigma-Aldrich, ON, Canada) in half of the recipients, one week prior to transplantation. Mice were considered diabetic after two consecutive blood glucose measurements ≥11.3 mmol/L (350 mg/dL).

Two groups of mice (diabetics and non-diabetics, n=25/group) were transplanted with approximately $7x10^6$ PE cells using the DL technique. Animals in the diabetic group also received two consecutive insulin-releasing pellets (LinBit®; LinShin Canada Inc. Toronto, ON, Canada - ~0.1U insulin/24 h/30 d) to maintain health for the duration of the study (160 days). A separate group of four mice (2 diabetics and 2 non-diabetics) were transplanted with same amount of PE cells and sacrificed four weeks post-transplant for early assessment of the graft. All mice were continuously monitored for general health, weight gain and non-fasting blood glucose, as well as the occurrence of tumor formation.

7.3.3. - C-peptide Measurements

Blood samples were also obtained at post-transplant week 4, 8, 12, 16, 20 and 22 to quantify stimulated human C-peptide concentration in plasma. Mice from both groups were fasted overnight and whole blood was collected after intraperitoneal injection of glucose (2 g/kg).

Quantification of C-peptide was performed using human-specific ultrasensitive ELISA (Mercodia, Uppsala, Sweden. Detection range: 5 - 280 pmol/L (0.015 – 0.85 ng/mL).

7.3.4. - Histology

Engrafted cells were analyzed at early (4 weeks post-transplant) and at the end of the study. Hematoxylin and eosin (H&E) and Masson's trichrome stains were used to visualize the grafts on abdominal wall sections and to assess tumor boundaries. Immunofluorescence was used to evaluate endocrine secretory function of long-term engrafted cells using anti-insulin (Dako A0082 – Alexa 568) and anti-glucagon (Abcam – Vector Fl-1000) antibodies. The procedure followed previously established methodology [9] and it included deparaffinization, primary and secondary antibody treatment and counter stain with DAPI (Invitrogen Molecular Probes. Eugene, Oregon). Slides were visualized using a fluorescent microscope with appropriate filters and AxioVision imaging software (Carl Zeiss Microscopy GmbH. Jena, Germany).

7.3.5. - Transplantation of Human Islets

In parallel, human islets were transplanted into 8-12 week B6.129S7-Rag1^{tm1Mom} diabetic mice and monitored for 22 weeks to compare human C-peptide secretion levels to those achieved by the study PE cells. The Clinical Islet Transplantation laboratory at the University of Alberta kindly provided human islets after the process of donation, isolation and culture, as reported in previous publications (12). Permission for these studies was granted by the Health Research Ethics Board of the University of Alberta, Edmonton, Alberta, Canada, and after written permission was obtained from donor families.

Mice were rendered diabetic by intraperitoneal injection of 180mg/kg streptozotocin (STZ, Sigma-Aldrich, ON, Canada). Animals were considered diabetic after two consecutive blood glucose measurements ≥11.3 mmol/L (350 mg/dL). Recipients (n=6 per group) received 0 IEQ (sham), 1,000 IEQ and 3,000 IEQ human islets from 3 different isolations. Islets from each isolation were randomly allocated to each group and transplanted under the kidney capsule as previously described (13). Mice in the Sham group were not diabetic and underwent the transplant procedure, but only received a saline solution under the kidney capsule. Animals in the STZ group were chemically induced diabetics and did not receive transplant, remaining diabetic throughout the entire study.

Animals were periodically monitored for general health, weight and blood glucose until endpoint (22 weeks) when blood samples were taken to determine basal and stimulated human C-peptide levels.

7.3.6. - Statistical Analysis

Data are represented as means ± standard error of the mean (SEM). Differences between groups were analyzed using t-test and one-way ANOVA with Tukey's post-hoc test. Z-score test was used to compare proportions between groups. All comparisons between groups were performed with a 95% confidence interval and a p-value <0.05 was considered significant. Analysis was performed using GraphPad Prism (GraphPad Software, La Jolla, CA, USA).

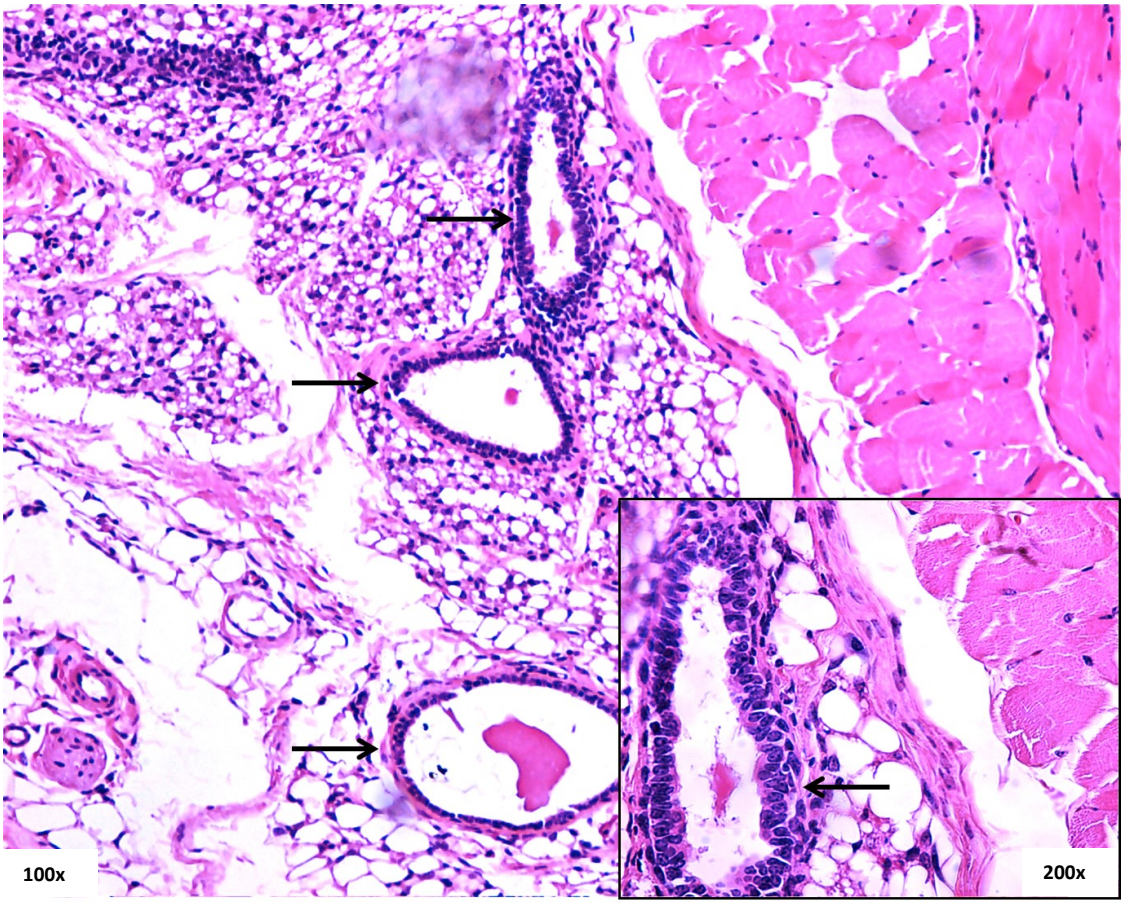


Figure 7.1 Early (4 week) graft visualization with hematoxylin & eosin stain, demonstrating formation of ductal structures (arrows) in the DL space. Inset: higher magnification (200x) microphotograph for better visualization.

7.4. – RESULTS

A total of 50 mice received $7x10^6$ PE cells/mouse subcutaneously, using the DL technique. Four weeks after transplantation, a viable graft was found in all four sacrificed animals, showing clear features of ductal formations (**Figure 7.1**). However, all animals remained diabetic and no detectable human C-peptide was found at this early time point (data not shown).

Twenty weeks after transplantation stimulated C-peptide was detected in both groups and continued to increase until the end of the study. Positive stimulated C-peptide was found in 12 of 25 (48%) mice in the diabetic group vs. 9 of 25 (36%), in non-diabetic (p=0.19). Mean stimulated C-peptide concentrations at 22 weeks were higher in the diabetic group although differences were not statistically significant (0.32 ± 0.15 ng/mL vs. 0.13 ± 0.09 ng/mL, p=0.30) (**Figure 7.2A**).

As expected, transplants with human islets rapidly reduced blood glycemia in mice, reaching normoglycemia at 12 days (1,000 IEQ) and 2 days (3000 IEQ), respectively. Animals in the STZ group remained hyperglycemic throughout the study period (**Figure 7.3**). When PE transplanted animals were compared to mice receiving a minimal and full mass of human islets, their C-peptide secretory profile was reduced (**Figure 7.2B**). PE transplanted in diabetic mice secreted on average 0.32 ng/mL, at 22 week of engraftment whereas mice receiving 1,000 IEQ and 3,000 IEQ secreted on average 2.46 ng/mL and 102.7 ng/mL respectively, after the same period of engraftment (3000IEQ vs. PE: p<0.001). C-peptide levels in both, Sham and STZ groups were under the detection limit for the assay.

By week 22 hESC were adequately engrafted and endocrine features were detected by immunofluorescence, with positive staining for glucagon and fewer cells containing insulin

(**Figure 7.4A-D**). Nonetheless, only one mouse in the diabetic group successfully achieved normoglycemia (1/25: 4%). The reduced amount of insulin in these cells contrasted with a significantly higher concentration in engrafted human islets, consistent with blood glucose normalization and positive stimulated C-peptide (**Figure 7.5**).

Monomorphic cystic changes were clinically detected in 3 of 25 (12%) diabetic mice vs. 2 of 25 (8%) non-diabetics (p=0.32). All cases presented with simple cysts without any clinical repercussion or malignant teratoma transformation. Upon microscopic examination, all cysts appeared fully surrounded and contained by the collagen wall delimiting the DL space and no ductal or endocrine structures were found outside the DL perimeter (**Figure 7.6**).

Table 7.1 summarizes the outcomes of the two study groups marking their corresponding similarities and differences, in terms of secretory function and cyst formation.

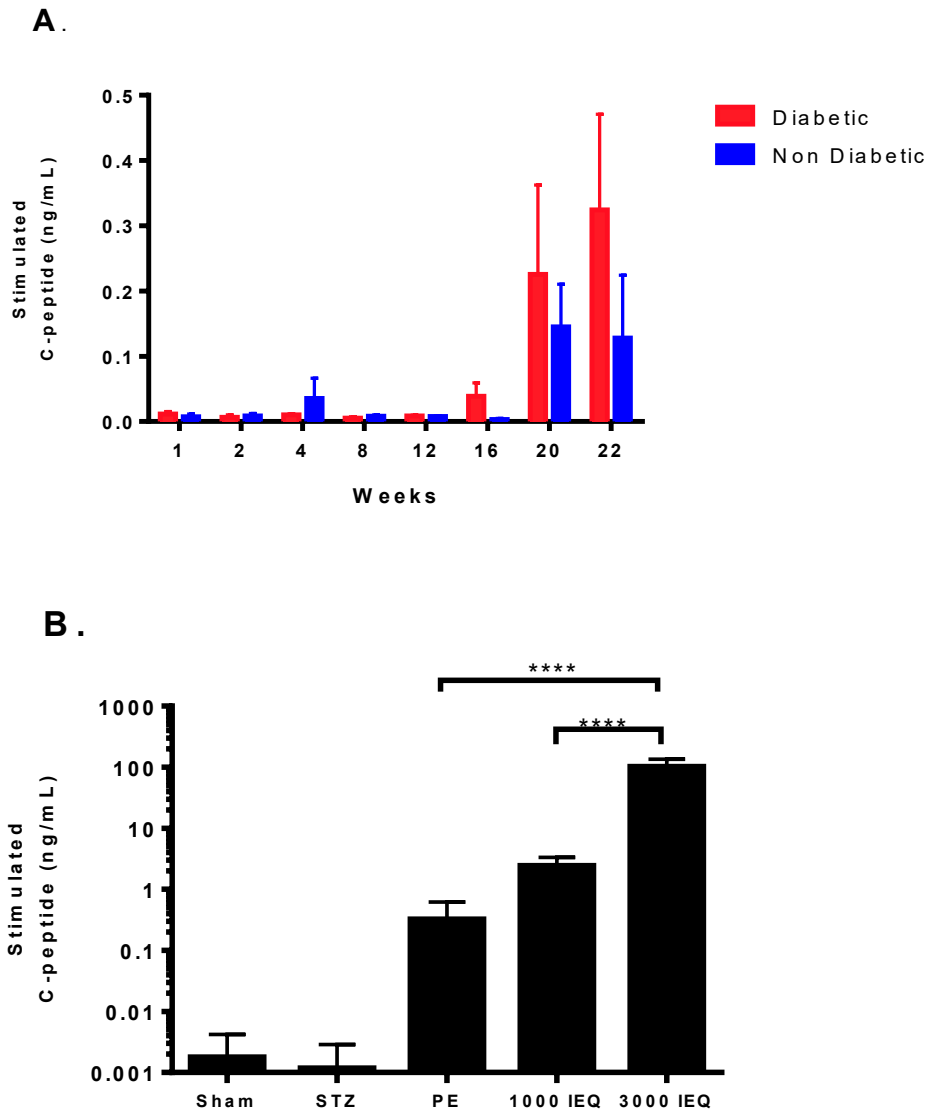


Figure 7.2 Stimulated C-peptide secretion from transplanted mice. A. Secretory profile over time for diabetic (red) and non-diabetic mice (blue), showing detectable C-peptide beyond 20 weeks post-transplant. **B**. Comparison of C-peptide secretory capabilities of transplanted PE vs. human islets at minimal and full dose transplanted in immunodeficient mice. Sham: non-diabetic mice with sham operation (n=6), STZ: diabetic animal with no transplant (n=6), PE: diabetic mice receiving hESC-derived PE cells (n=25). 1000 and 3000 IEQ: diabetic mice transplanted with minimal and full mas of human islets, respectively (n=6 each). Data represented as means ± s.e.m.

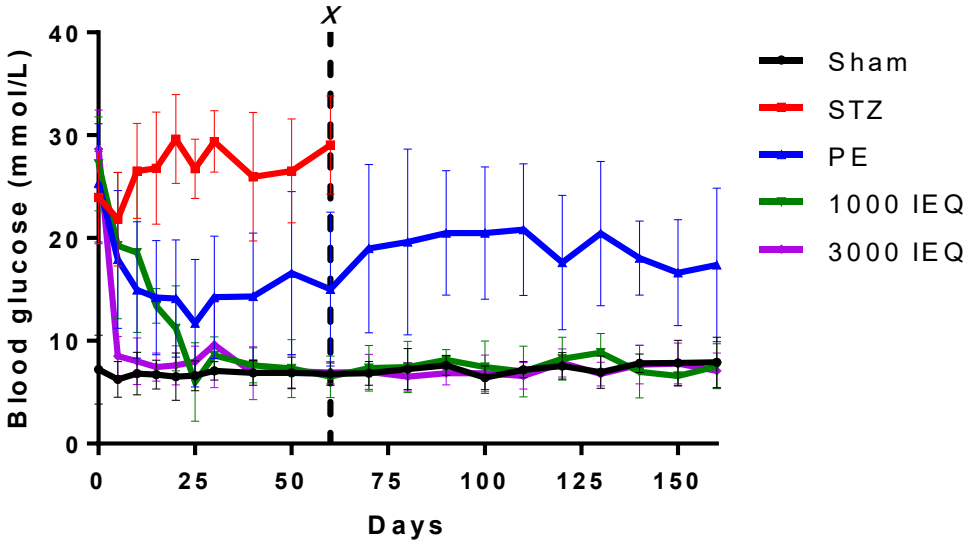


Figure 7.3 Blood glucose profiles from study groups. Day 0 represents time of transplant. Sham (black line, n=6) corresponds to non-diabetic animals receiving sham operation without transplant. STZ (red line, n=6) refers to chemically-induced diabetic mice with no transplant (shorter follow-up due to morbidity). PE (blue line, n=25) includes diabetic mice transplanted with PE cells. 1000 and 3000 IEQ (n=6 each, red and purple line, respectively) correspond to animals transplanted with human islets. Dotted line marked with X indicates the end of exogenous insulin (LinBit®) treatment for PE group. Data represented as means ± s.e.m.

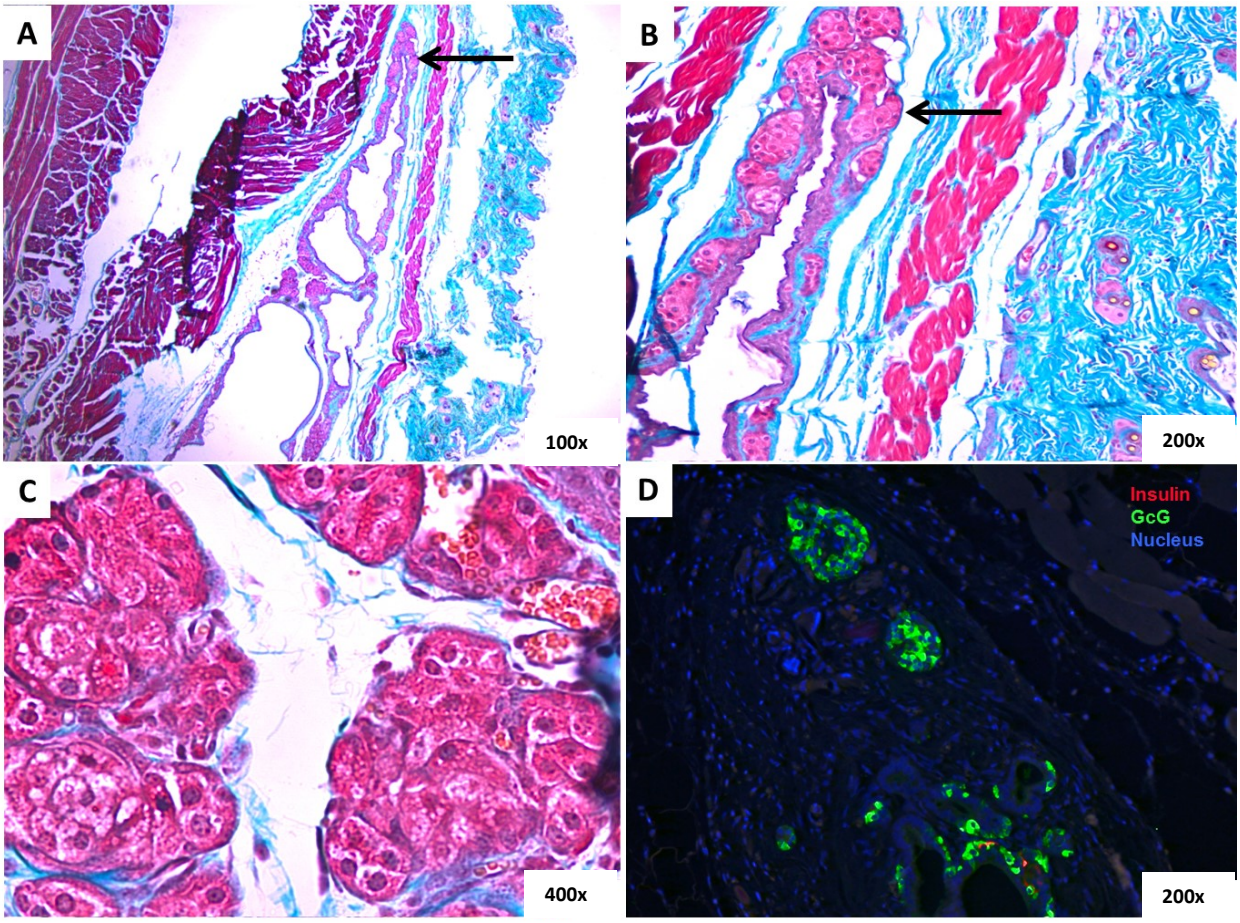


Figure 7.4 Engrafted hESC after 22 weeks of transplantation using the pre-vascularized subcutaneous DL site (Masson's Trichrome). **A – C**. Show islet-like cells around a ductal structure. **D**. Representative immunofluorescent slide staining the graft (arrows) for glucagon (GcG, green), insulin (red) and DAPI (blue).

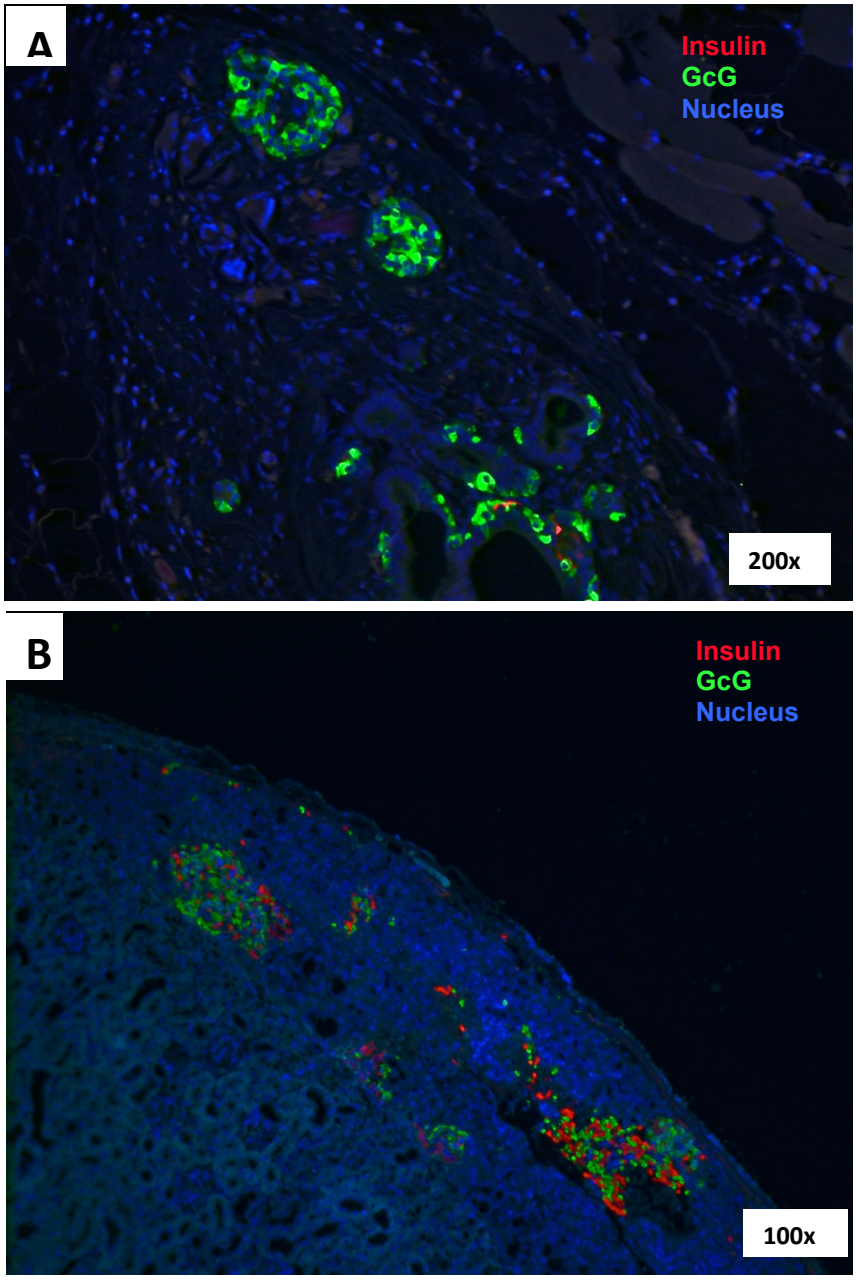


Figure 7.5. **Comparative endocrine staining of engrafted PE and human islets 22 weeks after transplant. A.** Representative immunofluorescent slide of engrafted PE staining for glucagon (GcG, green), insulin (red) and DAPI (blue). **B.** Representative immunofluorescent slide of human islet graft staining for glucagon (GcG, green), insulin (red) and DAPI (blue).

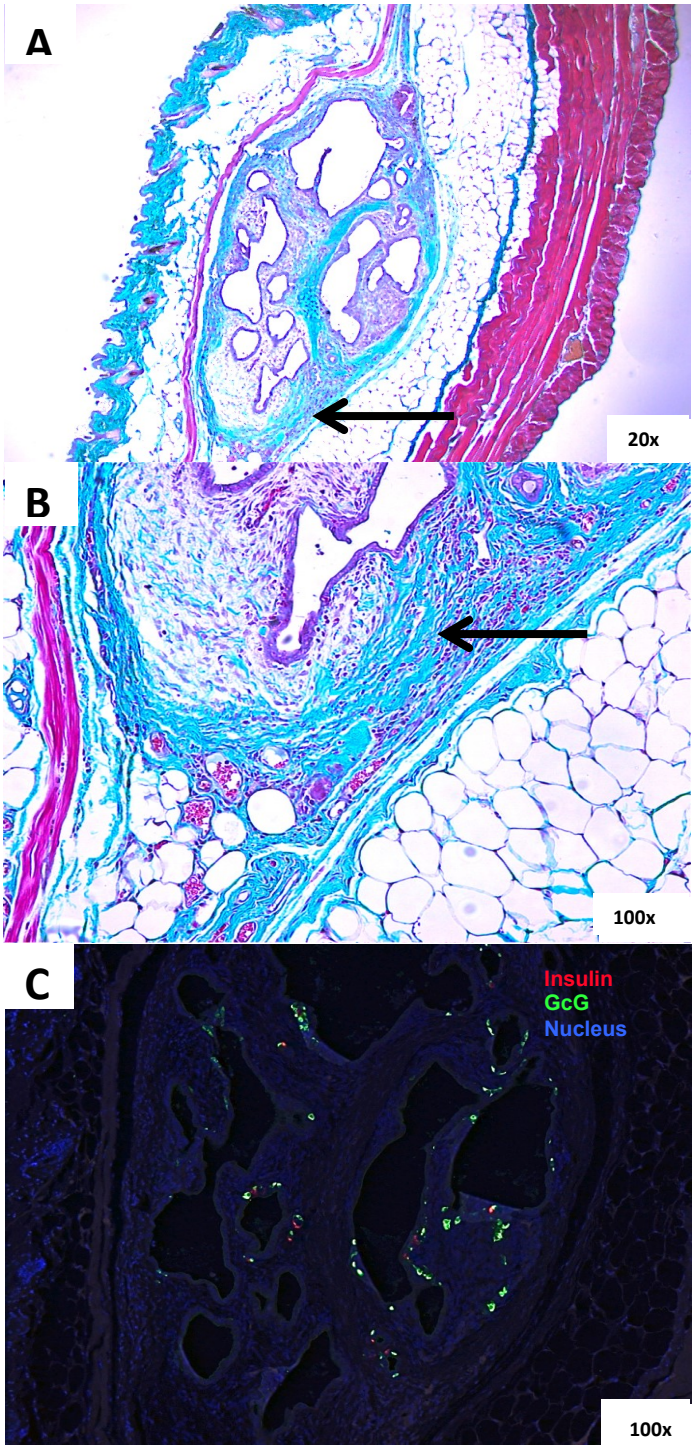


Figure 7.6 Benign cystic formations in one of the study animals. A and **B** show developing graft and surrounding tissue in the abdominal wall section. Notice blue collagen wall (arrows) around the DL site perimeter containing cystic structures. **C**. Corresponding immunofluorescent image of this cyst showing poor staining for endocrine markers at 15 weeks post-transplant.

7.5. – DISCUSSION

Our findings corroborate the utility of the DL technique to facilitate cell therapies. In this case, the aim was to engraft and mature PE cells derived from hESC, and measure indicators towards diabetes reversal.

In our study design we evaluated the potential effect of underlying hyperglycemia for engraftment and maturation of PE cells based on published evidence for accelerated maturation under a chronic hyperglycemic environment (14). Results confirmed an increased trend in both, maturation and mean concentration of stimulated human C-peptide levels measured at 22 weeks in diabetic mice. Differences however, did not reach statistical significance.

The process of effective differentiation of hESC is very complex and yet to be fully elucidated. Many authors agree on the multiple hurdles these cells encounter in the process of maturation and only recently, successful *in vivo* maturation have been reported with adequate glucose-response and occasionally, diabetes reversal (15-19).

Consistent with previous studies, the PE cells we tested in our experiments require a long (more than 5 months) *in vivo* maturation period (10, 17, 20, 21).

Despite observing adequate engraftment in almost half of the animals, glucagon staining was predominant in most of the histology samples and positive insulin cells were only occasionally found, which is consistent with the low levels of stimulated c-peptide detected at week 22, as well as failure to correct hyperglycemia. We speculate that longer in vivo maturation or a higher number of hESC-derived PE cells at the time of transplantation may be required to normalize glucose control in diabetic mice, although a more prominent insulin staining has been previously

Table 7.1 Outcome summary of 50 animals transplanted with insulin-producing stem cells. Maturation seems to occur more rapidly in diabetic mice, although differences did not reach statistical significance (95% confidence interval).

	Diabetic	Non-diabetic	p-value
Sample size	25	25	-
Proportion of detectable stimulated C-peptide	12/25 (48%)	9/25 (36%)	0.19
Mean stimulated C-peptide (ng/mL)	0.32 ± 0.15	0.13± 0.09	0.30
Monomorphic cystic formation (clinically detected)	3/25 (12%)	2/25 (8%)	0.32

reported when transplanting these cells in the kidney subcapsular space and mammary fat pad (10) (**Figure 7.7**). Further experimentation will definitely be required to fully understand the maturation process in this new transplant site.

One of the main limitations for the use of hESC is the inherit risk for teratoma transformation (7). This is one of the rationales for using alternative transplant sites like the DL technique where a dysfunctional/transforming graft may be easily retrieved. Current differentiation protocols are now focused on producing hESC preparations with a high grade of purity to avoid residual undifferentiated cells, which could potentially lead to tumor formation (22). In our series of transplants benign monomorphic cystic formations were present in 8 - 12% of cases and no teratoma was detected.

An interesting finding was that resulting cysts were successfully contained by the peripheral collagen wall present in the DL space during the 22-week observation period. This resulted in a restrictive effect similar to that present in other physical devices (14, 18). However, the real restraining capabilities in the settings of a true teratoma formation are still to be proven.

In conclusion, our subcutaneous DL technique has proven to be an adequate host for these human embryonic stem cells-derived pancreatic endoderm, allowing effective engraftment, maturation and added protection against tumor formations. This is certainly an important field of application for this technique and a starting point for further experimentation with improved cell preparation and transplant protocols.

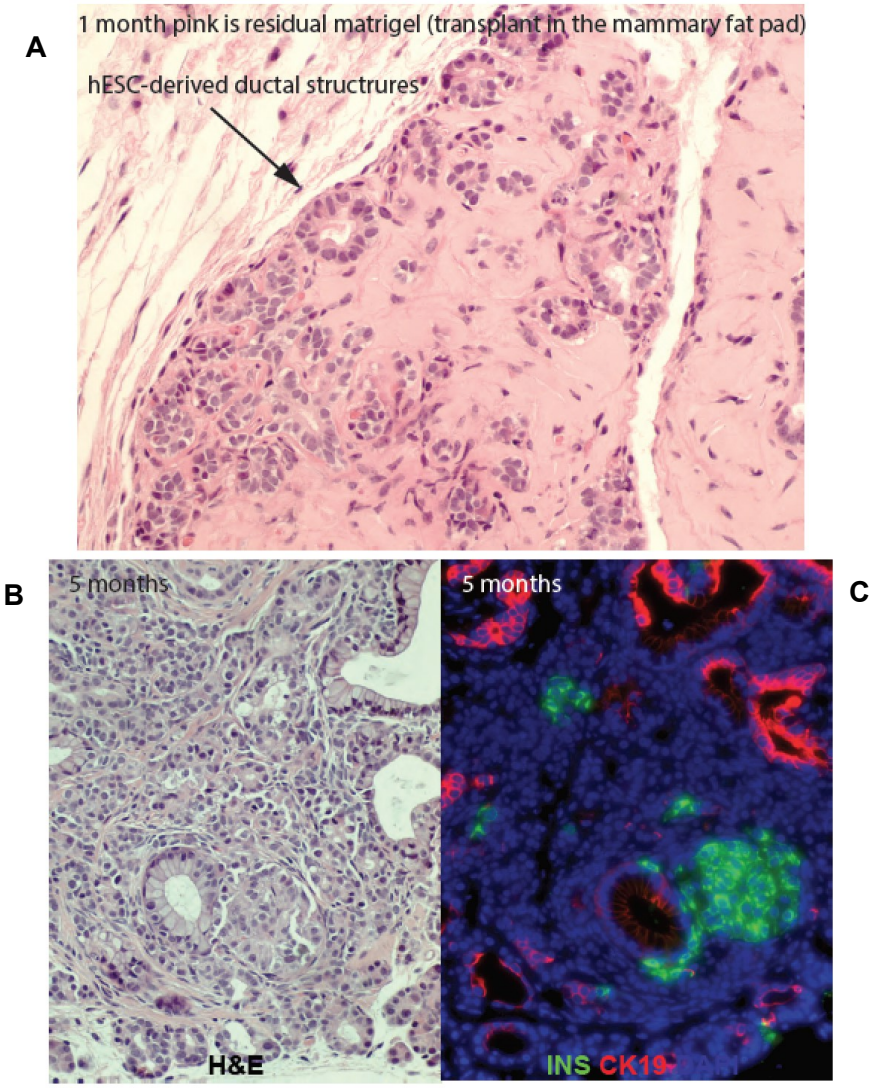


Figure 7.7 Engrafted pancreatic endoderm cells in the mammary fat pad at different time points. A. 1-month histology of the graft showing ductal structures. **B.** H&E stain of the graft, 5 month after transplantation, showing islet-like structure. **C.** Corresponding immunohistochemistry of the 5-month engrafted PE. Insulin (INS, green), CK-19 (ductal structure, red) and DAPI (blue). (Courtesy of MC.Nostro. Reproduced with permission).

7.6. – REFERENCES

1. Shapiro AM. Immune antibody monitoring predicts outcome in islet transplantation. Diabetes 2013;62(5):1377-1378.

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

3. Hua XF, Wang YW, Tang YX, Yu SQ, Jin SH, Meng XM et al. Pancreatic insulin-producing cells differentiated from human embryonic stem cells correct hyperglycemia in SCID/NOD mice, an animal model of diabetes. PloS One 2014;9(7):e102198.

4. Gioviale MC, Bellavia M, Damiano G, Lo Monte AI. Beyond islet transplantation in diabetes cell therapy: from embryonic stem cells to transdifferentiation of adult cells. Transplant Proc 2013;45(5):2019-2024.

5. Bose B, Shenoy SP, Konda S, Wangikar P. Human embryonic stem cell differentiation into insulin secreting beta-cells for diabetes. Cell Biol Int 2012;36(11):1013-1020.

6. Hrvatin S, O'Donnell CW, Deng F, Millman JR, Pagliuca FW, DiIorio P et al. Differentiated human stem cells resemble fetal, not adult, beta cells. Proc Natl Acad Sci U S A 2014;111(8):3038-3043.

7. McCall MD, Toso C, Baetge EE, Shapiro AM. Are stem cells a cure for diabetes? Clin Sci (Lond) 2010;118(2):87-97.

8. 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.

9. 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.

10. Nostro MC, Sarangi F, Yang C, Holland A, Elefanty AG, Stanley EG et al. Efficient generation of NKX6-1+ pancreatic progenitors from multiple human pluripotent stem cell lines. Stem Cell Reports 2015;4(4):591-604.

11. Korytnikov R, Nostro MC. Generation of polyhormonal and multipotent pancreatic progenitor lineages from human pluripotent stem cells. Methods 2016; 101:56-64.

12. Gala-Lopez BL, Pepper AR, Pawlick RL, O'Gorman D, Kin T, Bruni A et al. Antiaging Glycopeptide Protects Human Islets Against Tacrolimus-Related Injury and Facilitates Engraftment in Mice. Diabetes 2016;65(2):451-462.

13. Li D-s, Hao J, Yuan Y-H, Yun SH, Feng J-B, Dai L-J et al. Pancreatic islet transplantation to the renal subcapsule in mice. Protoc exch 2011. doi:10.1038/protex.2011.221.

14. Bruin JE, Rezania A, Xu J, Narayan K, Fox JK, O'Neil JJ et al. Maturation and function of human embryonic stem cell-derived pancreatic progenitors in macroencapsulation devices following transplant into mice. Diabetologia 2013;56(9):1987-1998.

15. Nostro MC, Keller G. Generation of beta cells from human pluripotent stem cells: Potential for regenerative medicine. Semin Cell Dev Biol 2012;23(6):701-710.

16. Pagliuca FW, Millman JR, Gurtler M, Segel M, Van Dervort A, Ryu JH et al. Generation of functional human pancreatic beta cells in vitro. Cell 2014;159(2):428-439.

17. Rezania A, Bruin JE, Arora P, Rubin A, Batushansky I, Asadi A et al. Reversal of diabetes with insulin-producing cells derived in vitro from human pluripotent stem cells. Nat Biotechnol 2014;32(11):1121-1133.

18. D'Amour KA, Bang AG, Eliazer S, Kelly OG, Agulnick AD, Smart NG et al. Production of pancreatic hormone-expressing endocrine cells from human embryonic stem cells. Nat Biotechnol 2006;24(11):1392-1401.

19. Russ HA, Parent AV, Ringler JJ, Hennings TG, Nair GG, Shveygert M et al. Controlled induction of human pancreatic progenitors produces functional beta-like cells in vitro. EMBO J 2015;34(13):1759-1772.

20. Rezania A, Bruin JE, Riedel MJ, Mojibian M, Asadi A, Xu J et al. Maturation of human embryonic stem cell-derived pancreatic progenitors into functional islets capable of treating pre-existing diabetes in mice. Diabetes 2012;61(8):2016-2029.

21. Kroon E, Martinson LA, Kadoya K, Bang AG, Kelly OG, Eliazer S et al. Pancreatic endoderm derived from human embryonic stem cells generates glucose-responsive insulin-secreting cells in vivo. Nat Biotechnol 2008;26(4):443-452.

22. Cunningham JJ, Ulbright TM, Pera MF, Looijenga LH. Lessons from human teratomas to guide development of safe stem cell therapies. Nat Biotechnol 2012;30(9):849-857.

CHAPTER 8

CONCLUSIONS AND FUTURE DIRECTIONS

8.1. - OVERVIEW

Type 1 diabetes is a chronic, progressive autoimmune disease that results from the immune-mediated destruction of the insulin-producing β-cells within the pancreatic islets. Chronic micro and macrovascular complications are a major source of morbidity and mortality in diabetic patients, with data suggesting that better glucose management results in significant declines in renal failure and total mortality (1-4).

Significant progress has occurred in the outcome of clinical islet transplantation, reflecting improvement in non-diabetogenic immunosuppression and preparation of sufficient quantities of highly viable islets for transplantation (5). Solitary islet transplantation has now become an accepted modality to stabilize frequent hypoglycemia or severe glycemic lability in highly selected subjects with poor diabetic control, resistant to standard, intensive or insulin-pump based therapies (3, 4). Sustained C-peptide production and successful insulin independence after pancreatic islet transplantation in type 1 diabetic patients was reported years ago by the Edmonton group (5). This reality became possible with the use of newer, more potent immunosuppressive agents, avoidance of corticosteroids, and high-quality islet preparations, although typically two islet infusions are still necessary to attain insulin independence on a routine basis.

A decade of research working to improve intrahepatic islet delivery has 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.

However, the hepatic portal vasculature may be considered as a hostile environment that limits islet engraftment and function (6).

Since many more islets must be transplanted to reverse diabetes, a significant portion of the transplanted islets fail to engraft. It has been estimated that up to 70% of the transplanted β-cell mass may be destroyed in the early post-transplant period. The major factor that negatively influences islet survival likely reflects a nonimmune-mediated physiological stress, namely prolonged hypoxia during the revascularization process, which can take up to 2 weeks (7). This phenomenon is also seen in islet autotransplantation after total pancreatectomy as described in **Appendix C**, where there is a presumption that allo- and autoimmunity do not play a role in the demise of transplanted islets (8). Tissue factor expression and release in isolated islets may also negatively influence the engraftment of transplanted islets through the instant blood-mediated inflammatory reaction and subsequent platelet activation, clot formation, and lymphocyte recruitment (9).

During this engraftment period, the islets are continuously exposed to immunosuppressive drugs, including tacrolimus and sirolimus, which are known to adversely impact β-cell survival and function (10, 11). 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 (12).

Given the limited supply of cadaveric donor pancreata and the prevalence of type 1 diabetes, considerable efforts have been made to prevent the loss of islet mass in the immediate post-transplant period. Many studies targeted at enhancing islet survival during the early post-transplant period have been published, and a variety of different strategies have been tested (13, 14).

An important contribution has come from participating centers in the Clinical Islet Transplantation Consortium (CIT), which is a scientific network to conduct studies on islet transplantation. This organization has performed studies to establish the IT as a valid therapeutic method to treat selected T1DM patients. In Europe and in Alberta, Canada, islet transplantation is covered by their federal health care systems. However, in the United States, clinical islet transplantation remains experimental and investigational. The Clinical Islet Transplant Consortium is conducting two pivotal Phase III clinical trials (CIT-06 & CIT-07, Clinical Trials.gov NCT00468117 and NCT00434811, respectively) in selected specialized islet transplantation centers (Universities of: Minnesota, Pennsylvania, Miami, Emory, Northwestern, Chicago, California San Francisco and Alberta), to support the FDA biological license application mandate (15). The anticipated results from these two trials should be published in the near future and will likely lead to successful licensure, allowing islet transplantation to be recognized as reimbursable therapy for patients with type 1 diabetes. The outcome of the biological licensing applications and in light of the significant advances that have been made in the new era of islet transplantation (2007-2010), which now demonstrated improved primary efficacy and safety outcomes with a 3 year insulin independent rate of 44%, with fewer islet infusions and adverse events per patient (1, 2, 16).

Of importance is also the experience presented in **Appendix C**, where the option of islet autotransplantation after total pancreatectomy is expanded beyond the boundaries of chronic pancreatitis to enter the controversial field of cancer. This case report does not pretend to be evidence for changing clinical practice, but a great opportunity to engage in discussions to evaluate this indication in very selected patients.

This chapter presents my insight on the new strategies to overcome many of the factors currently limiting the success of islet transplantation, from isolation to engraftment, and the potential mechanisms interfering in their successful survival. The chapter also includes a personal analysis on the value of implementing many of the current scientific breakthroughs and successfully incorporating a safe clinical transplantation with surrogate insulin-producing cells. Finally, general conclusions are provided.

8.2. – EMPOWERING DURABLE ISLET SURVIVAL

Successful islet transplantation relies on the ability to isolate healthy and durable islets, and in the availability of a well vascularized environment to allow engraftment and prolonged survival. Throughout this thesis, we have discussed the many phases of islet transplantation and the multiple hurdles islets endure along the process. New technologies and novel approaches allow the refinements of many of these steps to allow a significant improvement of beta cell health (**Figure 8.1**). I now present the most significant breakthroughs towards lasting islet engraftment and my personal view of the future directions of research to further improve transplantation.

8.2.1. - Donor selection

Identifying donor-based specific markers of islet isolation success may indeed provide a means of improving the success rates of the subsequent islet transplant. Previous single-center retrospective studies have identified several donor-related variables affecting islet isolation outcome; including but not limited to donor age, cause of death, body mass index (BMI), cold ischemia time, length of hospitalization, use of vasopressors, and blood glucose levels (17-22). O'Gorman and colleagues developed a scoring system based on donor characteristics that can predict islet isolation outcomes (23). This scoring system has proven to be effective in assessing whether a pancreas should be processed for islet isolation (21).

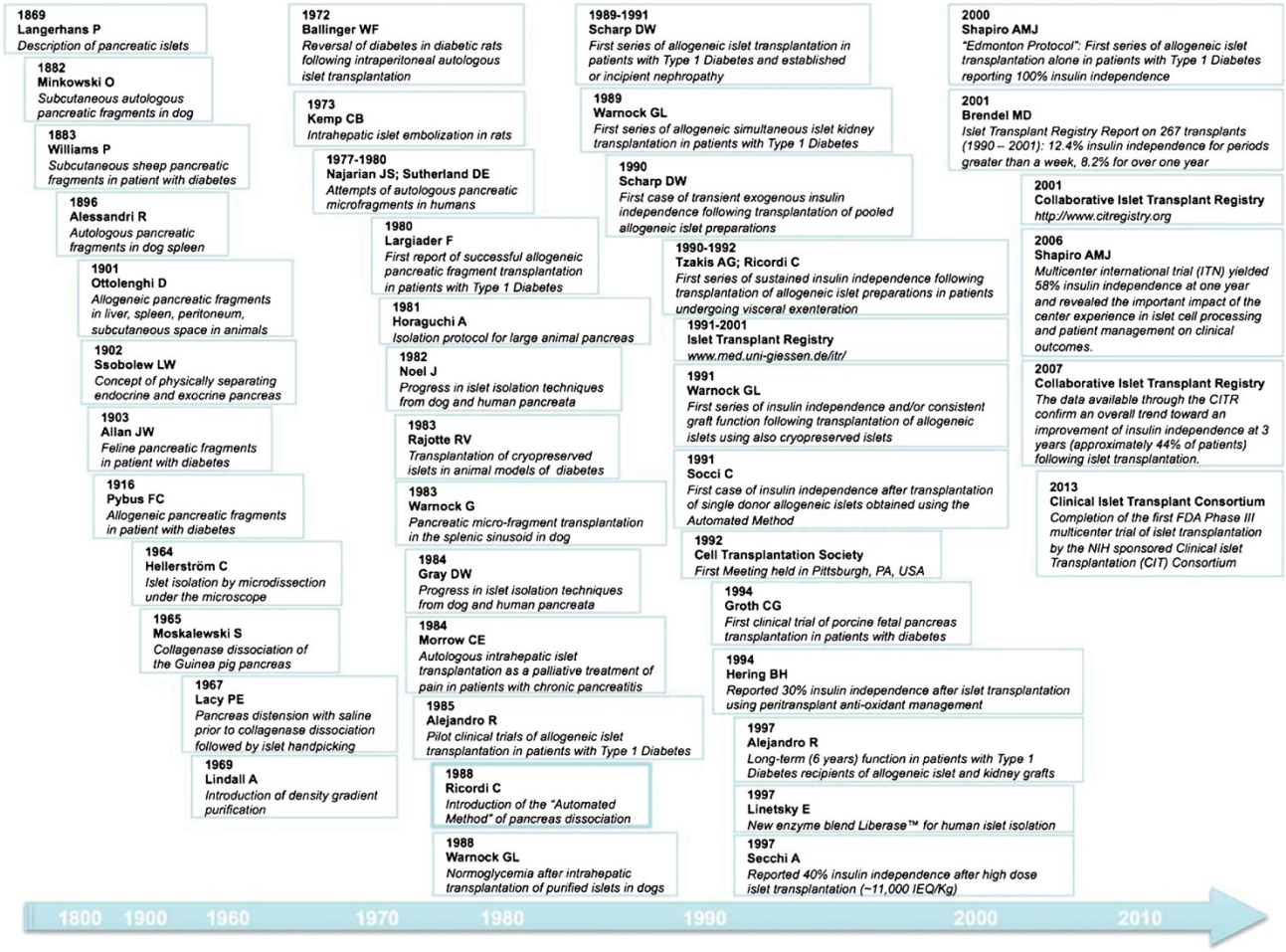


Figure 8.1 The evolution of pancreatic islet transplantation. Reproduced with permission from Piemonti L, Pileggi A. 25 Years of the Ricordi Automated Method for Islet Isolation. *CellR4* 2013; 1: e128.

It also allows for better management of the islet processing facility, as the cost of islet isolations is high. However, the actual impact of donor score on transplantation outcome is still unclear as it was developed solely based on islet isolation outcome.

Donation after cardiac death (DCD) is now one of the new challenges for islet transplantation. In experimental settings, islet yield and function derived from DCD pancreases seem to be comparable with those from their brain dead counterparts (24). However, in clinical settings, results are variable. Japan has one of the most extensive experiences using DCD donors for organ transplant. The Japanese groups have optimized retrieval in these type of donors, as well as the Kyoto preservation solution and the two-layer preservation method (25). Their most recent report for islet transplantation from this source shows that overall graft survival was 76.5%, 47.1%, and 33.6% at 1, 2, and 3 years, respectively; whereas corresponding graft survival after multiple transplantations was 100%, 80.0%, and 57.1%, respectively. All recipients remained free of severe hypoglycemia while three achieved insulin independence for 14, 79, and 215 days (26).

Our own experience at University of Alberta shows comparable results when using pancreases from both, neurological determination death donors and DCDs, with similar decrease in insulin requirements at 1 month post-transplant (64.8% vs. 60.2%, p=0.52, respectively) (24).

This is a clear indication of the potential benefits of DCD as an alternative source if used under strict releasing criteria, particularly in countries where heart-beating donors may not be readily available.

The long-term function of islets isolated from DCD however, remains unknown. Multiple experimental works report the significant injury resulting from the initial warm ischemia during withdrawal of support measures in those donors. This state promotes activation of different

injury pathways including danger-associated molecular patterns, accumulation of free radicals, caspase activation and ultimately, cell death, directly proportional to the total warm ischemia time after cessation of ventilation (27, 28). Prolonged accumulation of damaging metabolites may be associated with organ dysfunction after transplantation. This is one of the main reasons to establish a threshold at 30 minutes to accept organs for transplant, including pancreases for islets. Our recent series of isolations using pancreata from 15 DCD donors showed no significant differences in isolation yield or graft performance immediately after transplant, when donors progressed to asystole before the 45 minute wait period (24). The most appropriate wait period for warm ischemia in islet-dedicated pancreas is still unknown. The question now is, if results do worsen when warm ischemia is prolonged to the point where other organs cannot longer be used. Our unpublished observations with a modified mouse DCD model showed significant structural damage to the pancreas beyond 30 minutes, with a corresponding decreased yield after digestion. Unfortunately, the mouse DCD model for islet isolation has proven to be peculiar and not completely translatable to large animal and human islet isolation. I therefore believe that standard preservation of the pancreas may not be sufficient to expand the use of more marginal cases. New and more dynamic strategies are needed to expand the donor pool in this direction, and the option of using continuous preservation systems seems to be very attractive for this purpose.

8.2.2. - Pancreas preservation prior to islet isolation

Organs and tissue for transplantation are normally preserved in cold solutions to maintain tissue viability until transplantation. Pancreases for whole-organ transplant were traditionally perfused and stored with University of Wisconsin (UW). With the advent of Histidine-Tryptophane-Ketoglutarate (HTK) and Celsior solution for solid organ transplantation, multiple studies have compared their preservation outcomes, which are comparable for preservation periods under 12h - 15h. Above this period, HTK and Celsior are both associated with allograft edema, pancreatitis and vascular thrombosis (29-31).

According to a report from the National Islet Cell Resource Center Consortium in the USA, UW solution is the standard preservation solution prior to islet isolation (32), but during the last years, more pancreata are being stored in HTK solution. At the present time, there is no evidence that HTK solution is superior to UW regarding islet isolation outcome. However, cost advantages in utilization of HTK have granted popularity for this solution in organ preservation (29, 33-35).

Other modalities of static preservation include the two-layer method (TLM) using pre-oxygenated perfluorocarbon, which failed to demonstrate superiority to standard solutions in terms of pancreatic adenosine triphosphate level, islet yield, in vitro functional viability, and in vivo function after clinical transplantation (36) (20, 37). Another successful preservation agent is the Kyoto solution, originally developed by the Matsumoto group, containing trehalose and ulinastatin as distinct components, which are effective cytoprotective against again stress and inhibits trypsin, respectively (38). This solution has demonstrated favorable results in solid organ

transplantation and also in human islet isolation outcomes, such that it has become the standard preservation solution for Baylor's clinical islet transplant program (39, 40).

Recently, machine perfusion systems have been gaining increasing acceptance as a preservation method initially for kidneys from marginal donor and now is extended to every solid organ (41). Machine perfusion has several advantages over static cold storage. First, preservation solution can be continuously supplied directly to all cells at pressures and flow rates similar to a physiologic state. In addition, machine perfusion systems also allow real-time assessment of graft quality can by analysis measuring vascular pressures and resistance, as well as by analyzing various injury biomarkers in the perfusate. Moreover, these systems permit *ex vivo* pharmacologic manipulation of the organ, opening infinite possibilities of organ improvement before transplantation (42-45).

One of the main differences among the currently available systems is the temperature of preservation. The original works in machine perfusion were performed in hypothermic conditions based on lessons learned from static cold preservation and overwhelming evidence towards adequate tissue preservation when metabolic demands were significantly decreased. Early reports confirmed the value of this strategy with better islet yield and stimulation index compared to static preservation. Our center at the University of Alberta performed machine perfusion in 12 human pancreata using a LifePort^{TM} Kidney Transporter (Organ Recovery Systems, Des Plaines, IL, USA) (46). The first 4 pancreata were placed on the machine, after 10 hours of static preservation in UW, for up to 24 hours; metabolic and histologic changes of pancreata were assessed. It was found that tissue energy charge was maintained during the first 3 hours in the machine perfusion and thereafter it gradually decreased. Histologic analysis revealed that tissue edema became evident at 24 hours. The next eight pancreata were processed

for islet isolation after 6 hours of machine perfusion. Islet recovery and viability tended to be higher in pancreata preserved with the machine perfusion than in matched pancreata stored in static UW. These results are in accordance with the work of Leeser and colleagues who showed a feasibility of pump perfusion of human pancreata prior to islet isolation (47).

More recent evidence indicates that preserving organs in normothermic or subnormothermic state may be more beneficial and new systems are now developed to allow prolonged preservation of solid organs with novel perfusates designed to cope with the metabolic demands of organs now kept at room temperature (42). Various research groups are elucidating the multiple events that occur during normothermic or sub-normothermic *ex vivo* organ perfusion and trying to understand the potential parameters that may serve as predictors of function after transplant.

A very satisfying element is that many of these systems are now commercially available and are continuously being approved by regulatory agencies for human use. TransMedic, OrganOx and XVivo perfusion systems are one of the companies in the forefront for *ex vivo* organ perfusion systems. Our university is fully engaged in this exciting research within the scope of the CNTRP, launching multiple clinical trials in the field of liver, heart, lung, kidney and pancreas preservation. The preliminary results are indeed encouraging in terms of safety for patients. The next step would be to design strategies to treat those organs ex vivo with any or a combination of agents known to improve cell viability. A fully operational system for normothermic or subnormothermic preservation for pancreas is not commercially available yet. However, the current research will certainly result in a low-pressure system especially design for this gland, allowing longer periods for effective treatment with caspase inhibitors, antioxidants, AAGP or any other drug with proven benefits. The clear advantage is that pancreatic endocrine function

can be easily demonstrated while the organ is being perfused, allowing the clinical/technical team to choose the most appropriate time for transplantation or isolation depending on the purpose.

Another recent advancement in organ preservation is the use of persufflation. This technique uses gaseous oxygen perfusion to augment oxygen diffusion through the pancreatic tissue (48-53). In preclinical studies, this technique has demonstrated improved yields and viability (54). Based on this evidence we are currently evaluating the utility and efficacy of persufflation for clinical islet transplantation at the University of Alberta with the collaboration of McGill University and University of Toronto. Preliminary results are encouraging and should be reported in the coming months.

8.2.3. – Islet culture

Clinical islet transplantations rely on successful isolation of the human islets from donors followed by the in vitro culturing of the cells to maintain functionality until transplantation can be performed. Preservation of human islets in culture provides many benefits to clinical islet transplantation. First, it allows travel time for patients living away from transplant centers, as these procedures are conducted in specialized centers. Moreover, pre-transplant culture can provide attainment of therapeutic levels of immunosuppression before islet infusion. During the culture period, additional quality control testing can be undertaken, including microbiological and pyrogenic tests. In addition to these practical advantages, modification or treatment of islets through culture provides a strategic opportunity to promote islet survival after transplantation.

A number of issues may affect culture conditions and the quality of the final preparation, being the type of medium and the culture temperature, the two main determinants to avoid the risk of islet loss. Connaught Medical Research Laboratory 1066, originally designed for use with fibroblasts and kidney epithelial cells, appears to be the most widely used base medium for islet culture. Other supplements and additives vary depending on each center's preference and experience, and the final result should be a specific culture medium, which can adequately preserve islet quality. Examples of center-specific culture medium include: the Miami-Modified Medium-1 (MM1), Edmonton culture medium, the Memphis SFM medium (M-SFM) and the hCell OCZEM-SF/AF, among others (55, 56).

Albumin is known to contain many components with beneficial effect on cell survival. Animal serum such as fetal calf serum is traditionally added to culture media in experimental settings. However, when islets are destined for clinical transplantation, use of animal sera has been considered unacceptable because of potential risk associated with viral or prion-related disease transmission (57). Other potential problems of animal sera are evoking immune or inflammatory reactions in host against animal proteins (58-60), which cannot be diminished even by several washing steps (61). Therefore adding human serum albumin as an alternative is the current standard in clinical islet culture.

One of the major concerns with culturing islets is the uncertainty of islet recovery rate after culture. There is ample evidence of a reduction in the islet mass during culture. Bottino and colleagues reported that there was at most 80% reduction in DNA content in islet preparations following 24 hours culture (62). A very important area of investigation is the use of compounds capable of preventing cell loss during culture by improving islet health and enhancing their

function after transplant. This topic will be expanded further ahead when cytoprotective strategies are discussed.

Another area of investigation focuses in methods for reducing anoxia during culture. Isolated islets are especially susceptible to damage from anoxia due to their large size relative to single cells, high oxygen consumption rate, and low levels of enzymes necessary for energy production under anaerobic conditions (63). Islets cultured at high surface densities in standard T-flasks also exhibit low viable tissue recovery, viability, and potency, due to anoxic conditions. These effects have been prevented by culturing islets in gas-permeable devices, which increase oxygen availability to islets with clear benefits for clinical islet culture and shipment (63).

We had the opportunity to work with various prototypes of gas-permeable containers for culture based on the mounting evidence of the benefits of improved oxygen diffusion during islet culture. We were able to witness first-hand the advantages of incorporating such devices to experimental and clinical islet transplantation. First, gas-permeable flasks allowed larger seeding densities, still maintaining same islet quality (**Figure 8.2**). This effect provides the ability to accommodate large preparations in fewer flask and storage. Second, this technology was found to be convenient for transporting islets between centers, while preserving their integrity and function after transplantation. These findings are very encouraging and will allow further exchange of islets and potentially insulin-producing stem cells from manufacturing to transplanting centers.

8.2.4. - Assessment of islet preparations

In addition to the quantity of islets, the functional viability of an islet preparation is critical in predicting the success of islet transplantation. To date, there lacks a consensus within the islet transplantation field as to which assays accurately assess islet potency prior to transplantation and predicts their subsequent function post-transplant. The viability of an islet preparation is currently assessed with the use of fluorescent stains based on dye exclusion polarity. For example, fluorescein diacetate (FDAc) is a non-polar dye and passes through the plasma membrane of living cells, whereas propidium iodide (PI) can only enter cells that have a compromised membrane.

Figure 8.2 Gas permeable G-Rex culture flasks (Wilson Wolf, New Brighton, MN, USA) used in experimental and clinical transplantation at the University of Alberta, with shipments to University of Arizona. Photograph kindly provided by Wilson Wolf, reproduced with permission.

Using these two dyes together, the proportion of viable (green, FDA-positive) versus dead (red, PI-positive) cells can be assessed. FDA/PI is currently a widely used method for viability determination of the islet preparation prior to transplantation. These tests can be rapidly performed and are less labor intensive, making them attractive for use just prior to transplantation. However, there are several problems, making them of limited value. The main problem is that membrane integrity tests cannot distinguish between islets and non-islets. Another problem with the tests is the difficulty in assessing live/dead cells within a three-dimensional structure. In addition, these tests fail to measure the metabolic capacity of the islet preparation. Nevertheless, it is important to acknowledge that viability estimated by membrane integrity tests is predictive of some outcome measurements in clinical transplantation, according to an annual report from Collaborative Islet Transplant Registry (64).

Another important method to assess the quality of the islet preparation is the fractional beta cell viability, first described by Ichii and colleagues (65). The assay uses dissociated islets stained with a zinc specific dye, Newport Green (Molecular Probes, Eugene, OR, USA), and a mitochondrial dye, tetramethylrhodamine ethyl ester. Double positive cells are quantified using flow cytometry after dead cells are excluded using a DNA-binding dye (7-Aminoactinomycin D). Original reports showed that the beta cell specific viability of human islet preparations was a useful marker of the outcome of a mouse transplant assay. However, no hard evidence has been produced to associate this measurement with outcomes in clinical transplantation. The major limitation of this method is that dispersed single cells are not likely representative of the original islets, and the dissociation of the islet also may contribute to beta cell death, resulting in a false negative outcome. Moreover, necrotic cells or late-stage apoptotic cells are not counted as non-viable cells, thereby leading to overestimation.

A potentially more efficacious marker to determine the islet functional capacity is assaying for mitochondrial activity. Mitochondrial integrity is central to islet quality because mitochondria play a crucial role for glucose stimulated insulin secretion (66) and islet cell apoptosis (67). Mitochondrial activity can be evaluated using a variety of methods. These include OCR, detection of mitochondrial membrane potential using dyes, release of cytochrome c, and measurement of redox state. Papas and colleagues assessed OCR of human islet preparations normalized per DNA content (68). They were able to demonstrate that OCR/DNA assay predicts efficacy of human islets grafted into mice. A clear limitation for this assay however, is that it is not specific for islet, as all cells in the preparation will consume oxygen. Like membrane integrity test, the purity of the islet preparations significantly influences the assays precision. To circumvent this limitation, Sweet and colleagues developed a flow culture system (69) that allows to measure the OCR response in human islets, against glucose stimulation. They demonstrated that glucose stimulated changes in OCR were well correlated with in vivo function of human islet grafts, whereas glucose stimulation hardly increased OCR in non-islet tissue (70, 71).

Finally, an alternative approach for assessing islet preparation potency is to monitor the function of the engrafted beta cell mass. Matsumoto et al have developed the Secretory Unit of Islet Transplant Objects (SUITO) index using fasting C-peptide and fasting glucose, 1month post-transplant. This index has been efficacious in evaluating hypoglycemia post-transplant, the differences in outcome after living donor vs. cadaveric donors, or fresh vs. cultured islets, as well as single donor success rates, and overall clinical outcome after auto- and allografts (72-78). Despite the multiple assays to evaluate the quality of islets *in vitro*, there is no specific test to adequately predict islet potency and durability after transplant. Perhaps, the reason lies on the

multifactorial nature of islet engraftment and survival in humans. A good approach could be a combination of the multiple indexes currently available for each of the phases of islet transplantation. A multivariate analysis using donor index, fractional viability, OCR/DNA and PRA, along with an *in vitro* determination of the potential magnitude of IBMIR by pre-mixing recipient's blood with the islet preparation and measurement of: C-peptide released into the media (as an expression of cell death), thrombin-antithrombin complex, and a panel of pro-inflammatory cytokines. By combining all factors into the regression model, we could reach a predictor of function and durability of islets, close to what actually is happening in the patient. Such information could be a powerful tool to modify strategies to allow better outcome.

8.2.5. - Cytoprotective strategies during islet isolation

During the isolation procedure, the islets are exposed to numerous types of stress induced by non-physiologic stimuli. These include ischemic stress during organ, procurement, preservation and islet isolation, mechanical and enzymatic stress during digestion, and osmotic stress during purification. All these cumulative injury results in activation of various cellular damage pathways including danger-associated molecular patterns, accumulation of free radicals by oxidative stress and caspase activations, among others. These mechanisms will promote the release of a variety of pro-inflammatory cytokines, leading to a perpetuation of cellular damage and eventually, cell death.

A number of investigators have explored strategies to confer islet resistance to stress-induced damage. Most investigations have focused on modification to the isolation procedure, or treating islets during culture to protect the final product. Strategies are designed to modify some or various mechanisms of injury, while preserving islet viability and function *ex vivo*. Some of the

most interesting strategies include: the increased viability of dog islets when supplementing the isolation with beraprost sodium, a prostaglandin (PG) I_2 analogue (79); the inactivation of endogenous pancreatic enzymes during digestion by using pefabloc, which is also known to have anti-apoptotic effect (80) (81-85). Nicotinamide has been shown to protect islets from injury induced by cytokines (86). Ichii and colleagues added nicotinamide into the processing medium during islet isolation (87). They found nicotinamide supplementation increased human islet yields. They also showed a significant increase in c-peptide levels in patients transplanted with nicotinamide treated islets.

As mentioned in **Chapter 2**, the use of anti-oxidants during islet isolation to protect islets from oxidative cell injury is a rational approach, because islet cells harbor poor endogenous antioxidant defense systems (88). Oxidative stress is initiated by the excessive production of reactive oxygen species, which are potent inducers of pro-inflammatory stress responses often marked by proinflammatory cytokines (TNF- a, IL-1β, IL-6, and IFN-γ), and chemokine synthesis (89, 90).

The current directions of using antioxidants in islet transplantation rely in treating islets with metabolites, vitamins, trace elements, herbal products and enzymatic antioxidants to ameliorate the aforementioned deleterious effects. Glutamine was found to reduce human islet cell apoptosis and to improve islet yield and function, when pancreata was treated via the duct prior to (91). Along these lines, our group recently published similar findings with glutathione-ethyl-ester, showing increased viability, protection for apoptosis and better engraftment (92).

Similarly, mimetics of superoxide dismutase have demonstrated to be beneficial in improving islet survival in culture (62). Their ability to catalytically modulate oxidation-reduction reactions within a cell may control signaling cascades necessary for generating inflammation and provide

therapeutic benefit targeted at down regulation of the immune response. Within this group, metalloporphyrin-based antioxidants have been particularly successful in scavenging a broad range of oxidants (93-97). The utility of these antioxidants to ameliorate other inflammatory-mediated disease processes has been demonstrated in a T1DM model of adoptive transfer, apoptosis, and blocking of hydrogen peroxide-induced mitochondrial DNA damage, and partial rescue of a lethal phenotype in a manganese superoxide dismutase knockout mouse (98, 99). Furthermore, Piganelli and collaborators have demonstrated that redox modulation protects islets from both the stresses involved in the isolation procedure as well as transplant related injury (100, 101). It is conceivable that islet-sparing agents, which decrease the production of free radicals and inflammatory cytokines, may have a positive impact on islet function post-transplant by reducing the prevalence of primary non-function and, potentially increase the incidence of insulin independence from single islet infusions.

Based on this evidence and in close collaboration with BioMimetix Pharmaceutical Inc., we performed a series of pre-clinical experiments utilizing two mangano-metalloporphyrin formulations (BMX-001 and BMX-010) to evaluate potential benefits in islet transplantation. Our preliminary findings support the available evidence and those results prompted us to launch a clinical trial, initially with BMX-010 at a concentration of 34 mM. Our preliminary clinical results showed no toxicity to human islets, but fell short to provide significant improvement in yield, viability or functional parameters (OCR, insulin release). However, the percentage of islet utilization for transplantation was higher in the BMX group. We believe that our results may have been designed with a sub-optimal dose and as such, we are now modifying the trial to accommodate a higher dose and BMX-001, a more powerful metalloporphyrin formulation.

Another powerful injury mechanism for islets is the toxic effect resulting from exposure chronic immunosuppression. Anti-rejection medication is fundamental in preventing allo- and auto-immunity after islet transplantation. The mere existence of immunosuppression protocols is the reason why organ transplantation is successful in treating organ dysfunctions for a long term. However, this protection comes with added side effects from some of the most important agents. Multiple reports have provided evidence of CNI-induced (in particular tacrolimus) islet toxicity (102-104). Our group had the opportunity to corroborate first-hand this effect when demonstrating nil insulin secretion in islets exposed to tacrolimus both, *in vitro* and *in vivo*. As such, we tested different strategies to overcome this limitation and experiments with Anti-aging Glycopeptide in particular resulted very promising. AAGP is a synthetic analogue of anti-freeze proteins, with notable cytoprotective capabilities. Our experimental design (**Chapter 3**) included treating islets in culture and the protective results were consistent *in vitro* and also in a syngeneic transplant model in mice. AAGP-supplemented islets were able to function similarly to control islets despite being treated with high dose tacrolimus (~20 ng/mL) (105).

The specific pathways for tacrolimus-induced injury are still unclear. Mounting evidence suggests the inhibition of key nuclear factors and subsequent decrease in insulin synthesis (106), whereas other reports suggests that tacrolimus potentiates insulin resistance damage in beta cells (107) (**Figure 8.3**). Our own work provided evidence of decreased insulin release in islets treated with tacrolimus, probably due to membrane transport mechanisms, suggested by altered membrane capacitance, while insulin cellular content remained constant (105). Previous work has reported the CNIs may inhibit P-glycoprotein, a powerful efflux pump in cell membrane, responsible for avoiding cell toxicity mechanisms (108-111).

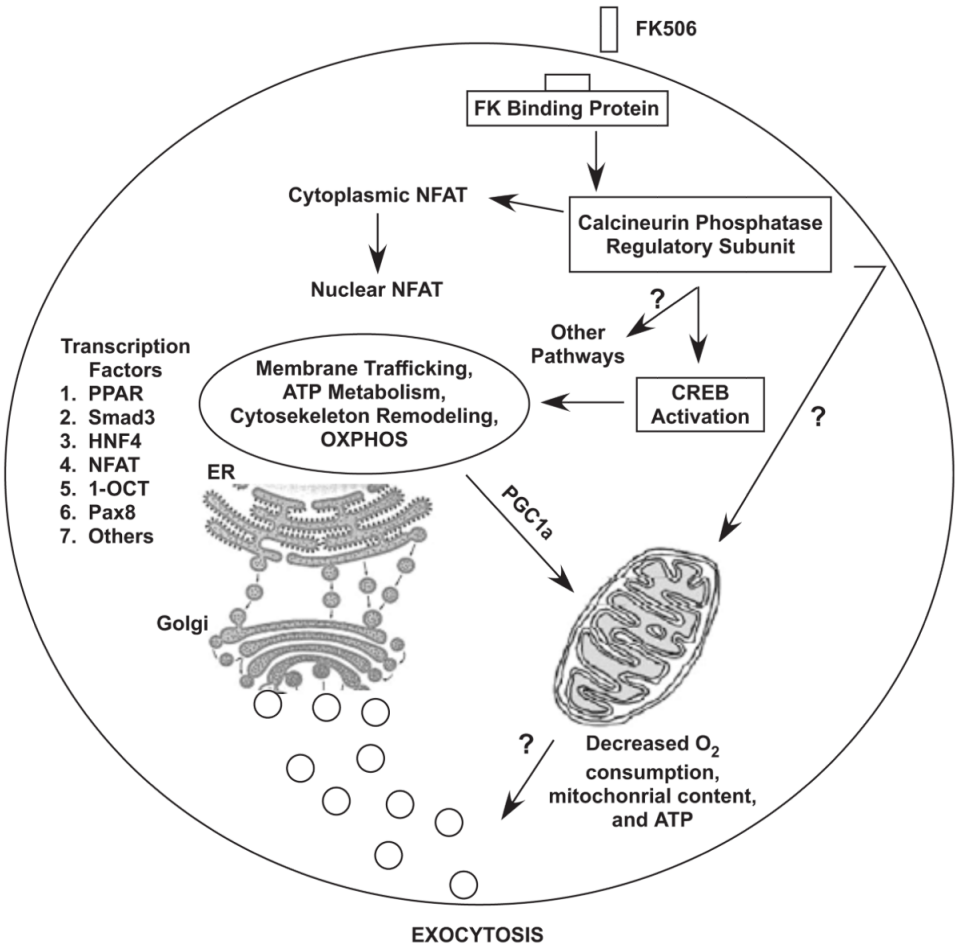


Figure 8.3 Schematic representation of pathways implicated in the effect of tacrolimus on islet cell function. Reproduced from Rostambeigi *et al*. Transplantation 2011 27; 91(6): 615-623, with permission.

We speculate AAGP may have a stabilizing effect by protecting P-glycoprotein from tacrolimus blockade or my mimicking its function. However, further experimentation will be required to accurately identify the mechanism for this protective effect. Applications for these findings are seemingly infinite and expand beyond the field of islet transplantation given that tacrolimus and CNIs in general are key immunossupressives for most transplant programs.

8.2.6. – ISLET ENGRAFTMENT

The site of transplantation of islets influences graft performance and eventually, graft survival (112). The various sites that have been evaluated for islet transplantation include the liver (113), spleen (114), abdominal cavity (in the omentum) (115), testes (116), and renal subcapsular space (117), the anterior eye chamber (118), the subcutaneous space (5), among others. The rationale for selecting different sites in experimental or clinical setting often depends on multiple factors including the type of donors, the volume of the islet preparation, and the need for close monitoring or eventual retrievability.

Initial experience largely attempted the subcutaneous and the intraperitoneal site of animal models and humans. Although results were encouraging, this method soon proved to be inefficient and related to limited islet survival (119). Kemp and collaborators found and described the superiority of the intraportal injection of cells compared to these traditional sites (120, 121). Portal embolization was thus recognized to be the most efficient site for implantation, with the benefit of high vascularity, proximity to islet-specific nutrient factors, and physiological first pass insulin delivery to the liver (122). Today, intra-portal islet transplantation is by far the current standard for clinical transplantation. The method has been refined and improved over the

years with the introduction of modern medical technology, which necessarily results in safer practice.

Various studies have proven the angiogenesis occurring after the islet intraportal implantation. In the mouse, capillary sprouts and arterioles arise within 2 to 4 days, interconnect by day 6, and the process is completed by day 10 to 14. These vessels are of host origin, pierce the islet, and branch into capillaries within the center of the graft (123). Once the islets are infused into the portal vein they are entrapped as a result of size restriction and promote the formation of an islet-thrombus, which in time is incorporated in the vessel wall eventually receiving vascularization from the surrounding tissues (124).

Even though the portal embolization has been universally embraced for its simplicity and good results, it is still far from perfect due to possible complications, e.g., portal thrombosis or subcapsular hematomas, and is hampered by a significant early loss of transplanted islets (125). Other studies in rodents have clearly shown that transplantation under the kidney capsule produce similar or superior long-term results compared with intraportal transplantation, and results in dogs have shown superior long-term outcomes after intrasplenic compared to intraportal transplantation (112). Other reports in the literature refer to experiences with islet transplantation on intramural small bowel site (126), gastric submucosa (127) and muscle (128), with different outcomes.

Immediately after transplantation, islets depend on diffusion of oxygen and nutrients from the surrounding environment for their survival and function. In order to regain proper islet function, new capillaries and blood vessels have to form and basically rebuild their old capillary network. The new network derives from both, the recipient blood vessels but also from the remnant donor islet endothelium (124). This revascularization process may initiate as soon as 1 – 3 days post-

transplant and may conclude round day (123). Multiple factors combine and contribute to apoptosis and cell death, resulting in islet tissue loss The most important factors related to early islet survival are (13):

1. Donor-related factors
2. Damage to islets during isolation and preservation
3. Technical problems at transplantation
4. Hypoxia following transplantation
5. Instant blood mediated immune reaction (IBMIR) upon exposure of isolated islets to allogeneic blood
6. immunogenicity in the implanted islets
7. Toxic effects of immunosuppressive drugs

Strategies to overcome these factors are diverse and often require synergy among them. A valid approach is to enhance revascularization by increasing the action of agents promoting angiogenesis or by inhibiting anti-angiogenic factors by using known and new therapeutic agents. More recently, co-culture and co-transplantation of islets with mesenchymal stem cells (MSC) has proven to be an effective tool to facilitate rapid re-oxygenation of islets shortly after transplant (129, 130). MSCs also contribute to modulate immediate inflammatory response eliciting a dual protective mechanism for newly-transplanted islets. Similarly, reduction of the deleterious effect of IBMIR is another area with potential benefits towards engraftment. Current experimental strategies to prevent IBMIR include using nicotidamine (131), low molecular weight dextran sulfate (132), thrombin inhibitor (133) and heparin coating islets (9). Despite these strategies, IBMIR remains a limiting factor on β cell function with the intra-portal site in addition to other vascular sites.

Recently, the subcutaneous space has been retaken as a candidate for islet and insulin producing stem Cell Transplant, based on the need to expand sources for islet transplantation and accommodate cells that may require a closer monitoring. The new approach demanded to create a vascularized space to allow cells to successfully engraft in a location known to have insufficient oxygen diffusion to harbor implanted cells (**Table 8.1**). Some of the new strategies to implement subcutaneous Cell Transplant include the use of devices to promote neovascularization resulting from foreign body reaction. In our pre-clinical and clinical laboratory we have worked with two device-based technologies to allow subcutaneous transplantation. The TheraCyte™ and Sernova Cell Pouch™ systems were specifically designed to achieve this purpose.

Table 8.1 Oxygen tension in different transplant sites and their ability to oxygenate transplanted islet. Modified from Moore *SJ et al*. *World J Transplant* 2015 March 24; 5(1): 1-10. Reproduced with permission.

Site	Oxygen tension of native tissue (mmHg)	Oxygen tension of transplanted islets (mmHg)
Pancreas	Approximately 40	n/a
Portal vein	Approximately 40	Approximately 5
Spleen	n/a	Approximately 5
Kidney capsule	15	Approximately 5
Peritoneal lining	Approximately 50	n/a
Intramuscular space	15	25
Subcutaneous	8	n/a

The first, providing a pre-loaded cell container with the possibility of immunoisolating features based on advanced design; the latter, a device designed to elicit vascularization and cell loading at a later point. Both systems have been successful in hosting islets in animal models, but their main purpose is to facilitate engraftment of insulin-producing stem cells in an accessible location under the skin.

Chapter 5 describes the results of our pilot study using the Sernova Cell Pouch^{TM}. Despite the observed biocompatibility in rodents and large animals of experimentation, we encountered seroma fluid collections in all our patients. Islets transplanted in these devices failed to decrease insulin requirements and produced a peak release of C-peptide in the first 24h after transplantation, consistent with beta cell death. At a later time point, devices were explanted and islets were found in patchy areas, not surrounded by immune cell infiltrates. A possible explanation for this behavior is that the resulting seeding density for islets infused inside Cell Pouch^{TM} chambers resulted in central ischemia and significant cell death. Moreover, the fact that all patients developed fluid collections may be an indicator of insufficient new vessel ingrowth and subsequent engraftment failure.

Pre-clinical and clinical experimentation with the TheraCyte^{TM} system is ongoing and currently coupled with insulin-producing stem cells, which are known to be more resilient than islets. Long-term durability of these systems is however, unknown. The use of the device-based concept has an inherited risk of persistent foreign body reaction and eventually, a collagen wall formation around the device, defeating the purpose of free exchange of oxygen, hormones and nutrients (134). To overcome this important limitation, our group designed a system using foreign body reaction only for a limited period followed by infusion of cells in a device-free scenario. The “Device-Less” technique was successfully validated for islets in syngeneic,

allogeneic and xenogeneic transplant models (5), and the benefits of this approach are now been tested in stem cell and cancer research (unpublished data). The success of any subcutaneous transplant technology is obviously dependent in effective vascularization, optimal oxygen diffusion across the graft and controlled collagen formation.

Other transplant sites are now being developed and tested in humans to attain engraftment of human islets in a site reachable by minimal invasive techniques, while providing direct monitoring capabilities. The University of Pittsburgh has recently published a new technique for endoscopic implantation of islets in the gastric submucosa. The technique is very simple and the risk for patients is almost non-existing (127). University of Alberta has now launched a clinical trial to evaluate the potential benefits of this technique. Another exiting breakthrough was the announcement of DRI BioHub, a novel concept to engraft islets in the omentum, by using a scaffold to provide a matrix for the cells in two variants, a biodegradable scaffold made of thrombin and the recipient's own plasma or a bioengineered scaffold with a silicone base. Results in the first patient are very encouraging and prompt for further clinical testing.

8.2.7. - IMMUNOSUPPRESSIVE STRATEGIES TOWARDS TOLERANCE

One of the major impediments to the clinical success of islet transplantation is the immune destruction of transplanted islets. Immunological challenges to islet survival, engraftment, and function post-transplantation are 2-fold: alloimmune destruction and autoimmune rejection. Although the former is common to all organ and tissue transplantation situations, type I diabetes offers additional challenges because it is autoimmune in origin (14).

Among the biological strategies used to overcome immune rejection are the use of novel immunosuppressive agents and regimens, and donor-specific induction of immune tolerance in the host (**Appendices A and B**). Immunosuppressive agents are typically delivered systemically to address the underlying autoimmunity as well as the allo-immune responses to transplanted islets. These immunosuppressants are permanently needed following transplantation and have multiple unavoidable side effects, such as an increased vulnerability to infection for the patient and cytotoxicity to the transplanted islets (135). Many of these immunosuppressants are diabetogenic by producing either transient dysfunctional insulin synthesis or secretion, or by inducing beta-cell death (105). However, anti-rejection drugs are required to allow islets to last and avoid recipient sensitization. This paradox requires a continuous optimization of available drugs and individualized therapeutic schemes. Experimental strategies like using AAGP, as discussed in **Chapter 3**, may provide an alternative to use immunosuppressants safely, at effective doses without jeopardizing the survival of the graft (105).

One of the requirements of immunosuppressive protocols in islet transplantation is the effectiveness in preventing any cytodestructive host immune response. This is critical taking into account that the marginal mass of engrafted islets has virtually no excess capacity to tolerate β-cell injury without reaching the tipping point causing metabolic dysfunction (14). Although immunosuppressive therapy has improved over time, with the identification of new drugs and combinations, targeted modulation of the innate and adaptive immune response to transplanted islets may provide a pathway to reduce or eliminate systemic immunosuppression. An inspiration toward the possibility of avoiding immunosuppression of the recipient without accompanying graft loss came from early observations of selective graft acceptance of twin animals that share common placental circulation during gestation (136).

Immune tolerance to an allogeneic or xenogenic islet transplant can be achieved at various stages in immune system development. These approaches target the graft, the graft donor, or the host. A wide range of experimental studies are now focused in finding improved results in islet transplant by introducing tolerance-inducing medication. Immunologic tolerance is lack of response to a specific antigens prompted by previous exposure to the specific antigen. It is considered to be a "training" process for T cells and occurs centrally and peripherally (137, 138). Immune tolerance has been accomplished in animals of experimentation, but remains a dream for transplant clinicians. In the setting of organ transplantation, tolerance refers to lack of rejection without active immunosuppression.

Early works in tolerance were based in observations of fewer or no events of rejection when transplants were performed between closely related animals, especially, between identical or fraternal twins. Further studies demonstrated that induction of tolerance was actually possible when the immune system was immature, at the fetal or newborn state. It involves clonal deletion of alloreactive T-cells, T cell anergy, immune deviation, and induction of regulatory T cells at both, central and peripheral stages (139). Donor-specific tolerance can be experimentally induced by intrathymic inoculation of recipient's APCs pulsed with allopeptides. The clinical application of this technique is very remote. An alternative route to generate central tolerance would be by means of a bone marrow transplant, which would allow recipient's hematopoietic reconstitution with donor stem cells (139). Mixed allogeneic chimerism is a variant for this strategy, including sublethal total body irradiation, combined with costimulatory blockade, or dual anti-CD4 and anti-CD8 antibody; followed by bone marrow infusion (140, 141).

The combination of bone marrow and islet transplantation could be used to induce donor-specific tolerance to islet allografts. However, the inherent risks - infection, graft-versus-host disease

(GVHD) and malignancies - have limited wide-spread clinical applications. One important discovery in this specific area of research is the induction of transplant tolerance using facilitating cells (FC), a cell population of bone marrow origin, initially described by Ildstad S. and collaborators as an engraftment facilitator for bone marrow stem cells in MHC-disparate allogeneic recipients without the risk of graft-versus-host disease (GVHD) (142). FC have been later characterized as CD8+, CD3+, CD45R+, Thy 1+, class IIdim/intermediate but alpha beta-TCR- and gamma delta-TCR-; with a distinctive subpopulation of plasmacytoid precursors dendritic cells (143).

FC have successfully allowed bone marrow transplantation using the aforementioned mixed chimerism strategy. More recently, this concept have been incorporated into an FDA-approved phase 2 study performed by Leventhal J and collaborators, with the utilization of a tolerance-promoting FC-based hematopoietic stem cell (HSC) to induce chimerism and tolerance in HLA-mismatched living donor renal transplant recipients (144, 145). In this innovative study, donors were mobilized with granulocyte colony stimulating factor 2 weeks prior to transplantation to facilitate cell collection during the donor procedure. 8 living donor kidney transplant recipients received pre-transplant treatment with fludarabine (an immunosuppressant usually used as a conditioning agent prior to HSC transplant), 200 cGy total body irradiation and cyclophosphamide. Donor-derived cryopreserved FC/HCS were infused on day one post-transplant and maintenance IS was a combination of Tac and MMF. Five patients showed evidence of durable macrochimerism and IS was weaned off one year post-procedure. Two subjects exhibited transient chimerism with reduction of IS to low-dose Tac monotherapy. The remaining patient lost the graft due to arterial thrombosis in the context of sepsis (145). A follow-up publication from this study reported a total of 14/15 (93%) subjects with demonstrated

chimerism, which was lost in time in 3 patients at 2, 3, and 6 months post-transplant, due to either incomplete conditioning, reduced cell dose or high PRA. All patients with durable chimerism 10/14 (10/12 receiving complete conditioning and optimal cell dose) successfully achieved complete IS withdrawal one year post-transplant without signs of allograft rejection or GVHD (146). The last publication for this trial reported 27 patients transplanted, with 12 subjects showing signs of persistent chimerism, normal renal function, absence of donor-specific antibodies and normal kidney biopsy, currently off immunosuppression. Five subjects exhibited transient chimerism, currently on low dose Tac monotherapy, and two subjects lost their grafts (144). Results from this trial provoked a generalized enthusiasm in the scientific community and have now prompted for further clinical studies, now sponsored by Novartis/Regenerex LLC. The potential benefits of this strategy clearly expand beyond bone marrow and kidney transplant to all transplant modalities, including IT. Performing immunosuppression-free transplants has always been the dream for clinicians. Facilitating cells clearly pose an opportunity for potential implementation in islet transplantation.

T-cell depletion is another alternative to seek tolerance. It involves depletion of $CD4^+$ and $CD8^+$ populations prior to transplant, to avoid responsiveness towards donor-specific antigens. The clear advantage is that many of the agents used for this purpose are currently used in clinical practice and offer a lesser risk than total body irradiation and bone marrow transplant. The benefits of agents such as Anti-CD3, antithymocyte globulin, CD20 mAb, and others are extensively discussed in **Appendices A and B**. They offer a clear opportunity to induce tolerance but fail to achieve this purpose in monotherapy regimens. As consequence, T-cell depletion is usually coupled with other agents blocking different pathways. Interesting results

come from the combination of T-cell depletion agents with granulocyte colony stimulating factor due to its ability to promote regulatory T cells and facilitate tolerance (139).

Very promising results are also coming from the use of costimulatory blockade as an alternative to contribute to tolerance. These agents exploit the mechanism of interfering with the secondary signal to complete T-cell activation, rendering the cells anergic (139). CTLA4-Ig is one of the most popular costimulatory blockade agents, used in pre-clinical experimentation. Treatment with this CLTA4 mimetic has resulted in immunomodulation in allogeneic islet transplantation, but fails to induce tolerance by itself. Our laboratory has extensively used this drug in different transplant models and the best results come from the combination of CTLA4-Ig with other agents (147).

Similar results come from CD40-CD154 blockade, which has been associated with induction of dominant tolerance and prevention of spontaneous autoimmune diabetes in animals. This strategy affects a crucial costimulatory pathway implicating the binding of CD40 on antigen-presenting cells to its ligand (CD154), expressed by T cells (148). Experimentation with this strategy is met with mixed results. Some reports associate the use of anti-CD154 with significant prolonged survival in islets with little side effects, whereas other authors showed increase formation of donor-specific antibodies and thromboembolic complications in non-human primates (139).

Very exciting news come from using regulatory T cells to induce tolerance. It is known that the presence of $CD4^{+}CD25^{+}Foxp3^{+}$ Tregs is associated with better outcomes after transplantation and tolerance, including the challenging situation of an ABO incompatible transplant (149, 150). Therefore, the ideal scenario would be to provide sufficient immunosuppression to inhibit allorejection while expanding Treg population. The dilemma is that many of our current arsenals

also deplete Tregs. Only rapamycin has been identified as an agent able to promote expansion and activation of Tregs, when used with appropriate mechanistic combination (151).
Another mechanism to potentially induce tolerance is by cotransplanting islets with stromal cells with immunomodulatory characteristics like hepatic stellate cells (152), sertoli cells (153) and mesenchymal stem cells (130). All these cells have demonstrated immunomodulatory capabilities and tolerogenic effects, although their durability and long-term effect is still questionable. Of special interest are the works by de Almeida and collaborators. (154) reporting tolerance with induced pluripotent stem cells (iPSCs). Mature patient's cells are reprogrammed to become pluripotent through transfection (155). The advantage is that iPCSs are recognized as "self" and have the potential to restore dysfunctional tissue without the need for immunosuppression.

8.3. – USING STEM CELLS IN CLINICAL TRANSPLANTATION. HOW CLOSE ARE WE?

The increasing demand for islet transplantation is met with increasing limitation in donor availability. Current alternatives to islet transplantation are the use of xenografts and using bioengineered human stem cells. The practice of islet xenotransplantation has been studied extensively and results are now more encouraging after new and more efficient immunosuppressive protocols have been developed for this purpose (156, 157). Two clinical trials are currently active in New Zealand (DiaBCell) and in Russia using porcine islets, encapsulated in alginate-based capsules as a mechanical barrier to immune cell engagement.

Another important alternative to transplantation of pancreatic islets from human and xenogenic sources is the generation of insulin-producing β-cells either from pre-existing β-cells, or from non-β-cell precursors (158). In the former approach, a patient's own β-cells can be extracted and made to divide in culture before retransplantation into the patient. Various non-β-cells may also be used to generate β-cells: 1) the patient's own (adult) stem cells can be made to differentiate; 2) the patient's own terminally differentiated cells, e.g., pancreatic ductal cells, can be made to dedifferentiate into stem cells, followed by transformation into the β-cell phenotype, or 3) embryonic stem cells can be differentiated into insulin-producing cells. The use of a patient's own cells has the distinct advantage of circumventing alloimmunity, which is one of the most challenging barriers to successful islet transplantation. Although many details of our understanding of the differentiation pathway, as well as of the intricate mechanisms of islet function, remain unknown, significant progress has been made by several groups in generating cells of the desirable phenotype (158).

Advantages to using stem cells include an unlimited supply from specialized center using reproducible differentiation protocols to provide sufficient number of cells upon request of the transplant center. Also, bioengineered stem cells tolerate hypoxia better and create a new network of blood vessels more rapidly, compare to human islets (159). Bruin and collaborators make a striking analogy in a recent paper by recognizing that two donated pancreases are needed for one recipient (~ 5,000 – 10,000 IEQ/Kg) to render normoglycemia and insulin independence; whereas a single vial of stem cells may be used to treat approximately 2000 diabetic patients at a ratio of $5x10^8$ differentiated cells per patient (160). Stem Cell Transplant, however, is not without limitations. Time-to-mature beta cell is probably the major limitation for stem cell researchers, with the corresponding delay from transplantation to full diabetes reversal in

animals. Delay in maturation also poses a significant challenge in graft monitoring, which can only be achieved by measuring C-peptide in blood, in a transplant model also susceptible to early silent rejection. The latest reports on the subject present more sophisticated and efficient maturation sequences resulting in quasi-mature beta cells and a significant reduction in the time required for cells to revert diabetes (**Figure 8.4**) (161-164).

My experience with insulin-producing stem cells began while collaborating with M. Cristina Nostro and Gordon Keller, from the McEwan Centre for Regenerative Medicine. As presented in **Chapter 7**, we utilized our recently developed Device-less technique to implant pancreatic endoderm cells derived from embryonic stem cells (**Figure 8.4B**). The idea was to validate this transplant site for insulin-producing stem cells and multiple experiments were performed to evaluate engraftment and maturation success. This pre-vascularized subcutaneous approach was adequate for these cells to engraft and follow a maturation process beyond 22 weeks. As previously published, we realized that the maturation process (expressed as secretion of human C-peptide and positive endocrine staining) was more efficient in diabetic mice, supporting the observation that effect hyperglycemia positively influence endocrine differentiation (165). However, we had a concern with the duration of the maturation process, the ability to reach mature beta-cell state with full and regulated insulin production and the lack of diabetes reversal after 5 months.

This is a common limitation of many of the differentiation protocols, probably caused by using schemes based on polyhormonal cells. Recent investigations have shown that indeed, polyhormonal cell differentiation may result in beta cell formation, but with low efficiency since this process results mainly in alpha cells (160). Fortunately, new breakthroughs are now allowing more efficient differentiation *in vitro* to a more advanced stage; where cells are very close to

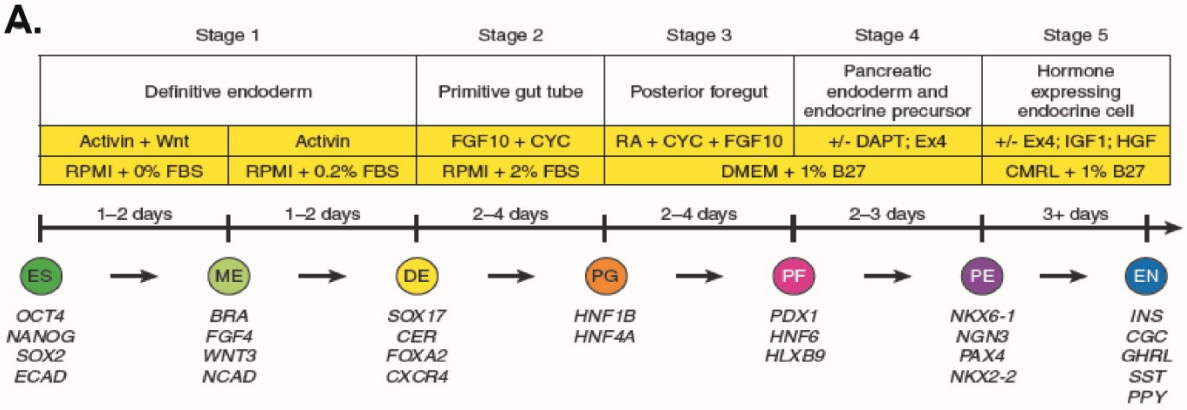
become mature beta cells. Kroon and colleagues successfully reached mature beta cells after transplanting stage-4 pancreatic progenitors into non-diabetic immunodeficient mice (**Figure 8.4A**) (161). New and more efficient differentiation protocols have now been reported by Kieffer's (164) and Melton's (163) groups (**Figure 8.4C and D**). These protocols promote full differentiation of human pluripotent stem cells into pancreatic progenitors and ultimately, stage-7 NKX6.1+/PDX1+ cells, capable of effectively secreting insulin upon glucose stimulation. Even though these Stage-7 cells do not show similar insulin release patterns (assessed by insulin perifusion and intracellular calcium imaging) to those observed in mature human beta cells, their function is sufficient to produce hypoglycemia. Transplantation of these quasi-mature beta cells has successfully reversed diabetes in STZ-induced diabetic mice with a significantly shorter wait period (166), and also prevented hyperglycemic state in NRG-Akita (NOD-Rag1$^{-/-}$ IL-2r$\gamma^{-/-}$ Ins2^{Akita}) mice, which spontaneously develop diabetes as a result of insulin misfolding (163). The advantage is clear, shorter maturation periods *in vivo* and more effective diabetes reversal, which will eventually lead to a more realistic clinical application. An alternative would be to design protocols that allow full differentiation into more mature Stage-8 (or beyond) beta cells *in vitro* capable of producing insulin in just a few days, upon engraftment. This strategy may allow more control on lineage fate, preventing de-differentiation or teratoma formation (**Figure 8.5**) (160). However, there may be an increase in metabolic and oxygen demands for these more mature cells jeopardizing the possibility of using subcutaneous devices as a transplant vehicle. FDA and Health Canada have recently approved phase 1/2 clinical trials with macroencapsulated hESC-derived pancreatic progenitors cells (Viacyte, Inc.) to evaluate safety and in-human maturation profiles. University of Alberta is one of the participating centers and we are eagerly

waiting for the preliminary results and the potential expansion of this technology within the clinical environment.

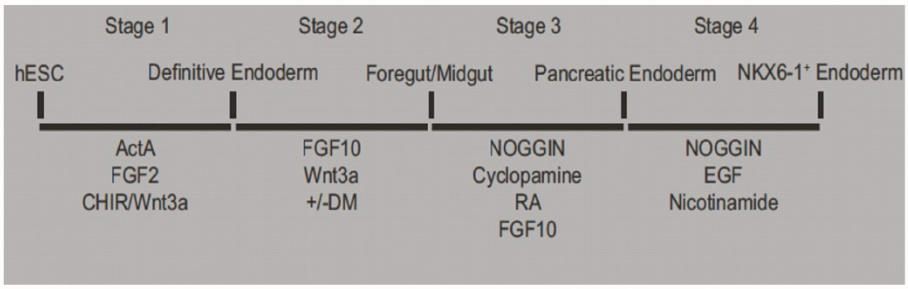
Another caveat of stem Cell Transplant is the possibility of tumor transformation, which is often associated with incomplete purification protocol. Cystic formations are frequent in stem Cell Transplant given the histogenesis process occurring immediately after engraftment. Classic teratomas however, remaining a concerning complication of this transplant procedure. They are usually detected when they cause morbidity either through a mass effect or through the release of hormones from functional endocrine tissue. Given that the process of teratoma formation is not fully understood, alternative transplant sites have been implemented to host stem cell-derived preparations. The subcutaneous space is by far one of the most popular sites due to its accessibility for transplant, direct monitoring and retrieval procedures, if required.

These encouraging engraftment reports paired with the new revolutionary protocols are now prompting centers to initiate clinical trials to evaluate safety and efficacy in humans. With specific strategies to induce patient own cells to de-differentiate into beta cells, we are now a step closer to personalized transplant medicine. Based on all these elements we can assure that the possibility of treating type 1 diabetes mellitus with insulin-producing stem cells is no longer in the distant future.

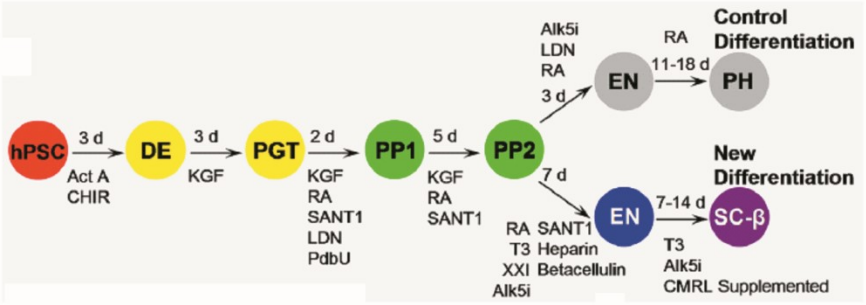
A.


Stage 1
Stage 2
Stage 3
Stage 4
Stage 5
Definitive endoderm
Primitive gut tube
Posterior foregut
Pancreatic endoderm and endocrine precursor
Hormone expressing endocrine cell
Activin + Wnt
Activin
FGF10 + CYC
RA + CYC + FGF10
+/- DAPT; Ex4
+/- Ex4; IGF1; HGF
RPMI + 0% FBS
RPMI + 0.2% FBS
RPMI + 2% FBS
DMEM + 1% B27
CMRL + 1% B27
1–2 days
1–2 days
2–4 days
2–4 days
2–3 days
3+ days
ES
ME
DE
PG
PF
PE
EN
OCT4 NANOG SOX2 ECAD
BRA FGF4 WNT3 NCAD
SOX17 CER FOXA2 CXCR4
HNF1B HNF4A
PDX1 HNF6 HLXB9
NKX6-1 NGN3 PAX4 NKX2-2
INS CGC GHRL SST PPY

B.


Stage 1
Stage 2
Stage 3
Stage 4
hESC
Definitive Endoderm
Foregut/Midgut
Pancreatic Endoderm
NKX6-1+ Endoderm
ActA
FGF2
CHIR/Wnt3a
FGF10
Wnt3a
+/-DM
NOGGIN
Cyclopamine
RA
FGF10
NOGGIN
EGF
Nicotinamide

C.


hPSC
3 d
Act A
CHIR
DE
3 d
KGF
PGT
2 d
KGF
RA
SANT1
LDN
PdbU
PP1
5 d
KGF
RA
SANT1
PP2
Alk5i
LDN
RA
3 d
EN
RA
11-18 d
PH
Control Differentiation
7 d
EN
7-14 d
SC-β
RA SANT1
T3 Heparin
XXI Betacellulin
Alk5i
T3
Alk5i
CMRL Supplemented
New Differentiation

D.

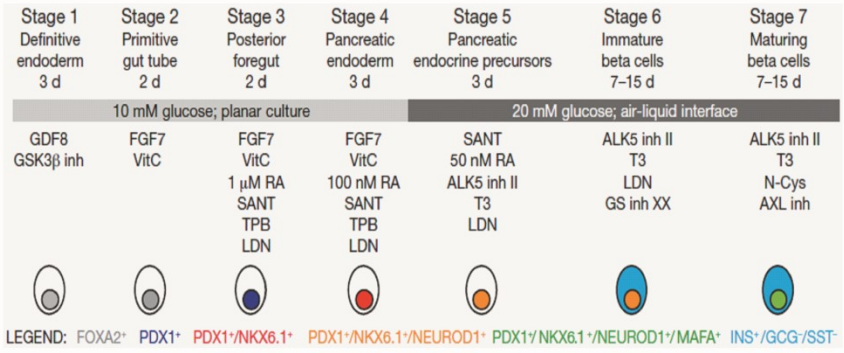

Stage 1
Definitive endoderm
3 d
Stage 2
Primitive gut tube
2 d
Stage 3
Posterior foregut
2 d
Stage 4
Pancreatic endoderm
3 d
Stage 5
Pancreatic endocrine precursors
3 d
Stage 6
Immature beta cells
7–15 d
Stage 7
Maturing beta cells
7–15 d
10 mM glucose; planar culture
20 mM glucose; air-liquid interface
GDF8
GSK3β inh
FGF7
VitC
FGF7
VitC
1 μM RA
SANT
TPB
LDN
FGF7
VitC
100 nM RA
SANT
TPB
LDN
SANT
50 nM RA
ALK5 inh II
T3
LDN
ALK5 inh II
T3
LDN
GS inh XX
ALK5 inh II
T3
N-Cys
AXL inh
LEGEND: FOXA2+ PDX1+ PDX1+/NKX6.1+ PDX1+/NKX6.1+/NEUROD1+ PDX1+/NKX6.1+/NEUROD1+/MAFA+ INS+/GCG-/SST-

Figure 8.4 Different differentiation protocols to reach endocrine cells. The diagrams present maturation stages and associated markers to identify each phase. **(A)** Differentiation protocol reported by D'Amour KA *et al*. *Nat Biotech* 2006 24(11): 1392-1401. The protocol is divided into five stages and the growth factors, medium and range of duration for each stage are shown. Several markers characteristic of each cell population are listed. CYC, KAAD-cyclopamine; RA, all-*trans* retinoic acid; DAPT, γ-secretase inhibitor; Ex4, exendin-4; ES, hES cell; ME, mesoendoderm; DE, definitive endoderm; PG, primitive gut tube; PF, posterior foregut endoderm; PE, pancreatic endoderm and endocrine precursor; EN, hormone-expressing endocrine cells. **(B)** Schematic of the protocol used by Nostro MC *et al*. to differentiate hESCs toward pancreatic endoderm. At the final stage of differentiation, cells were treated with NOGGIN, EGF, and nicotinamide, singly or in combinations (Nostro MC *et al. Stem Cell Reports* 2015, Vol 4: 591-604. **(C)** Schematic of directed differentiation from hPSC into INS+ cells via new or previously published control differentiations (Pagliuca FW, *et al. Cell* 2014 Vol 159:428-439). (D) Overview of differentiation protocol and characterization of S4–S6, reported by Rezania A. *et al*. Summary of seven-stage differentiation protocol, including the important growth factors and small molecules that were added at each stage. Key markers of the differentiating pancreatic endocrine cells are also illustrated (INS, insulin; GCG, glucagon; SST, somatostatin) (Rezania A. *et al*. *Nat Biotech* 2014; 32(11): 1121-33. All figures are reproduced from the originals with permission.

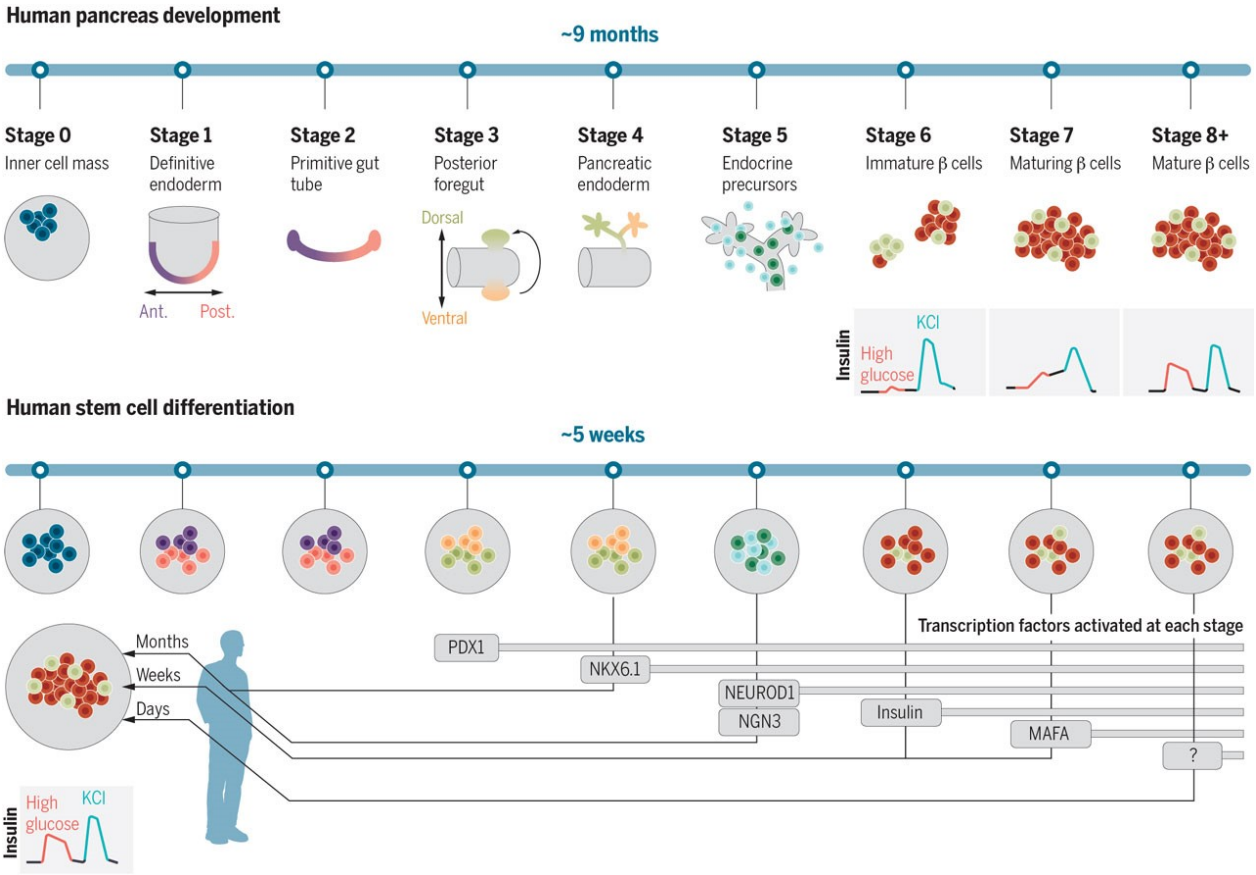


Figure 8.5 Emulating pancreatic development. Shown is a schematic of an in vitro differentiation protocol for human pluripotent stem cells (dark blue cells) that is designed to mimic embryonic development of the human pancreas. The key stages of human pancreas development can be mimicked in vitro by addition of small molecules and growth factors to pluripotent stem cells in culture. Additional iterations to in vitro differentiation protocols (stage 8+) are required to generate mature human β cells with the appropriate insulin secretion kinetics. Although stage 8+ cells are appealing for transplantation because they would theoretically only require several days to become fully functional after transplant, it is also feasible to consider transplanting immature cells between stages 4 and 7 into patients with diabetes. Stages 4 and 5 cells will likely require months to become fully functional in vivo, whereas stage 6 to 7 cells should reach maturity within weeks. Reproduced with permission from Bruin JE, Rezania A and Kieffer TJ. Replacing and safeguarding pancreatic beta cells for diabetes. *Sci Transl Med* 7 (316), 316:23.

8.4. – GENERAL CONCLUSIONS

Islet transplantation has come a long way since its first attempts by Watson-Williams almost two centuries ago to today's technologically advanced clinical practice. Many generations of great scientist and courageous patients have contributed to bring islet transplantation to its rightful place within the arsenal to treat diabetes. Nowadays, the procedure is highly successful, with >80% of the recipients being protected from hypoglycemic episodes for as much as 5 years after transplantation, and 5-year insulin independence rates now rivaling those obtain with whole organ pancreas transplantation. This has become a possibility through the discovery and implementation of new and more potent immunosuppression. Along with substantial advancements in human islet isolation technology that have provided high quality islets. Despite this tremendous progress, a large number of islets is still required to obtain durable insulin independence in clinical islet transplantation. Preventing the early post-transplant cell death would definitely have an immediate impact on islet transplantation. There are multiple opportunities for intervention throughout the entire process, from pancreas procurement, islet isolation and culture, through to strategies for enhanced islet survival after implantation. In addition to prevention of β-cell dysfunction and death post-transplantation to reduce the number of islets required per patient, islets from different species (xenotransplantation), stem cells (neogenesis), or pre-existing islets (regeneration therapy) are being evaluated to overcome the shortage.

The goal now should be to sharply focus on routinely obtaining a large number of viable islets that provide full functional survival for the long-term. Once met, this goal will undoubtedly enhance the long-term rates of insulin independence from single-donor recipients in the clinical

setting. Indeed, much works remains to be done to achieve this goal; but it is clear that there is scope and tangible path for significant improvements that will permit islet transplantation to be a practical therapy for all patients type 1 diabetes.

8.5. - REFERENCES

1. (CITR) TCITR. Seventh Annual Data Report In., 2011.

2. Barton FB, Rickels MR, Alejandro R, Hering BJ, Wease S, Naziruddin B et al. Improvement in outcomes of clinical islet transplantation: 1999-2010. Diabetes Care 2012;35(7):1436-1445.

3. Ryan EA, Bigam D, Shapiro AM. Current indications for pancreas or islet transplant. Diabetes Obes Metab 2006;8(1):1-7.

4. Shapiro AM, Ricordi C, Hering BJ, Auchincloss H, Lindblad R, Robertson RP et al. International trial of the Edmonton protocol for islet transplantation. N Engl J Med 2006;355(13):1318-1330.

5. 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.

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

7. Emamaullee JA, Shapiro AM. Interventional strategies to prevent beta-cell apoptosis in islet transplantation. Diabetes 2006;55(7):1907-1914.

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

9. Cabric S, Sanchez J, Lundgren T, Foss A, Felldin M, Kallen R et al. Islet surface heparinization prevents the instant blood-mediated inflammatory reaction in islet transplantation. Diabetes 2007;56(8):2008-2015.

10. Gillard P, Ling Z, Mathieu C, Crenier L, Lannoo M, Maes B et al. Comparison of sirolimus alone with sirolimus plus tacrolimus in type 1 diabetic recipients of cultured islet cell grafts. Transplantation 2008;85(2):256-263.

11. Kloster-Jensen K, Sahraoui A, Vethe NT, Korsgren O, Bergan S, Foss A et al. Treatment with Tacrolimus and Sirolimus Reveals No Additional Adverse Effects on Human Islets In Vitro Compared to Each Drug Alone but They Are Reduced by Adding Glucocorticoids. J Diabetes Res 2016;2016:4196460.

12. Desai NM, Goss JA, Deng S, Wolf BA, Markmann E, Palanjian M et al. Elevated portal vein drug levels of sirolimus and tacrolimus in islet transplant recipients: local immunosuppression or islet toxicity? Transplantation 2003;76(11):1623-1625.

13. Al-Adra DP, Gill RS, Imes S, O'Gorman D, Kin T, Axford SJ et al. Single-donor islet transplantation and long-term insulin independence in select patients with type 1 diabetes mellitus. Transplantation 2014;98(9):1007-1012.

14. Shapiro AM. Strategies toward single-donor islets of Langerhans transplantation. Curr Opin Organ Transplant 2011;16(6):627-631.

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

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

17. Ihm SH, Matsumoto I, Sawada T, Nakano M, Zhang HJ, Ansite JD et al. Effect of donor age on function of isolated human islets. Diabetes 2006;55(5):1361-1368.

18. Lakey JR, Warnock GL, Rajotte RV, Suarez-Alamazor ME, Ao Z, Shapiro AM et al. Variables in organ donors that affect the recovery of human islets of Langerhans. Transplantation 1996;61(7):1047-1053.

19. Matsumoto I, Sawada T, Nakano M, Sakai T, Liu B, Ansite JD et al. Improvement in islet yield from obese donors for human islet transplants. Transplantation 2004;78(6):880-885.

20. Ponte GM, Pileggi A, Messinger S, Alejandro A, Ichii H, Baidal DA et al. Toward maximizing the success rates of human islet isolation: influence of donor and isolation factors. Cell Transplant 2007;16(6):595-607.

21. Witkowski P, Liu Z, Cernea S, Guo Q, Poumian-Ruiz E, Herold K 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.

22. 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.

23. O'Gorman D, Kin T, Murdoch T, Richer B, McGhee-Wilson D, Ryan E et al. The standardization of pancreatic donors for islet isolation. Transplant Proc 2005;37(2):1309-1310.

24. Andres A, Kin T, O'Gorman D, Livingstone S, Bigam D, Kneteman N 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.

25. Noguchi H. Pancreas procurement and preservation for islet transplantation: personal considerations. J Transplant 2011;2011:783168.

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

27. Slegtenhorst BR, Dor FJ, Elkhal A, Rodriguez H, Yang X, Edtinger K et al. Mechanisms and consequences of injury and repair in older organ transplants. Transplantation 2014;97(11):1091-1099.

28. Damman J, Bloks VW, Daha MR, van der Most PJ, Sanjabi B, van der Vlies P et al. Hypoxia and Complement-and-Coagulation Pathways in the Deceased Organ Donor as the Major Target for Intervention to Improve Renal Allograft Outcome. Transplantation 2015;99(6):1293-1300.

29. Fridell JA, Mangus RS, Powelson JA. Organ preservation solutions for whole organ pancreas transplantation. Curr Opin Organ Transplant 2011;16(1):116-122.

30. Schneeberger S, Biebl M, Steurer W, Hesse UJ, Troisi R, Langrehr JM et al. A prospective randomized multicenter trial comparing histidine-tryptophane-ketoglutarate versus University of Wisconsin perfusion solution in clinical pancreas transplantation. Transpl Int : 2009;22(2):217-224.

31. Stewart ZA, Cameron AM, Singer AL, Dagher NN, Montgomery RA, Segev DL. Histidine-tryptophan-ketoglutarate (HTK) is associated with reduced graft survival in pancreas transplantation. Am J Transplant 2009;9(1):217-221.

32. Kaddis JS, Hanson MS, Cravens J, Qian D, Olack B, Antler M et al. Standardized transportation of human islets: an islet cell resource center study of more than 2,000 shipments. Cell Transplant 2013;22(7):1101-1111.

33. Latchana N, Peck JR, Whitson BA, Henry ML, Elkhammas EA, Black SM. Preservation solutions used during abdominal transplantation: Current status and outcomes. World J Transplant 2015;5(4):154-164.

34. Nashan B, Spetzler V, Schemmer P, Kirste G, Rahmel A. Regarding "Compared Efficacy of Preservation Solutions in Liver Transplantation: A Long-Term Graft Outcome Study From the European Liver Transplant Registry". Am J Transplant 2015;15(12):3272-3273.

35. Juang SE, Huang HW, Kao CW, Chen CL, Lu HF, Cheng KW et al. Effect of university of wisconsin and histidine-tryptophan-ketoglutarate preservation solutions on blood potassium levels of patients undergoing living-donor liver transplantation. Transplant Proc 2012;44(2):366-368.

36. Kin T, Mirbolooki M, Salehi P, Tsukada M, O'Gorman D, Imes S et al. Islet isolation and transplantation outcomes of pancreas preserved with University of Wisconsin solution versus two-layer method using preoxygenated perfluorocarbon. Transplantation 2006;82(10):1286-1290.

37. Caballero-Corbalan J, Eich T, Lundgren T, Foss A, Felldin M, Kallen R et al. No beneficial effect of two-layer storage compared with UW-storage on human islet isolation and transplantation. Transplantation 2007;84(7):864-869.

38. Noguchi H, Ueda M, Nakai Y, Iwanaga Y, Okitsu T, Nagata H et al. Modified two-layer preservation method (M-Kyoto/PFC) improves islet yields in islet isolation. Am J Transpl 2006;6(3):496-504.

39. Noguchi H, Naziruddin B, Onaca N, Jackson A, Shimoda M, Ikemoto T et al. Comparison of modified Celsior solution and M-kyoto solution for pancreas preservation in human islet isolation. Cell Transplant 2010;19(6):751-758.

40. Noguchi H, Naziruddin B, Jackson A, Shimoda M, Fujita Y, Chujo D et al. Comparison of ulinastatin, gabexate mesilate, and nafamostat mesilate in preservation solution for islet isolation. Cell Transplant 2012;21(2-3):509-516.

41. Sung RS, Christensen LL, Leichtman AB, Greenstein SM, Distant DA, Wynn JJ et al. Determinants of discard of expanded criteria donor kidneys: impact of biopsy and machine perfusion. Am J Transplant 2008;8(4):783-792.

42. 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.

43. Bruns H, Schemmer P. Machine perfusion in solid organ transplantation: where is the benefit? Langenbecks Arch Surg 2014;399(4):421-427.

44. 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.

45. Yuan X, Theruvath AJ, Ge X, Floerchinger B, Jurisch A, Garcia-Cardena G et al. Machine perfusion or cold storage in organ transplantation: indication, mechanisms, and future perspectives. Transpl Int 2010;23(6):561-570.

46. Kin T, Mirbolooki M, Brassil J, Shapiro AM, Lakey J. Machine perfusion for prolonged pancreas preservation prior to islet isolation (Abstract). In. Annual Scientific Meeting. Canadian Society of Transplantation. 2006.

47. Leeser DB, Bingaman AW, Poliakova L, Shi Q, Gage F, Bartlett ST et al. Pulsatile pump perfusion of pancreata before human islet cell isolation. Transplant Proc 2004;36(4):1050-1051.

48. Suszynski TM, Rizzari MD, Scott WE, Eckman PM, Fonger JD, John R et al. Persufflation (gaseous oxygen perfusion) as a method of heart preservation. J Cardiothorac Surg 2013;8:105.

49. Suszynski TM, Rizzari MD, Scott WE, 3rd, Tempelman LA, Taylor MJ, Papas KK. Persufflation (or gaseous oxygen perfusion) as a method of organ preservation. Cryobiology 2012;64(3):125-143.

50. Nagai K, Yagi S, Afify M, Bleilevens C, Uemoto S, Tolba RH. Impact of venous-systemic oxygen persufflation with nitric oxide gas on steatotic grafts after partial orthotopic liver transplantation in rats. Transplantation 2013;95(1):78-84.

51. Srinivasan PK, Yagi S, Doorschodt B, Nagai K, Afify M, Uemoto S et al. Impact of venous systemic oxygen persufflation supplemented with nitric oxide gas on cold-stored, warm ischemia-damaged experimental liver grafts. Liver Transpl 2012;18(2):219-225.

52. Tolba RH, Schildberg FA, Schnurr C, Glatzel U, Decker D, Minor T. Reduced liver apoptosis after venous systemic oxygen persufflation in non-heart-beating donors. J Invest Surg 2006;19(4):219-227.

53. Minor T, Tolba R, Akbar S, Dombrowski T, Muller A. The suboptimal donor: reduction of ischemic injury in fatty livers by gaseous oxygen persufflation. Transplant Proc 2000;32(1):10.

54. Scott WE, 3rd, O'Brien TD, Ferrer-Fabrega J, Avgoustiniatos ES, Weegman BP, Anazawa T et al. Persufflation improves pancreas preservation when compared with the two-layer method. Transplant Proc 2010;42(6):2016-2019.

55. Carter J, Karmiol S, Nagy M, McElreath R, Calloway C, Motley A et al. Pretransplant islet culture: a comparison of four serum-free media using a murine model of islet transplantation. Transplant Proc 2005;37(8):3446-3449.

56. Murdoch TB, McGhee-Wilson D, Shapiro AM, Lakey JR. Methods of human islet culture for transplantation. Cell Transplant 2004;13(6):605-617.

57. Will RG, Ironside JW, Zeidler M, Cousens SN, Estibeiro K, Alperovitch A et al. A new variant of Creutzfeldt-Jakob disease in the UK. Lancet 1996;347(9006):921-925.

58. Johnson LF, deSerres S, Herzog SR, Peterson HD, Meyer AA. Antigenic cross-reactivity between media supplements for cultured keratinocyte grafts. J Burn Care Rehabil 1991;12(4):306-312.

59. Mackensen A, Drager R, Schlesier M, Mertelsmann R, Lindemann A. Presence of IgE antibodies to bovine serum albumin in a patient developing anaphylaxis after vaccination with human peptide-pulsed dendritic cells. Cancer Immunol Immunother 2000;49(3):152-156.

60. Meyer AA, Manktelow A, Johnson M, deSerres S, Herzog S, Peterson HD. Antibody response to xenogeneic proteins in burned patients receiving cultured keratinocyte grafts. J Trauma 1988;28(7):1054-1059.

61. Spees JL, Gregory CA, Singh H, Tucker HA, Peister A, Lynch PJ et al. Internalized antigens must be removed to prepare hypoimmunogenic mesenchymal stem cells for cell and gene therapy. Mol Ther 2004;9(5):747-756.

62. 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.

63. Papas KK, Avgoustiniatos ES, Tempelman LA, Weir GC, Colton CK, Pisania A et al. High-density culture of human islets on top of silicone rubber membranes. Transplant Proc 2005;37(8):3412-3414.

64. Registry CIT. Annual Report. 2008 November 27, 2008.

65. Ichii H, Inverardi L, Pileggi A, Molano RD, Cabrera O, Caicedo A et al. A novel method for the assessment of cellular composition and beta-cell viability in human islet preparations. Am J Transplant 2005;5(7):1635-1645.

66. Maechler P. Mitochondria as the conductor of metabolic signals for insulin exocytosis in pancreatic beta-cells. Cell Mol Life Sci 2002;59(11):1803-1818.

67. Aikin R, Rosenberg L, Paraskevas S, Maysinger D. Inhibition of caspase-mediated PARP-1 cleavage results in increased necrosis in isolated islets of Langerhans. J Mol Med (Berl) 2004;82(6):389-397.

68. Papas KK, Colton CK, Nelson RA, Rozak PR, Avgoustiniatos ES, Scott WE, 3rd et al. Human islet oxygen consumption rate and DNA measurements predict diabetes reversal in nude mice. Am J Transplant 2007;7(3):707-713.

69. Sweet IR, Khalil G, Wallen AR, Steedman M, Schenkman KA, Reems JA et al. Continuous measurement of oxygen consumption by pancreatic islets. Diabetes Technol Ther 2002;4(5):661-672.

70. Sweet IR, Gilbert M, Jensen R, Sabek O, Fraga DW, Gaber AO et al. Glucose stimulation of cytochrome C reduction and oxygen consumption as assessment of human islet quality. Transplantation 2005;80(8):1003-1011.

71. Sweet IR, Gilbert M, Scott S, Todorov I, Jensen R, Nair I et al. Glucose-stimulated increment in oxygen consumption rate as a standardized test of human islet quality. Am J Transplant 2008;8(1):183-192.

72. Takita M, Matsumoto S, Qin H, Noguchi H, Shimoda M, Chujo D et al. Secretory unit of islet transplant objects (SUITO) index can predict severity of hypoglycemic episodes in clinical islet Cell Transplant. Cell Transplant 2012;21(1):91-98.

73. Matsumoto S, Noguchi H, Takita M, Shimoda M, Tamura Y, Olsen G et al. Super-high-dose islet transplantation is associated with high SUITO index and prolonged insulin independence: a case report. Transplant Proc 2010;42(6):2156-2158.

74. Takita M, Matsumoto S, Noguchi H, Shimoda M, Chujo D, Itoh T et al. Secretory unit of islet transplant objects (SUITO) index can predict outcome of intravenous glucose tolerance test. Transplant Proc 2010;42(6):2065-2067.

75. Matsumoto S, Noguchi H, Takita M, Shimoda M, Tamura Y, Olsen G et al. Excellence of suito index for assessing clinical outcome of islet transplantation. Transplant Proc 2010;42(6):2062-2064.

76. Matsumoto S, Noguchi H, Hatanaka N, Shimoda M, Kobayashi N, Jackson A et al. SUITO index for evaluation of efficacy of single donor islet transplantation. Cell Transplant 2009;18(5):557-562.

77. Matsumoto S, Noguchi H, Hatanaka N, Kobayashi N, Jackson A, Naziruddin B et al. Evaluation of engraftment after single islet transplantation from a brain-dead donor by the secretory unit of islet transplant objects (SUITO) index. Transplant Proc 2008;40(2):364-366.

78. Matsumoto S, Yamada Y, Okitsu T, Iwanaga Y, Noguchi H, Nagata H et al. Simple evaluation of engraftment by secretory unit of islet transplant objects for living donor and cadaveric donor fresh or cultured islet transplantation. Transplant Proc 2005;37(8):3435-3437.

79. Arita S, Une S, Ohtsuka S, Kawahara T, Kasraie A, Smith CV et al. Increased islet viability by addition of beraprost sodium to collagenase solution. Pancreas 2001;23(1):62-67.

80. Basir I, van der Burg MP, Scheringa M, Tons A, Bouwman E. Improved outcome of pig islet isolation by Pefabloc inhibition of trypsin. Transplant Proc 1997;29(4):1939-1941.

81. Matsumoto S, Rigley TH, Reems JA, Kuroda Y, Stevens RB. Improved islet yields from Macaca nemestrina and marginal human pancreata after two-layer method preservation and endogenous trypsin inhibition. Am J Transplant 2003;3(1):53-63.

82. Lakey JR, Helms LM, Kin T, Korbutt GS, Rajotte RV, Shapiro AM et al. Serine-protease inhibition during islet isolation increases islet yield from human pancreases with prolonged ischemia. Transplantation 2001;72(4):565-570.

83. Dong Z, Saikumar P, Patel Y, Weinberg JM, Venkatachalam MA. Serine protease inhibitors suppress cytochrome c-mediatedcaspase-9 activation and apoptosis during hypoxia-reoxygenation. Biochem J 2000;347 Pt 3:669-677.

84. Kagaya S, Kitanaka C, Noguchi K, Mochizuki T, Sugiyama A, Asai A et al. A functional role for death proteases in s-Myc- and c-Myc-mediated apoptosis. Mol Cell Biol 1997;17(11):6736-6745.

85. Park IC, Park MJ, Choe TB, Jang JJ, Hong SI, Lee SH. TNF-alpha induces apoptosis mediated by AEBSF-sensitive serine protease(s) that may involve upstream caspase-3/CPP32 protease activation in a human gastric cancer cell line. Int J Oncol 2000;16(6):1243-1248.

86. 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.

87. Ichii H, Wang X, Messinger S, Alvarez A, Fraker C, Khan A et al. Improved human islet isolation using nicotinamide. Am J Transplant 2006;6(9):2060-2068.

88. 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.

89. 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.

90. 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.

91. 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.

92. 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.

93. Day BJ, Shawen S, Liochev SI, Crapo JD. A metalloporphyrin superoxide dismutase mimetic protects against paraquat-induced endothelial cell injury, in vitro. J Pharmacol Exp Ther 1995;275(3):1227-1232.

94. Ferrer-Sueta G, Vitturi D, Batinic-Haberle I, Fridovich I, Goldstein S, Czapski G et al. Reactions of manganese porphyrins with peroxynitrite and carbonate radical anion. J Biol Chem 2003;278(30):27432-27438.

95. Day BJ, Fridovich I, Crapo JD. Manganic porphyrins possess catalase activity and protect endothelial cells against hydrogen peroxide-mediated injury. Arch Biochem Biophys 1997;347(2):256-262.

96. Day BJ, Batinic-Haberle I, Crapo JD. Metalloporphyrins are potent inhibitors of lipid peroxidation. Free Radic Biol Med 1999;26(5-6):730-736.

97. Batinic-Haberle I, Benov L, Spasojevic I, Fridovich I. The ortho effect makes manganese(III) meso-tetrakis(N-methylpyridinium-2-yl)porphyrin a powerful and potentially useful superoxide dismutase mimic. J Biol Chem 1998;273(38):24521-24528.

98. Melov S, Schneider JA, Day BJ, Hinerfeld D, Coskun P, Mirra SS et al. A novel neurological phenotype in mice lacking mitochondrial manganese superoxide dismutase. Nat Genet 1998;18(2):159-163.

99. Piganelli JD, Flores SC, Cruz C, Koepp J, Batinic-Haberle I, Crapo J 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.

100. Sklavos MM, Bertera S, Tse HM, Bottino R, He J, Beilke JN et al. Redox modulation protects islets from transplant-related injury. Diabetes 2010;59(7):1731-1738.

101. 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.

102. Rostambeigi N, Lanza IR, Dzeja PP, Deeds MC, Irving BA, Reddi HV et al. Unique cellular and mitochondrial defects mediate FK506-induced islet beta-cell dysfunction. Transplantation 2011;91(6):615-623.

103. Uchizono Y, Iwase M, Nakamura U, Sasaki N, Goto D, Iida M. Tacrolimus impairment of insulin secretion in isolated rat islets occurs at multiple distal sites in stimulus-secretion coupling. Endocrinology 2004;145(5):2264-2272.

104. Malinowski M, Pratschke J, Lock J, Neuhaus P, Stockmann M. Effect of tacrolimus dosing on glucose metabolism in an experimental rat model. Ann Transplant 2010;15(3):60-65.

105. Gala-Lopez BL, Pepper AR, Pawlick RL, O'Gorman D, Kin T, Bruni A et al. Antiaging Glycopeptide Protects Human Islets Against Tacrolimus-Related Injury and Facilitates Engraftment in Mice. Diabetes 2016;65(2):451-462.

106. Hernandez-Fisac I, Pizarro-Delgado J, Calle C, Marques M, Sanchez A, Barrientos A et al. Tacrolimus-induced diabetes in rats courses with suppressed insulin gene expression in pancreatic islets. Am J Transplant 2007;7(11):2455-2462.

107. Rodriguez-Rodriguez AE, Trinanes J, Velazquez-Garcia S, Porrini E, Vega Prieto MJ, Diez Fuentes ML et al. The higher diabetogenic risk of tacrolimus depends on pre-existing insulin resistance. A study in obese and lean Zucker rats. Am J Transplant 2013;13(7):1665-1675.

108. Naesens M, Kuypers D, Sarwal MM. The bumpy road of genomic medicine in transplantation: lessons from studies on calcineurin inhibitor nephrotoxicity. Transplantation 2012;93(6):578-579.

109. Metalidis C, Lerut E, Naesens M, Kuypers DR. Expression of CYP3A5 and P-glycoprotein in renal allografts with histological signs of calcineurin inhibitor nephrotoxicity. Transplantation 2011;91(10):1098-1102.

110. Kuypers DR, Naesens M, de Jonge H, Lerut E, Verbeke K, Vanrenterghem Y. Tacrolimus dose requirements and CYP3A5 genotype and the development of calcineurin inhibitor-associated nephrotoxicity in renal allograft recipients. Therapeutic drug monitoring 2010;32(4):394-404.

111. Naesens M, Kuypers DR, Sarwal M. Calcineurin inhibitor nephrotoxicity. Clin J Am Soc Nephrol 2009;4(2):481-508.

112. 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.

113. Pipeleers-Marichal MA, Pipeleers DG, Cutler J, Lacy PE, Kipnis DM. Metabolic and morphologic studies in intraportal-islet-transplanted rats. Diabetes 1976;25(11):1041-1051.

114. Finch DR, Wise PH, Morris PJ. Successful intra-splenic transplantation of syngeneic and allogeneic isolated pancreatic islets. Diabetologia 1977;13(3):195-199.

115. Gustavson SM, Rajotte RV, Hunkeler D, Lakey JR, Edgerton DS, Neal DW et al. Islet auto-transplantation into an omental or splenic site results in a normal beta cell but abnormal alpha cell response to mild non-insulin-induced hypoglycemia. Am J Transplant 2005;5(10):2368-2377.

116. Ar'Rajab A, Dawidson IJ, Harris RB, Sentementes JT. Immune privilege of the testis for islet xenotransplantation (rat to mouse). Cell Transplant 1994;3(6):493-498.

117. Napoli R, Davalli AM, Hirshman MF, Weitgasser R, Weir GC, Horton ES. Islet transplantation under the kidney capsule fully corrects the impaired skeletal muscle glucose transport system of streptozocin diabetic rats. J Clin Invest 1996;97(6):1389-1397.

118. Mojibian M, Harder B, Hurlburt A, Bruin JE, Asadi A, Kieffer TJ. Implanted islets in the anterior chamber of the eye are prone to autoimmune attack in a mouse model of diabetes. Diabetologia 2013;56(10):2213-2221.

119. Juang JH, Hsu BR, Kuo CH. Islet transplantation at subcutaneous and intramuscular sites. Transplant Proc 2005;37(8):3479-3481.

120. Kemp CB, Knight MJ, Scharp DW, Ballinger WF, Lacy PE. Effect of transplantation site on the results of pancreatic islet isografts in diabetic rats. Diabetologia 1973;9(6):486-491.

121. Kemp CB, Scharp DW, Knight MJ, Ballinger WF, Lacy PE. Importance of implantation site of pancreatic islet isografts in treatment of experimental diabetes. Surgical forum 1973;24:297-299.

122. Meier JJ, Hong-McAtee I, Galasso R, Veldhuis JD, Moran A, Hering BJ et al. Intrahepatic transplanted islets in humans secrete insulin in a coordinate pulsatile manner directly into the liver. Diabetes 2006;55(8):2324-2332.

123. 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.

124. Brissova M, Fowler M, Wiebe P, Shostak A, Shiota M, Radhika A et al. Intraislet endothelial cells contribute to revascularization of transplanted pancreatic islets. Diabetes 2004;53(5):1318-1325.

125. Kawahara T, Kin T, Kashkoush S, Gala-Lopez B, Bigam DL, Kneteman NM et al. Portal vein thrombosis is a potentially preventable complication in clinical islet transplantation. Am J Transplant 2011;11(12):2700-2707.

126. Kakabadze Z, Gupta S, Brandhorst D, Korsgren O, Berishvili E. Long-term engraftment and function of transplanted pancreatic islets in vascularized segments of small intestine. Transpl Int 2011;24(2):175-183.

127. Echeverri GJ, McGrath K, Bottino R, Hara H, Dons EM, van der Windt DJ et al. Endoscopic gastric submucosal transplantation of islets (ENDO-STI): technique and initial results in diabetic pigs. Am J Transpl 2009;9(11):2485-2496.

128. Espes D, Eriksson O, Lau J, Carlsson PO. Striated muscle as implantation site for transplanted pancreatic islets. J Transplant 2011;2011:352043.

129. Wu H, Wen D, Mahato RI. Third-party mesenchymal stem cells improved human islet transplantation in a humanized diabetic mouse model. Mol Ther 2013;21(9):1778-1786.

130. Figliuzzi M, Bonandrini B, Silvani S, Remuzzi A. Mesenchymal stem cells help pancreatic islet transplantation to control type 1 diabetes. World J Stem Cells 2014;6(2):163-172.

131. Moberg L, Olsson A, Berne C, Felldin M, Foss A, Kallen R et al. Nicotinamide inhibits tissue factor expression in isolated human pancreatic islets: implications for clinical islet transplantation. Transplantation 2003;76(9):1285-1288.

132. Goto M, Johansson H, Maeda A, Elgue G, Korsgren O, Nilsson B. Low molecular weight dextran sulfate prevents the instant blood-mediated inflammatory reaction induced by adult porcine islets. Transplantation 2004;77(5):741-747.

133. 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.

134. Socarras TO, Vasconcelos AC, Campos PP, Pereira NB, Souza JP, Andrade SP. Foreign body response to subcutaneous implants in diabetic rats. PloS one 2014;9(11):e110945.

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

136. Wood KJ, Bushell AR, Jones ND. The discovery of immunological tolerance: now more than just a laboratory solution. J Immunol 2010;184(1):3-4.

137. Donckier V, Sanchez-Fueyo A, Craciun L, Lucidi V, Buggenhout A, Troisi R et al. Induction of tolerance in solid organ transplantation: the rationale to develop clinical protocols in liver transplantation. Transplant Proc 2009;41(2):603-606.

138. Fehr T, Sykes M. Tolerance induction in clinical transplantation. Transpl Immunol 2004;13(2):117-130.

139. Bhatt S, Fung JJ, Lu L, Qian S. Tolerance-inducing strategies in islet transplantation. Int J Endocrinol 2012;2012:396524.

140. Im KI, Park MJ, Kim N, Lim JY, Park HS, Lee SH et al. Induction of mixed chimerism using combinatory cell-based immune modulation with mesenchymal stem cells and regulatory T cells for solid-organ transplant tolerance. Stem Cells Dev 2014;23(19):2364-2376.

141. Bradley JA. Induction of transplant tolerance through mixed hematopoietic chimerism. Am J Transpl 2012;12(5):1073-1074.

142. Kaufman CL, Colson YL, Wren SM, Watkins S, Simmons RL, Ildstad ST. Phenotypic characterization of a novel bone marrow-derived cell that facilitates engraftment of allogeneic bone marrow stem cells. Blood 1994;84(8):2436-2446.

143. Fugier-Vivier IJ, Rezzoug F, Huang Y, Graul-Layman AJ, Schanie CL, Xu H et al. Plasmacytoid precursor dendritic cells facilitate allogeneic hematopoietic stem cell engraftment. J Exp Med 2005;201(3):373-383.

144. Ildstad ST, Leventhal J, Wen Y, Yolcu E. Facilitating cells: Translation of hematopoietic chimerism to achieve clinical tolerance. Chimerism 2016:1-7.

145. Leventhal J, Abecassis M, Miller J, Gallon L, Ravindra K, Tollerud DJ et al. Chimerism and tolerance without GVHD or engraftment syndrome in HLA-mismatched combined kidney and hematopoietic stem Cell Transplant. Sci Transl Med 2012;4(124):124ra128.

146. Leventhal J, Abecassis M, Miller J, Gallon L, Tollerud D, Elliott MJ et al. Tolerance induction in HLA disparate living donor kidney transplantation by donor stem cell infusion: durable chimerism predicts outcome. Transplantation 2013;95(1):169-176.

147. Pawlick R, Gala-Lopez B, Pepper AR, McCall M, Ziff O, Shapiro AM. The combination of anti-NKG2D and CTLA-4 Ig therapy prolongs islet allograft survival in a murine model. Am J Transpl 2014;14(10):2367-2374.

148. Nanji SA, Hancock WW, Luo B, Schur CD, Pawlick RL, Zhu LF et al. Costimulation blockade of both inducible costimulator and CD40 ligand induces dominant tolerance to islet allografts and prevents spontaneous autoimmune diabetes in the NOD mouse. Diabetes 2006;55(1):27-33.

149. Dijke IE, Hoeppli RE, Ellis T, Pearcey J, Huang Q, McMurchy AN et al. Discarded Human Thymus Is a Novel Source of Stable and Long-Lived Therapeutic Regulatory T Cells. Am J Transpl 2016;16(1):58-71.

150. Dijke IE, Weimar W, Baan CC. Regulatory T cells after organ transplantation: where does their action take place? Hum Immunol 2008;69(7):389-398.

151. Battaglia M, Stabilini A, Tresoldi E. Expanding human T regulatory cells with the mTOR-inhibitor rapamycin. Methods Mol Biol 2012;821:279-293.

152. Zhang ZY, Zhou ZQ, Song KB, Kim SC, Zhou GW. Hepatic stellate cells induce immunotolerance of islet allografts. Transplant Proc 2014;46(5):1594-1600.

153. Suarez-Pinzon W, Korbutt GS, Power R, Hooton J, Rajotte RV, Rabinovitch A. Testicular sertoli cells protect islet beta-cells from autoimmune destruction in NOD mice by a transforming growth factor-beta1-dependent mechanism. Diabetes 2000;49(11):1810-1818.

154. de Almeida PE, Meyer EH, Kooreman NG, Diecke S, Dey D, Sanchez-Freire V et al. Transplanted terminally differentiated induced pluripotent stem cells are accepted by immune mechanisms similar to self-tolerance. Nat Commun 2014;5:3903.

155. Wertheim JA, Leventhal JR. Clinical implications of basic science discoveries: induced pluripotent stem cell therapy in transplantation--a potential role for immunologic tolerance. Am J Transpl 2015;15(4):887-890.

156. Cooper DK, Bottino R, Satyananda V, Wijkstrom M, Trucco M. Toward clinical islet xenotransplantation - are revisions to the IXA guidelines warranted? Xenotransplantation 2013;20(2):68-74.

157. van der Windt DJ, Bottino R, Kumar G, Wijkstrom M, Hara H, Ezzelarab M et al. Clinical islet xenotransplantation: how close are we? Diabetes 2012;61(12):3046-3055.

158. Moore SJ, Gala-Lopez BL, Pepper AR, Pawlick RL, Shapiro AJ. Bioengineered stem cells as an alternative for islet Cell Transplant. World J Transplant 2015;5(1):1-10.

159. Simon MC, Keith B. The role of oxygen availability in embryonic development and stem cell function. Nat Rev Mol Cell Biol 2008;9(4):285-296.

160. Bruin JE, Rezania A, Kieffer TJ. Replacing and safeguarding pancreatic beta cells for diabetes. Sci Transl Med 2015;7(316):316ps323.

161. D'Amour KA, Bang AG, Eliazer S, Kelly OG, Agulnick AD, Smart NG et al. Production of pancreatic hormone-expressing endocrine cells from human embryonic stem cells. Nat Biotechnol 2006;24(11):1392-1401.

162. Nostro MC, Sarangi F, Yang C, Holland A, Elefanty AG, Stanley EG et al. Efficient generation of NKX6-1+ pancreatic progenitors from multiple human pluripotent stem cell lines. Stem cell reports 2015;4(4):591-604.

163. Pagliuca FW, Millman JR, Gurtler M, Segel M, Van Dervort A, Ryu JH et al. Generation of functional human pancreatic beta cells in vitro. Cell 2014;159(2):428-439.

164. Rezania A, Bruin JE, Arora P, Rubin A, Batushansky I, Asadi A et al. Reversal of diabetes with insulin-producing cells derived in vitro from human pluripotent stem cells. Nat Biotechnol 2014;32(11):1121-1133.

165. Bruin JE, Rezania A, Xu J, Narayan K, Fox JK, O'Neil JJ et al. Maturation and function of human embryonic stem cell-derived pancreatic progenitors in macroencapsulation devices following transplant into mice. Diabetologia 2013;56(9):1987-1998.

166. Rezania A, Bruin JE, Riedel MJ, Mojibian M, Asadi A, Xu J et al. Maturation of human embryonic stem cell-derived pancreatic progenitors into functional islets capable of treating pre-existing diabetes in mice. Diabetes 2012;61(8):2016-2029.

GENERAL BIBLIOGRAPHY

GENERAL BIBLIOGRAPHY

1. Adverse events and their association with treatment regimens in the diabetes control and complications trial. Diabetes care 1995;18(11):1415-1427.

2. (CITR) TCITR. Seventh Annual Data Report In., 2011.

3. (SRTR) OPaTNOaSRoTR. OPTN/SRTR 2011 Annual Data Report. In: Health and Human Services HRaSA, Healthcare Systems Bureau, Division of Transplantation, (ed). Rockville, MD, 2012.

4. Adams AB, Shirasugi N, Jones TR, Durham MM, Strobert EA, Cowan S et al. Development of a chimeric anti-CD40 monoclonal antibody that synergizes with LEA29Y to prolong islet allograft survival. J Immunol 2005;174(1):542-550.

5. 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.

6. Aguayo-Albasini JL, Garcia-Garcia ML, Martin-Lorenzo JG, Cases-Baldo MJ, Campillo-Soto A, Mengual-Ballester M. Subtotal distal pancreatectomy for metachronous metastatic renal clear cell carcinoma. Rev Esp Enferm Dig 2012;104(11):613-614.

7. Aikin R, Rosenberg L, Paraskevas S, Maysinger D. Inhibition of caspase-mediated PARP-1 cleavage results in increased necrosis in isolated islets of Langerhans. J Mol Med (Berl) 2004;82(6):389-397.

8. Amir G, Rubinsky B, Basheer SY, Horowitz L, Jonathan L, Feinberg MS et al. Improved viability and reduced apoptosis in sub-zero 21-hour preservation of transplanted rat hearts using anti-freeze proteins. J Heart Lung Transplant 2005;24(11):1915-1929.

9. 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.

10. Anderson JM. Biological response to materials. Annu Rev Mater Res 2001;31:81-110.

11. Anderson JM, Rodriguez A, Chang DT. Foreign body reaction to biomaterials. Semin Immunol 2008;20(2):86-100.

12. Andres A, Kin T, O'Gorman D, Livingstone S, Bigam D, Kneteman N 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.

13. Andres A, Toso C, Morel P, Demuylder-Mischler S, Bosco D, Baertschiger R et al. Impairment of renal function after islet transplant alone or islet-after-kidney transplantation using a sirolimus/tacrolimus-based immunosuppressive regimen. Transpl Int 2005;18(11):1226-1230.

14. Aoyagi T, Yamashita K, Suzuki T, Uno M, Goto R, Taniguchi M et al. A human anti-CD40 monoclonal antibody, 4D11, for kidney transplantation in cynomolgus monkeys: induction and maintenance therapy. Am J Transplant 2009;9(8):1732-1741.

15. Arita S, Une S, Ohtsuka S, Kawahara T, Kasraie A, Smith CV et al. Increased islet viability by addition of beraprost sodium to collagenase solution. Pancreas 2001;23(1):62-67.

16. 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.

17. Arora S, Tangirala B, Osadchuk L, Sureshkumar KK. Belatacept : a new biological agent for maintenance immunosuppression in kidney transplantation. Expert Opin Biol Ther 2012;12(7):965-979.

18. 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.

19. Axelrod DA, Sung RS, Meyer KH, Wolfe RA, Kaufman DB. Systematic evaluation of pancreas allograft quality, outcomes and geographic variation in utilization. Am J Transplant 2010;10(4):837-845.

20. Badell IR, Thompson PW, Turner AP, Russell MC, Avila JG, Cano JA et al. Nondepleting anti-CD40-based therapy prolongs allograft survival in nonhuman primates. Am J Transplant 2012;12(1):126-135.

21. Baeke F, Van Belle TL, Takiishi T, Ding L, Korf H, Laureys J et al. Low doses of anti-CD3, ciclosporin A and the vitamin D analogue, TX527, synergise to delay recurrence of autoimmune diabetes in an islet-transplanted NOD mouse model of diabetes. Diabetologia 2012;55(10):2723-2732.

22. Baekkeskov S, Aanstoot HJ, Christgau S, Reetz A, Solimena M, Cascalho M 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.

23. Baidal DA, Froud T, Ferreira JV, Khan A, Alejandro R, Ricordi C. The bag method for islet cell infusion. Cell Transplant 2003;12(7):809-813.

24. Ballarin R, Spaggiari M, Cautero N, De Ruvo N, Montalti R, Longo C et al. Pancreatic metastases from renal cell carcinoma: the state of the art. World J Gastroenterol 2011;17(43):4747-4756.

25. Ballian N, Brunicardi FC. Islet vasculature as a regulator of endocrine pancreas function. World J Surg 2007;31(4):705-714.

26. Bang JK, Lee JH, Murugan RN, Lee SG, Do H, Koh HY et al. Antifreeze peptides and glycopeptides, and their derivatives: potential uses in biotechnology. Mar Drugs 2013;11(6):2013-2041.

27. Barbaro B, Salehi P, Wang Y, Qi M, Gangemi A, Kuechle J et al. Improved human pancreatic islet purification with the refined UIC-UB density gradient. Transplantation 2007;84(9):1200-1203.

28. Barnett MJ, McGhee-Wilson D, Shapiro AM, Lakey JR. Variation in human islet viability based on different membrane integrity stains. Cell Transplant 2004;13(5):481-488.

29. Barshes NR, Lee TC, Brunicardi FC, Mote A, Schock AP, Alejandro R et al. Lack of cytomegalovirus transmission after pancreatic islet transplantation. Cell Transplant 2004;13(7-8):833-838.

30. Barshes NR, Wyllie S, Goss JA. Inflammation-mediated dysfunction and apoptosis in pancreatic islet transplantation: implications for intrahepatic grafts. J Leukoc Biol 2005;77(5):587-597.

31. Barton FB, Rickels MR, Alejandro R, Hering BJ, Wease S, Naziruddin B et al. Improvement in outcomes of clinical islet transplantation: 1999-2010. Diabetes care 2012;35(7):1436-1445.

32. Basir I, van der Burg MP, Scheringa M, Tons A, Bouwman E. Improved outcome of pig islet isolation by Pefabloc inhibition of trypsin. Transplant Proc 1997;29(4):1939-1941.

33. Bassi C, Butturini G, Falconi M, Sargenti M, Mantovani W, Pederzoli P. High recurrence rate after atypical resection for pancreatic metastases from renal cell carcinoma. Br J Surg 2003;90(5):555-559.

34. Batinic-Haberle I, Benov L, Spasojevic I, Fridovich I. The ortho effect makes manganese(III) meso-tetrakis(N-methylpyridinium-2-yl)porphyrin a powerful and potentially useful superoxide dismutase mimic. J Biol Chem 1998;273(38):24521-24528.

35. Battaglia M, Stabilini A, Tresoldi E. Expanding human T regulatory cells with the mTOR-inhibitor rapamycin. Methods Mol Biol 2012;821:279-293.

36. Bazerbachi F, Selzner M, Boehnert MU, Marquez MA, Norgate A, McGilvray ID et al. Thymoglobulin versus basiliximab induction therapy for simultaneous kidney-pancreas transplantation: impact on rejection, graft function, and long-term outcome. Transplantation 2011;92(9):1039-1043.

37. Bazerbachi F, Selzner M, Marquez MA, Norgate A, Aslani N, McGilvray ID et al. Portal venous versus systemic venous drainage of pancreas grafts: impact on long-term results. Am J Transplant 2012;12(1):226-232.

38. Beckwith J, Nyman JA, Flanagan B, Schrover R, Schuurman HJ. A health economic analysis of clinical islet transplantation. Clin Transplant 2012;26(1):23-33.

39. Begum S, Chen W, Herold KC, Papaioannou VE. Remission of type 1 diabetes after anti-CD3 antibody treatment and transplantation of embryonic pancreatic precursors. Endocrinology 2009;150(10):4512-4520.

40. Beiras-Fernandez A, Thein E, Hammer C. Induction of immunosuppression with polyclonal antithymocyte globulins: an overview. Exp Clin Transplant2003;1(2):79-84.

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

42. Bellin MD, Beilman GJ, Dunn TB, Pruett TL, Chinnakotla S, Wilhelm JJ et al. Islet Autotransplantation to Preserve Beta Cell Mass in Selected Patients With Chronic Pancreatitis and Diabetes Mellitus Undergoing Total Pancreatectomy. Pancreas 2012.

43. Bellin MD, Freeman ML, Schwarzenberg SJ, Dunn TB, Beilman GJ, Vickers SM et al. Quality of life improves for pediatric patients after total pancreatectomy and islet autotransplant for chronic pancreatitis. Clin Gastroenterol Hepatol 2011;9(9):793-799.

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

45. Berney T, Bucher P, Mathe Z, Andres A, Bosco D, Mage R et al. Islet of Langerhans allogeneic transplantation at the University of Geneva in the steroid free era in islet after kidney and simultaneous islet-kidney transplantations. Transplant Proc 2004;36(4):1121-1122.

46. Bhatt S, Fung JJ, Lu L, Qian S. Tolerance-inducing strategies in islet transplantation. Int J Endocrinol 2012;2012:396524.

47. Biglarnia AR, Nilsson B, Nilsson T, von Zur-Muhlen B, Wagner M, Berne C et al. Prompt reversal of a severe complement activation by eculizumab in a patient undergoing intentional ABO-incompatible pancreas and kidney transplantation. Transpl Int 2011;24(8):e61-66.

48. Bingley PJ, Bonifacio E, Gale EA. Can we really predict IDDM? Diabetes 1993;42(2):213-220.

49. Blum B, Benvenisty N. The Tumorigenicity of Human Embryonic Stem Cells. In: George FVW, George K, (eds). Adv Cancer Res: Academic Press, 2008: 133-158.

50. Boettler T, von Herrath M. Immunotherapy of type 1 diabetes--how to rationally prioritize combination therapies in T1D. Int Immunopharmacol 2010;10(12):1491-1495.

51. Boggi U, Vistoli F, Egidi FM, Marchetti P, De Lio N, Perrone V et al. Transplantation of the pancreas. Curr Diab Rep 2012;12(5):568-579.

52. Borot S, Crowe LA, Toso C, Vallee JP, Berney T. Noninvasive imaging techniques in islet transplantation. Curr Diab Rep 2011;11(5):375-383.

53. Borot S, Niclauss N, Wojtusciszyn A, Brault C, Demuylder-Mischler S, Muller Y et al. Impact of the number of infusions on 2-year results of islet-after-kidney transplantation in the GRAGIL network. Transplantation 2011;92(9):1031-1038.

54. Bose B, Shenoy SP, Konda S, Wangikar P. Human embryonic stem cell differentiation into insulin secreting beta-cells for diabetes. Cell Biol Int 2012;36(11):1013-1020.

55. 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.

56. 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.

57. Bradley JA. Induction of transplant tolerance through mixed hematopoietic chimerism. Am J Transplant 2012;12(5):1073-1074.

58. Brekken RA, Overholser JP, Stastny VA, Waltenberger J, Minna JD, Thorpe PE. Selective inhibition of vascular endothelial growth factor (VEGF) receptor 2 (KDR/Flk-1) activity by a monoclonal anti-VEGF antibody blocks tumor growth in mice. Cancer Res 2000;60(18):5117-5124.

59. Brendle TA. Preventing surgically induced diabetes after total pancreatectomy via autologous islet cell reimplantation. AORN J 2010;92(2):169-181; quiz 182-163.

60. Brissova M, Powers AC. Revascularization of transplanted islets: can it be improved? Diabetes 2008;57(9):2269-2271.

61. Bristow AF. Assignment of quantities to biological medicines: an old problem re-discovered. Philos Trans A Math Phys Eng Sci 2011;369(1953):4004-4013.

62. Bristow AF, Barrowcliffe T, Bangham DR. Standardization of biological medicines: the first hundred years, 1900-2000. Notes Rec R Soc Lond 2006;60(3):271-289.

63. Bruin JE, Rezania A, Kieffer TJ. Replacing and safeguarding pancreatic beta cells for diabetes. Sci Transl Med 2015;7(316):316ps323.

64. Bruin JE, Rezania A, Xu J, Narayan K, Fox JK, O'Neil JJ et al. Maturation and function of human embryonic stem cell-derived pancreatic progenitors in macroencapsulation devices following transplant into mice. Diabetologia 2013;56(9):1987-1998.

65. Bruns H, Schemmer P. Machine perfusion in solid organ transplantation: where is the benefit? Langenbecks Arch Surg 2014;399(4):421-427.

66. Bucher PO, J.; Bosco, D.; Mathe, Z.; Toso,, C.; Buhler LHB, T.; Morel, P. Microbial surveillance during human pancreatic islet isolation. Transpl Int 2005;18:584 - 589.

67. Burke GW, 3rd, Vendrame F, Pileggi A, Ciancio G, Reijonen H, Pugliese A. Recurrence of autoimmunity following pancreas transplantation. Curr Diab Rep 2011;11(5):413-419.

68. Butler AE, Galasso R, Meier JJ, Basu R, Rizza RA, Butler PC. Modestly increased beta cell apoptosis but no increased beta cell replication in recent-onset type 1 diabetic patients who died of diabetic ketoacidosis. Diabetologia 2007;50(11):2323-2331.

69. Butler PC, Meier JJ, Butler AE, Bhushan A. The replication of beta cells in normal physiology, in disease and for therapy. Nat Clin Pract Endocrinol Metab 2007;3(11):758-768.

70. Caballero-Corbalan J, Eich T, Lundgren T, Foss A, Felldin M, Kallen R et al. No beneficial effect of two-layer storage compared with UW-storage on human islet isolation and transplantation. Transplantation 2007;84(7):864-869.

71. Cabrera O, Jacques-Silva MC, Berman DM, Fachado A, Echeverri F, Poo R et al. Automated, high-throughput assays for evaluation of human pancreatic islet function. Cell Transplant 2008;16(10):1039-1048.

72. Cabric S, Sanchez J, Lundgren T, Foss A, Felldin M, Kallen R et al. Islet surface heparinization prevents the instant blood-mediated inflammatory reaction in islet transplantation. Diabetes 2007;56(8):2008-2015.

73. Calne R, Moffatt SD, Friend PJ, Jamieson NV, Bradley JA, Hale G et al. Campath IH allows low-dose cyclosporine monotherapy in 31 cadaveric renal allograft recipients. Transplantation 1999;68(10):1613-1616.

74. Campbell PM, Salam A, Ryan EA, Senior P, Paty BW, Bigam D et al. Pretransplant HLA antibodies are associated with reduced graft survival after clinical islet transplantation. Am J Transplant 2007;7(5):1242-1248.

75. Campbell PM, Senior PA, Salam A, Labranche K, Bigam DL, Kneteman NM et al. High risk of sensitization after failed islet transplantation. Am J Transplant 2007;7(10):2311-2317.

76. Carroll PBR, C.; Fontes, P.; Rilo, H. R.; Phipps,, J.; Tzakis AGF, J. J.; Starzl, T. E. Microbiologic surveillance as part of human islet transplantation: Results of the first 26 patients. Transplant Proc 1992;24:2798–2799.

77. Carter J, Karmiol S, Nagy M, McElreath R, Calloway C, Motley A et al. Pretransplant islet culture: a comparison of four serum-free media using a murine model of islet transplantation. Transplant Proc 2005;37(8):3446-3449.

78. Caumo A, Maffi P, Nano R, Luzi L, Hilbrands R, Gillard P et al. Comparative evaluation of simple indices of graft function after islet transplantation. Transplantation 2011;92(7):815-821.

79. Cavallari G, Neri F, Nardo B. Combined Kidney-Islet Transplantation. InTech, 2011.

80. Cechin SR, Perez-Alvarez I, Fenjves E, Molano RD, Pileggi A, Berggren PO et al. Anti-Inflammatory Properties of Exenatide in Human Pancreatic Islets. Cell Transplant 2011.

81. Chand DH, Southerland SM, Cunningham RJ, 3rd. Tacrolimus: the good, the bad, and the ugly. Pediatr Transplant 2001;5(1):32-36.

82. Chentoufi AA, Geenen V, Giannokakis N, Amrani A. Type 1 diabetes immunological tolerance and immunotherapy. Clin Dev Immunol 2011;2011:103738.

83. Chhabra P, Brayman KL. Current status of immunomodulatory and cellular therapies in preclinical and clinical islet transplantation. J Transplant 2011;2011:637692.

84. Cho DC. Therapeutic challenges in advanced renal cell carcinoma. Clin Pract (Lond) 2013;10(1):39-46.

85. Cooper DK, Bottino R, Satyananda V, Wijkstrom M, Trucco M. Toward clinical islet xenotransplantation - are revisions to the IXA guidelines warranted? Xenotransplantation 2013;20(2):68-74.

86. Cooper JE, Wiseman AC. Novel immunosuppressive agents in kidney transplantation. Clin Nephrol 2010;73(5):333-343.

87. Costa PZ, Soares R. Neovascularization in diabetes and its complications. Unraveling the angiogenic paradox. Life Sci 2013;92(22):1037-1045.

88. Cryer PE. The barrier of hypoglycemia in diabetes. Diabetes 2008;57(12):3169-3176.

89. Cunningham JJ, Ulbright TM, Pera MF, Looijenga LH. Lessons from human teratomas to guide development of safe stem cell therapies. Nat Biotechnol 2012;30(9):849-857.

90. Cure P, Pileggi A, Faradji RN, Baidal DA, Froud T, Selvaggi G et al. Cytomegalovirus infection in a recipient of solitary allogeneic islets. Am J Transplant 2006;6(5 Pt 1):1089-1090.

91. Dai XQ, Plummer G, Casimir M, Kang Y, Hajmrle C, Gaisano HY et al. SUMOylation regulates insulin exocytosis downstream of secretory granule docking in rodents and humans. Diabetes 2011;60(3):838-847.

92. Damman J, Bloks VW, Daha MR, van der Most PJ, Sanjabi B, van der Vlies P et al. Hypoxia and Complement-and-Coagulation Pathways in the Deceased Organ Donor as the Major Target for Intervention to Improve Renal Allograft Outcome. Transplantation 2015;99(6):1293-1300.

93. D'Amour KA, Bang AG, Eliazer S, Kelly OG, Agulnick AD, Smart NG et al. Production of pancreatic hormone-expressing endocrine cells from human embryonic stem cells. Nat Biotechnol 2006;24(11):1392-1401.

94. Daugherty AL, Mrsny RJ. Formulation and delivery issues for monoclonal antibody therapeutics. Adv Drug Deliv Rev 2006;58(5-6):686-706.

95. Davis CL. Preemptive transplantation and the transplant first initiative. Current opinion in nephrology and hypertension 2010;19(6):592-597.

96. Day BJ, Batinic-Haberle I, Crapo JD. Metalloporphyrins are potent inhibitors of lipid peroxidation. Free Radic Biol Med 1999;26(5-6):730-736.

97. Day BJ, Fridovich I, Crapo JD. Manganic porphyrins possess catalase activity and protect endothelial cells against hydrogen peroxide-mediated injury. Arch Biochem Biophys 1997;347(2):256-262.

98. Day BJ, Shawen S, Liochev SI, Crapo JD. A metalloporphyrin superoxide dismutase mimetic protects against paraquat-induced endothelial cell injury, in vitro. J Pharmacol Exp Ther 1995;275(3):1227-1232.

99. de Almeida PE, Meyer EH, Kooreman NG, Diecke S, Dey D, Sanchez-Freire V et al. Transplanted terminally differentiated induced pluripotent stem cells are accepted by immune mechanisms similar to self-tolerance. Nat Commun 2014;5:3903.

100. de Koning EJ, van den Brand JJ, Mott VL, Charge SB, Hansen BC, Bodkin NL et al. Macrophages and pancreatic islet amyloidosis. Amyloid 1998;5(4):247-254.

101. Deller RC, Vatish M, Mitchell DA, Gibson MI. Synthetic polymers enable non-vitreous cellular cryopreservation by reducing ice crystal growth during thawing. Nat Commun 2014;5:3244.

102. Delmastro MM, Piganelli JD. Oxidative stress and redox modulation potential in type 1 diabetes. Clin Dev Immunol 2011;2011:593863.

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

104. Deng S, Markmann JF, Rickels M, Yeh H, Kim JI, Lian MM et al. Islet alone versus islet after kidney transplantation: metabolic outcomes and islet graft survival. Transplantation 2009;88(6):820-825.

105. Desai NM, Goss JA, Deng S, Wolf BA, Markmann E, Palanjian M et al. Elevated portal vein drug levels of sirolimus and tacrolimus in islet transplant recipients: local immunosuppression or islet toxicity? Transplantation 2003;76(11):1623-1625.

106. DeVries AL. Antifreeze peptides and glycopeptides in cold-water fishes. Annu Rev Physiol 1983;45:245-260.

107. Dezaki K, Kageyama H, Seki M, Shioda S, Yada T. Neuropeptide W in the rat pancreas: potentiation of glucose-induced insulin release and Ca2+ influx through L-type Ca2+ channels in beta-cells and localization in islets. Regul Pept 2008;145(1-3):153-158.

108. Diamantopoulos S, Allende G, Ferreira JM, Ciancio G, Burke GW, Pugliese A. Retrospective assessment of islet cell autoantibodies in pancreas organ donors. Diabetes care 2008;31(9):1741-1742.

109. Dijke IE, Hoeppli RE, Ellis T, Pearcey J, Huang Q, McMurchy AN 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.

110. Dijke IE, Weimar W, Baan CC. Regulatory T cells after organ transplantation: where does their action take place? Hum Immunol 2008;69(7):389-398.

111. 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.

112. Donckier V, Sanchez-Fueyo A, Craciun L, Lucidi V, Buggenhout A, Troisi R et al. Induction of tolerance in solid organ transplantation: the rationale to develop clinical protocols in liver transplantation. Transplant Proc 2009;41(2):603-606.

113. Dong M, Parsaik AK, Erwin PJ, Farnell MB, Murad MH, Kudva YC. Systematic review and meta-analysis: islet autotransplantation after pancreatectomy for minimizing diabetes. Clin Endocrinol 2011;75(6):771-779.

114. Dong Z, Saikumar P, Patel Y, Weinberg JM, Venkatachalam MA. Serine protease inhibitors suppress cytochrome c-mediatedcaspase-9 activation and apoptosis during hypoxia-reoxygenation. Biochem J 2000;347 Pt 3:669-677.

115. Dotta F, Censini S, van Halteren AG, Marselli L, Masini M, Dionisi S et al. Coxsackie B4 virus infection of beta cells and natural killer cell insulitis in recent-onset type 1 diabetic patients. Proc Natl Acad Sci U S A 2007;104(12):5115-5120.

116. Dou H, Wang C, Wu X, Yao L, Zhang X, Teng S et al. Calcium influx activates adenylyl cyclase 8 for sustained insulin secretion in rat pancreatic beta cells. Diabetologia 2015;58(2):324-333.

117. Durrbach A, Pestana JM, Pearson T, Vincenti F, Garcia VD, Campistol J et al. A phase III study of belatacept versus cyclosporine in kidney transplants from extended criteria donors (BENEFIT-EXT study). Am J Transplant 2010;10(3):547-557.

118. Echeverri GJ, McGrath K, Bottino R, Hara H, Dons EM, van der Windt DJ et al. Endoscopic gastric submucosal transplantation of islets (ENDO-STI): technique and initial results in diabetic pigs. Am J Transplant 2009;9(11):2485-2496.

119. Eckhard M, Martin I, Eich T, Weimer R, Zinn S, Bretzel RG et al. Incidence of cytomegalovirus infections after immunosuppression induction in clinical islet transplantation and impact on graft function. Transplant Proc 2002;34(5):1922-1924.

120. Eich T, Eriksson O, Lundgren T. Visualization of early engraftment in clinical islet transplantation by positron-emission tomography. N Engl J Med 2007;356(26):2754-2755.

121. Emamaullee JA, Merani S, Toso C, Kin T, Al-Saif F, Truong W et al. Porcine marginal mass islet autografts resist metabolic failure over time and are enhanced by early treatment with liraglutide. Endocrinology 2009;150(5):2145-2152.

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

123. Espes D, Eriksson O, Lau J, Carlsson PO. Striated muscle as implantation site for transplanted pancreatic islets. J Transplant 2011;2011:352043.

124. Faradji RN, Froud T, Messinger S, Monroy K, Pileggi A, Mineo D et al. Long-term metabolic and hormonal effects of exenatide on islet transplant recipients with allograft dysfunction. Cell Transplant 2009;18(10):1247-1259.

125. Farney AC, Cho E, Schweitzer EJ, Dunkin B, Philosophe B, Colonna J et al. Simultaneous cadaver pancreas living-donor kidney transplantation: a new approach for the type 1 diabetic uremic patient. Annals of surgery 2000;232(5):696-703.

126. Faye A, Van Den Abeele T, Peuchmaur M, Mathieu-Boue A, Vilmer E. Anti-CD20 monoclonal antibody for post-transplant lymphoproliferative disorders. Lancet 1998;352(9136):1285.

127. Federation ID. IDF Diabetes Atlas. Brussels, Belgium: International Diabetes Federation, 2013.

128. Fehr T, Sykes M. Tolerance induction in clinical transplantation. Transpl Immunol 2004;13(2):117-130.

129. Feng S, Goodrich NP, Bragg-Gresham JL, Dykstra DM, Punch JD, DebRoy MA et al. Characteristics associated with liver graft failure: the concept of a donor risk index. Am J Transplant 2006;6(4):783-790.

130. Ferrer-Sueta G, Vitturi D, Batinic-Haberle I, Fridovich I, Goldstein S, Czapski G et al. Reactions of manganese porphyrins with peroxynitrite and carbonate radical anion. J Biol Chem 2003;278(30):27432-27438.

131. Figliuzzi M, Bonandrini B, Silvani S, Remuzzi A. Mesenchymal stem cells help pancreatic islet transplantation to control type 1 diabetes. World J Stem Cells 2014;6(2):163-172.

132. Fiorina P, Shapiro AM, Ricordi C, Secchi A. The clinical impact of islet transplantation. Am J Transplant 2008;8(10):1990-1997.

133. Fischer A, Blanche S, Le Bidois J, Bordigoni P, Garnier JL, Niaudet P et al. Anti-B-cell monoclonal antibodies in the treatment of severe B-cell lymphoproliferative syndrome following bone marrow and organ transplantation. N Engl J Med 1991;324(21):1451-1456.

134. Forbes JM, Cooper ME. Mechanisms of diabetic complications. Physiol Rev 2013;93(1):137-188.

135. Ford O. Sernova gets approval to start Cell Pouch™ clinical trial in Canada. Medical Device Daily;16(90).

136. Forster S, Liu X, Adam U, Schareck WD, Hopt UT. Islet autotransplantation combined with pancreatectomy for treatment of pancreatic adenocarcinoma: a case report. Transplant Proc 2004;36(4):1125-1126.

137. Freeman RB, Paya C, Pescovitz MD, Humar A, Dominguez E, Washburn K et al. Risk factors for cytomegalovirus viremia and disease developing after prophylaxis in high-risk solid-organ transplant recipients. Transplantation 2004;78(12):1765-1773.

138. Friberg AS, Lundgren T, Malm H, Felldin M, Nilsson B, Jenssen T et al. Transplanted functional islet mass: donor, islet preparation, and recipient factors influence early graft function in islet-after-kidney patients. Transplantation 2012;93(6):632-638.

139. Fridell JA, Mangus RS, Hollinger EF, Taber TE, Goble ML, Mohler E et al. The case for pancreas after kidney transplantation. Clin Transplant 2009;23(4):447-453.

140. Fridell JA, Mangus RS, Powelson JA. Organ preservation solutions for whole organ pancreas transplantation. Curr Opin Organ Transplant 2011;16(1):116-122.

141. Froud T, Baidal DA, Faradji R, Cure P, Mineo D, Selvaggi G et al. Islet transplantation with alemtuzumab induction and calcineurin-free maintenance immunosuppression results in improved short- and long-term outcomes. Transplantation 2008;86(12):1695-1701.

142. Froud T, Yrizarry JM, Alejandro R, Ricordi C. Use of D-STAT to prevent bleeding following percutaneous transhepatic intraportal islet transplantation. Cell Transplant 2004;13(1):55-59.

143. Fugier-Vivier IJ, Rezzoug F, Huang Y, Graul-Layman AJ, Schanie CL, Xu H et al. Plasmacytoid precursor dendritic cells facilitate allogeneic hematopoietic stem cell engraftment. J Exp Med 2005;201(3):373-383.

144. Fujiwara N, Kobayashi K. Macrophages in inflammation. Curr Drug Targets Inflamm Allergy 2005;4(3):281-286.

145. Gabardi S, Martin ST, Roberts KL, Grafals M. Induction immunosuppressive therapies in renal transplantation. American journal of health-system pharmacy : Am J Health Syst Pharm 2011;68(3):211-218.

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

147. Gala-Lopez BL, Pepper AR, Pawlick RL, O'Gorman D, Kin T, Bruni A et al. Antiaging Glycopeptide Protects Human Islets Against Tacrolimus-Related Injury and Facilitates Engraftment in Mice. Diabetes 2016;65(2):451-462.

148. Gala-Lopez BL, Senior PA, Koh A, Kashkoush SM, Kawahara T, Kin T et al. Late cytomegalovirus transmission and impact of T-depletion in clinical islet transplantation. Am J Transplant 2011;11(12):2708-2714.

149. Gerber PA, Pavlicek V, Demartines N, Zuellig R, Pfammatter T, Wuthrich R et al. Simultaneous islet-kidney vs pancreas-kidney transplantation in type 1 diabetes mellitus: a 5 year single centre follow-up. Diabetologia 2008;51(1):110-119.

150. Ghofaili KA, Fung M, Ao Z, Meloche M, Shapiro RJ, Warnock GL et al. Effect of exenatide on beta cell function after islet transplantation in type 1 diabetes. Transplantation 2007;83(1):24-28.

151. 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.

152. Gioviale MC, Bellavia M, Damiano G, Lo Monte AI. Beyond islet transplantation in diabetes cell therapy: from embryonic stem cells to transdifferentiation of adult cells. Transplant Proc 2013;45(5):2019-2024.

153. Giuseppe Cavallari FNaBN. Combined Kidney-Islet Transplantation. In: Ortiz PJ, (ed). Understanding the Complexities of Kidney Transplantation: InTech, 2011.

154. Goto M, Eich TM, Felldin M, Foss A, Kallen R, Salmela K et al. Refinement of the automated method for human islet isolation and presentation of a closed system for in vitro islet culture. Transplantation 2004;78(9):1367-1375.

155. Goto M, Johansson H, Maeda A, Elgue G, Korsgren O, Nilsson B. Low molecular weight dextran sulfate prevents the instant blood-mediated inflammatory reaction induced by adult porcine islets. Transplantation 2004;77(5):741-747.

156. Grainger DW. All charged up about implanted biomaterials. Nat Biotechnol 2013;31(6):507-509.

157. Groth CG, Korsgren O, Tibell A, Tollemar J, Moller E, Bolinder J et al. Transplantation of porcine fetal pancreas to diabetic patients. Lancet 1994;344(8934):1402-1404.

158. Gruessner AC, Gruessner RW. Pancreas transplant outcomes for United States and non United States cases as reported to the United Network for Organ Sharing and the International Pancreas Transplant Registry as of December 2011. Clin Transpl 2012:23-40.

159. Gruessner AC, Sutherland DE. Pancreas transplant outcomes for United States (US) and non-US cases as reported to the United Network for Organ Sharing (UNOS) and the International Pancreas Transplant Registry (IPTR) as of June 2004. Clin Transplant 2005;19(4):433-455.

160. Gruessner AC, Sutherland DE. Access to pancreas transplantation should not be restricted because of age: invited commentary on Schenker et al. Transpl Int 2011;24(2):134-135.

161. Gruessner AC, Sutherland DE, Gruessner RW. Pancreas transplantation in the United States: a review. Curr Opin Organ Transplant 2010;15(1):93-101.

162. Gruessner RW, Kandaswamy R, Humar A, Gruessner AC, Sutherland DE. Calcineurin inhibitor- and steroid-free immunosuppression in pancreas-kidney and solitary pancreas transplantation. Transplantation 2005;79(9):1184-1189.

163. Hafiz MM, Poggioli R, Caulfield A, Messinger S, Geiger MC, Baidal DA et al. Cytomegalovirus prevalence and transmission after islet allograft transplant in patients with type 1 diabetes mellitus. Am J Transplant 2004;4(10):1697-1702.

164. Halloran PF. Immunosuppressive drugs for kidney transplantation. N Engl J Med 2004;351(26):2715-2729.

165. Han D, Berman DM, Willman M, Buchwald P, Rothen D, Kenyon NM et al. Choice of Immunosuppression Influences Cytomegalovirus DNAemia in Cynomolgus Monkey (Macaca fascicularis) Islet Allograft Recipients. Cell Transplant 2010.

166. Han DJ, Sutherland DE. Pancreas transplantation. Gut and liver 2010;4(4):450-465.

167. Hanley SC, Paraskevas S, Rosenberg L. Donor and isolation variables predicting human islet isolation success. Transplantation 2008;85(7):950-955.

168. Hariharan S, Pirsch JD, Lu CY, Chan L, Pesavento TE, Alexander S et al. Pancreas after kidney transplantation. J Am Soc Nephrol 2002;13(4):1109-1118.

169. Harlan DM, Kenyon NS, Korsgren O, Roep BO. Current advances and travails in islet transplantation. Diabetes 2009;58(10):2175-2184.

170. Hawthorne WJ, Salvaris EJ, Phillips P, Hawkes J, Liuwantara D, Burns H et al. Control of IBMIR in neonatal porcine islet xenotransplantation in baboons. Am J Transplant 2014;14(6):1300-1309.

171. Hellerstrom C, Andersson A, Korsgren O, Jansson L, Sandler S. Aspects of pancreatic islet transplantation in diabetes mellitus. Baillieres Clin Gastroenterol 1989;3(4):851-863.

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

173. Hering BJ, Kandaswamy R, Ansite JD, Eckman PM, Nakano M, Sawada T et al. Single-donor, marginal-dose islet transplantation in patients with type 1 diabetes. JAMA 2005;293(7):830-835.

174. Hering BJ, Kandaswamy R, Harmon JV, Ansite JD, Clemmings SM, Sakai T et al. Transplantation of cultured islets from two-layer preserved pancreases in type 1 diabetes with anti-CD3 antibody. Am J Transplant 2004;4(3):390-401.

175. Hernandez-Fisac I, Pizarro-Delgado J, Calle C, Marques M, Sanchez A, Barrientos A et al. Tacrolimus-induced diabetes in rats courses with suppressed insulin gene expression in pancreatic islets. Am J Transplant 2007;7(11):2455-2462.

176. Hilgert I, Franek F, Stefanova I, Kaslik J, Jirka J, Kristofova H et al. Therapeutic in vivo use of the A1-CD3 monoclonal antibody. Transplantation 1993;55(2):435-438.

177. Hrvatin S, O'Donnell CW, Deng F, Millman JR, Pagliuca FW, DiIorio P et al. Differentiated human stem cells resemble fetal, not adult, beta cells. Proc Natl Acad Sci U S A 2014;111(8):3038-3043.

178. Hua XF, Wang YW, Tang YX, Yu SQ, Jin SH, Meng XM et al. Pancreatic insulin-producing cells differentiated from human embryonic stem cells correct hyperglycemia in SCID/NOD mice, an animal model of diabetes. PloS One 2014;9(7):e102198.

179. Hughes SJ, Clark A, McShane P, Contractor HH, Gray DW, Johnson PR. Characterisation of collagen VI within the islet-exocrine interface of the human pancreas: implications for clinical islet isolation? Transplantation 2006;81(3):423-426.

180. Humar A, Mazzulli T, Moussa G, Razonable RR, Paya CV, Pescovitz MD et al. Clinical utility of cytomegalovirus (CMV) serology testing in high-risk CMV D+/R- transplant recipients. Am J Transplant 2005;5(5):1065-1070.

181. Humar A, Paya C, Pescovitz MD, Dominguez E, Washburn K, Blumberg E et al. Clinical utility of cytomegalovirus viral load testing for predicting CMV disease in D+/R- solid organ transplant recipients. Am J Transplant 2004;4(4):644-649.

182. Huurman VA, Kalpoe JS, van de Linde P, Vaessen N, Ringers J, Kroes AC et al. Choice of antibody immunotherapy influences cytomegalovirus viremia in simultaneous pancreas-kidney transplant recipients. Diabetes care 2006;29(4):842-847.

183. Hwang JW, Jung HS, Lee DY. Inhibition of platelet adhesion onto intrahepatically transplanted islets using PEGylation for attenuating instant blood-mediated inflammatory reaction (IBMIR). J Control Release : official journal of the Controlled Release Society 2011;152 Suppl 1:e213-214.

184. Ichii H, Inverardi L, Pileggi A, Molano RD, Cabrera O, Caicedo A et al. A novel method for the assessment of cellular composition and beta-cell viability in human islet preparations. Am J Transplant 2005;5(7):1635-1645.

185. Ichii H, Wang X, Messinger S, Alvarez A, Fraker C, Khan A et al. Improved human islet isolation using nicotinamide. Am J Transplant 2006;6(9):2060-2068.

186. Ihm SH, Matsumoto I, Sawada T, Nakano M, Zhang HJ, Ansite JD et al. Effect of donor age on function of isolated human islets. Diabetes 2006;55(5):1361-1368.

187. Ildstad ST, Leventhal J, Wen Y, Yolcu E. Facilitating cells: Translation of hematopoietic chimerism to achieve clinical tolerance. Chimerism 2016:1-7.

188. Im KI, Park MJ, Kim N, Lim JY, Park HS, Lee SH et al. Induction of mixed chimerism using combinatory cell-based immune modulation with mesenchymal stem cells and regulatory T cells for solid-organ transplant tolerance. Stem Cells Dev 2014;23(19):2364-2376.

189. Inc. P. AAGPs Overview. 2014; Available from: http://www.protokinetix.com/aagp/overview/

190. In't Veld P, Lievens D, De Grijse J, Ling Z, Van der Auwera B, Pipeleers-Marichal M et al. Screening for insulitis in adult autoantibody-positive organ donors. Diabetes 2007;56(9):2400-2404.

191. In't Veld P, Marichal M. Microscopic anatomy of the human islet of Langerhans. In: Islam MS, (ed). The Islets of Langerhans, Advances in Experimental Medicine and Biology: Springer Science, 2010: 1-19.

192. Jahansouz C, Kumer SC, Brayman KL. Evolution of beta-Cell Replacement Therapy in Diabetes Mellitus: Islet Cell Transplant. J Transplant 2011;2011:247959.

193. Jang HJ, Kim SC, Han DJ. Comparison of pancreas transplantation outcome between the cyclosporine and tacrolimus eras. Transplant Proc 2000;32(7):2470-2471.

194. Johnson JD, Ao Z, Ao P, Li H, Dai LJ, He Z et al. Different effects of FK506, rapamycin, and mycophenolate mofetil on glucose-stimulated insulin release and apoptosis in human islets. Cell Transplant 2009;18(8):833-845.

195. Johnson KH, O'Brien TD, Hayden DW, Jordan K, Ghobrial HK, Mahoney WC et al. Immunolocalization of islet amyloid polypeptide (IAPP) in pancreatic beta cells by means of peroxidase-antiperoxidase (PAP) and protein A-gold techniques. The Am J Pathol 1988;130(1):1-8.

196. Johnson LF, deSerres S, Herzog SR, Peterson HD, Meyer AA. Antigenic cross-reactivity between media supplements for cultured keratinocyte grafts. J Burn Care Rehabil 1991;12(4):306-312.

197. Juang JH, Bonner-Weir S, Ogawa Y, Vacanti JP, Weir GC. Outcome of subcutaneous islet transplantation improved by polymer device. Transplantation 1996;61(11):1557-1561.

198. Juang JH, Hsu BR, Kuo CH. Islet transplantation at subcutaneous and intramuscular sites. Transplant Proc 2005;37(8):3479-3481.

199. Juang SE, Huang HW, Kao CW, Chen CL, Lu HF, Cheng KW et al. Effect of university of wisconsin and histidine-tryptophan-ketoglutarate preservation solutions on blood potassium levels of patients undergoing living-donor liver transplantation. Transplant Proc 2012;44(2):366-368.

200. Kaddis JS, Hanson MS, Cravens J, Qian D, Olack B, Antler M et al. Standardized transportation of human islets: an islet cell resource center study of more than 2,000 shipments. Cell Transplant 2013;22(7):1101-1111.

201. Kagaya S, Kitanaka C, Noguchi K, Mochizuki T, Sugiyama A, Asai A et al. A functional role for death proteases in s-Myc- and c-Myc-mediated apoptosis. Mol Cell Biol 1997;17(11):6736-6745.

202. Kanak MA TM, Kunnatodi F, Lawrence MC, Levy M, Nazirudin B. Inflammatory Response in Islet Transplantation. Int J Endocrinol 2014.

203. Kandaswamy R, Stock PG, Skeans MA, Gustafson SK, Sleeman EF, Wainright JL et al. OPTN/SRTR 2011 Annual Data Report: pancreas. Am J Transplant 2013;13 Suppl 1:47-72.

204. Kantha SS. A centennial review; the 1890 tetanus antitoxin paper of von Behring and Kitasato and the related developments. Keio J Med 1991;40(1):35-39.

205. Kapur P, Pena-Llopis S, Christie A, Zhrebker L, Pavia-Jimenez A, Rathmell WK et al. Effects on survival of BAP1 and PBRM1 mutations in sporadic clear-cell renal-cell carcinoma: a retrospective analysis with independent validation. Lancet Oncol 2013;14(2):159-167.

206. Kaufman CL, Colson YL, Wren SM, Watkins S, Simmons RL, Ildstad ST. Phenotypic characterization of a novel bone marrow-derived cell that facilitates engraftment of allogeneic bone marrow stem cells. Blood 1994;84(8):2436-2446.

207. Kaufman DB, Iii GW, Bruce DS, Johnson CP, Gaber AO, Sutherland DE et al. Prospective, randomized, multi-center trial of antibody induction therapy in simultaneous pancreas-kidney transplantation. Am J Transplant 2003;3(7):855-864.

208. Kaufman DB, Leventhal JR, Gallon LG, Parker MA, Koffron AJ, Fryer JP et al. Risk factors and impact of cytomegalovirus disease in simultaneous pancreas-kidney transplantation. Transplantation 2001;72(12):1940-1945.

209. Kaufman DB, Shapiro R, Lucey MR, Cherikh WS, R TB, Dyke DB. Immunosuppression: practice and trends. Am J Transplant 2004;4 Suppl 9:38-53.

210. Kawahara T, Kin T, Kashkoush S, Gala-Lopez B, Bigam DL, Kneteman NM et al. Portal vein thrombosis is a potentially preventable complication in clinical islet transplantation. Am J Transplant 2011;11(12):2700-2707.

211. Kawahara T, Kin T, Shapiro AM. A comparison of islet autotransplantation with allotransplantation and factors elevating acute portal pressure in clinical islet transplantation. J Hepatobiliary Pancreat Sci 2012;19(3):281-288.

212. Kawakami Y, Iwata H, Gu Y, Miyamoto M, Murakami Y, Yamasaki T et al. Modified subcutaneous tissue with neovascularization is useful as the site for pancreatic islet transplantation. Cell Transplant 2000;9(5):729-732.

213. Kawakami Y, Iwata H, Gu YJ, Miyamoto M, Murakami Y, Balamurugan AN et al. Successful subcutaneous pancreatic islet transplantation using an angiogenic growth factor-releasing device. Pancreas 2001;23(4):375-381.

214. Kenneth Ward W. A review of the foreign-body response to subcutaneously-implanted devices: the role of macrophages and cytokines in biofouling and fibrosis. J Diabetes Sci Technol 2008;2(5):768-777.

215. Kenyon NS, Fernandez LA, Lehmann R, Masetti M, Ranuncoli A, Chatzipetrou M et al. Long-term survival and function of intrahepatic islet allografts in baboons treated with humanized anti-CD154. Diabetes 1999;48(7):1473-1481.

216. Kin T, Mirbolooki M, Brassil J, Shapiro AM, Lakey J. Machine perfusion for prolonged pancreas preservation prior to islet isolation (Abstract). In. Annual Scientific Meeting. Canadian Society of Transplantation. 2006.

217. Kin T, Mirbolooki M, Salehi P, Tsukada M, O'Gorman D, Imes S et al. Islet isolation and transplantation outcomes of pancreas preserved with University of Wisconsin solution versus two-layer method using preoxygenated perfluorocarbon. Transplantation 2006;82(10):1286-1290.

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

219. Kin T, O'Gorman D, Schroeder A, Onderka C, Richer B, Rosichuk S et al. Human islet distribution program for basic research at a single center. Transplant Proc 2011;43(9):3195-3197.

220. Kin T, Rosichuk S, Shapiro A, Lakey J. Detection of Microbial Contamination During Human Islet Isolation. Cell Transplant 2006;15:1–100.

221. 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.

222. King KM, Rubin G. A history of diabetes: from antiquity to discovering insulin. Br J Nurs 2003;12(18):1091-1095.

223. Kirk AD, Turgeon NA, Iwakoshi NN. B cells and transplantation tolerance. Nat Rev Nephrol 2010;6(10):584-593.

224. Kirschfink M. C1-inhibitor and transplantation. Immunobiology 2002;205(4-5):534-541.

225. Kitzmann JP, Pepper AR, Gala-Lopez B, Pawlick R, Kin T, O'Gorman D et al. Human islet viability and function is maintained during high-density shipment in silicone rubber membrane vessels. Transplant Proc 2014;46(6):1989-1991.

226. Kleinclauss F, Fauda M, Sutherland DE, Kleinclauss C, Gruessner RW, Matas AJ et al. Pancreas after living donor kidney transplants in diabetic patients: impact on long-term kidney graft function. Clin Transplant 2009;23(4):437-446.

227. Kloster-Jensen K, Sahraoui A, Vethe NT, Korsgren O, Bergan S, Foss A et al. Treatment with Tacrolimus and Sirolimus Reveals No Additional Adverse Effects on Human Islets In Vitro Compared to Each Drug Alone but They Are Reduced by Adding Glucocorticoids. J Diabetes Res 2016;2016:4196460.

228. Knoll GA, Nichol G. Dialysis, kidney transplantation, or pancreas transplantation for patients with diabetes mellitus and renal failure: a decision analysis of treatment options. J Am Soc Nephrol 2003;14(2):500-515.

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

230. Kohler G, Milstein C. Continuous cultures of fused cells secreting antibody of predefined specificity. Nature 1975;256(5517):495-497.

231. Korsgren O, Lundgren T, Felldin M, Foss A, Isaksson B, Permert J et al. Optimising islet engraftment is critical for successful clinical islet transplantation. Diabetologia 2008;51(2):227-232.

232. Korytnikov R, Nostro MC. Generation of polyhormonal and multipotent pancreatic progenitor lineages from human pluripotent stem cells. Methods 2015.

233. Kothary N, Diak IL, Brinker A, Bezabeh S, Avigan M, Dal Pan G. Progressive multifocal leukoencephalopathy associated with efalizumab use in psoriasis patients. J Am Acad Dermatol 2011;65(3):546-551.

234. Kriz J, Vilk G, Mazzuca DM, Toleikis PM, Foster PJ, White DJ. A novel technique for the transplantation of pancreatic islets within a vascularized device into the greater omentum to achieve insulin independence. Am J Surg 2012;203(6):793-797.

235. Kroon E, Martinson LA, Kadoya K, Bang AG, Kelly OG, Eliazer S et al. Pancreatic endoderm derived from human embryonic stem cells generates glucose-responsive insulin-secreting cells in vivo. Nat Biotechnol 2008;26(4):443-452.

236. Kuypers DR, Naesens M, de Jonge H, Lerut E, Verbeke K, Vanrenterghem Y. Tacrolimus dose requirements and CYP3A5 genotype and the development of calcineurin inhibitor-associated nephrotoxicity in renal allograft recipients. Ther Drug Monit 2010;32(4):394-404.

237. Lacy P KM. A method for th isolation of intact islets of Langerhans from the rat pancreas. Diabetes 1967;15(35).

238. Lakey JR, Helms LM, Kin T, Korbutt GS, Rajotte RV, Shapiro AM et al. Serine-protease inhibition during islet isolation increases islet yield from human pancreases with prolonged ischemia. Transplantation 2001;72(4):565-570.

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

240. Lakey JR, Warnock GL, Rajotte RV, Suarez-Alamazor ME, Ao Z, Shapiro AM et al. Variables in organ donors that affect the recovery of human islets of Langerhans. Transplantation 1996;61(7):1047-1053.

241. Lakey JR WG, Shapiro AM, Korbutt GS, Ao Z, Kneteman NM, et al. Intraductal collagenase delivery into the human pancreas using syringe loading or controlled perfusion. Cell Transplant 1999;8(3):285-292.

242. Lakey JRR, R. V.; Warnock, G. L. Microbial surveillance of human islet isolation, in vitro culture, and cryopreservation. Clin Invest Med 1995;18:168–176.

243. Lan MS, Wasserfall C, Maclaren NK, Notkins AL. IA-2, a transmembrane protein of the protein tyrosine phosphatase family, is a major autoantigen in insulin-dependent diabetes mellitus. Proc Natl Acad Sci U S A 1996;93(13):6367-6370.

244. Latchana N, Peck JR, Whitson BA, Henry ML, Elkhammas EA, Black SM. Preservation solutions used during abdominal transplantation: Current status and outcomes. World J Transplant 2015;5(4):154-164.

245. Latek R, Fleener C, Lamian V, Kulbokas E, 3rd, Davis PM, Suchard SJ et al. Assessment of belatacept-mediated costimulation blockade through evaluation of CD80/86-receptor saturation. Transplantation 2009;87(6):926-933.

246. Laughlin E, Burke G, Pugliese A, Falk B, Nepom G. Recurrence of autoreactive antigen-specific CD4+ T cells in autoimmune diabetes after pancreas transplantation. Clinical immunology 2008;128(1):23-30.

247. Leeser DB, Bingaman AW, Poliakova L, Shi Q, Gage F, Bartlett ST et al. Pulsatile pump perfusion of pancreata before human islet cell isolation. Transplant Proc 2004;36(4):1050-1051.

248. Lehmann R, Weber M, Berthold P, Zullig R, Pfammatter T, Moritz W et al. Successful simultaneous islet-kidney transplantation using a steroid-free immunosuppression: two-year follow-up. Am J Transplant 2004;4(7):1117-1123.

249. Lenisa L, Castoldi R, Socci C, Motta F, Ferrari G, Spotti D et al. Cost-effective treatment for diabetic end-stage renal disease: dialysis, kidney, or kidney-pancreas transplantation? Transplant Proc 1995;27(6):3108-3113.

250. Leventhal J, Abecassis M, Miller J, Gallon L, Ravindra K, Tollerud DJ et al. Chimerism and tolerance without GVHD or engraftment syndrome in HLA-mismatched combined kidney and hematopoietic stem Cell Transplant. Sci Transl Med 2012;4(124):124ra128.

251. Leventhal J, Abecassis M, Miller J, Gallon L, Tollerud D, Elliott MJ et al. Tolerance induction in HLA disparate living donor kidney transplantation by donor stem cell infusion: durable chimerism predicts outcome. Transplantation 2013;95(1):169-176.

252. Li D-s, Hao J, Yuan Y-H, Yun SH, Feng J-B, Dai L-J et al. Pancreatic islet transplantation to the renal subcapsule in mice. Protoc exch 2011.

253. Li F, Li Q. Effects of different dose of FK506 on endocrine function of pancreatic islets and damage of beta cells of pancreatic islets in a Wistar rat model. Immunopharmacol Immunotoxicol 2010;32(2):333-338.

254. Lifson N, Kramlinger KG, Mayrand RR, Lender EJ. Blood flow to the rabbit pancreas with special reference to the islets of Langerhans. Gastroenterology 1980;79(3):466-473.

255. Lloveras J, Farney AC, Sutherland DE, Wahoff D, Field J, Gores PF. Significance of contaminated islet preparations in clinical islet transplantation. Transplant Proc 1994;26(2):579-580.

256. Locke JE, Magro CM, Singer AL, Segev DL, Haas M, Hillel AT et al. The use of antibody to complement protein C5 for salvage treatment of severe antibody-mediated rejection. Am J Transplant 2009;9(1):231-235.

257. Lowe MC, Badell IR, Turner AP, Thompson PW, Leopardi FV, Strobert EA et al. Belatacept and sirolimus prolong nonhuman primate islet allograft survival: adverse consequences of concomitant alefacept therapy. Am J Transplant 2013;13(2):312-319.

258. Mackensen A, Drager R, Schlesier M, Mertelsmann R, Lindemann A. Presence of IgE antibodies to bovine serum albumin in a patient developing anaphylaxis after vaccination with human peptide-pulsed dendritic cells. Cancer Immunol Immunother 2000;49(3):152-156.

259. Maechler P. Mitochondria as the conductor of metabolic signals for insulin exocytosis in pancreatic beta-cells. Cell Mol Life Sci 2002;59(11):1803-1818.

260. Maffi P, Balzano G, Ponzoni M, Nano R, Sordi V, Melzi R et al. Autologous Pancreatic Islet Transplantation in Human Bone Marrow. Diabetes 2013;62(10):3523-3531.

261. Maffi P, Bertuzzi F, De Taddeo F, Magistretti P, Nano R, Fiorina P et al. Kidney function after islet transplant alone in type 1 diabetes: impact of immunosuppressive therapy on progression of diabetic nephropathy. Diabetes care 2007;30(5):1150-1155.

262. Malinowski M, Pratschke J, Lock J, Neuhaus P, Stockmann M. Effect of tacrolimus dosing on glucose metabolism in an experimental rat model. Ann Transplant 2010;15(3):60-65.

263. Marroquin CE, Edwards EB, Collins BH, Desai DM, Tuttle-Newhall JE, Kuo PC. Half-life analysis of pancreas and kidney transplants. Transplantation 2005;80(2):272-275.

264. Martin ST, Tichy EM, Gabardi S. Belatacept: a novel biologic for maintenance immunosuppression after renal transplantation. Pharmacotherapy 2011;31(4):394-407.

265. Matsumoto I, Sawada T, Nakano M, Sakai T, Liu B, Ansite JD et al. Improvement in islet yield from obese donors for human islet transplants. Transplantation 2004;78(6):880-885.

266. Matsumoto S, Matsusita M, Morita T, Kamachi H, Tsukiyama S, Furukawa Y et al. Effects of synthetic antifreeze glycoprotein analogue on islet cell survival and function during cryopreservation. Cryobiology 2006;52(1):90-98.

267. Matsumoto S, Noguchi H, Hatanaka N, Kobayashi N, Jackson A, Naziruddin B et al. Evaluation of engraftment after single islet transplantation from a brain-dead donor by the secretory unit of islet transplant objects (SUITO) index. Transplant Proc 2008;40(2):364-366.

268. Matsumoto S, Noguchi H, Hatanaka N, Shimoda M, Kobayashi N, Jackson A et al. SUITO index for evaluation of efficacy of single donor islet transplantation. Cell Transplant 2009;18(5):557-562.

269. Matsumoto S, Noguchi H, Takita M, Shimoda M, Tamura Y, Olsen G et al. Excellence of suito index for assessing clinical outcome of islet transplantation. Transplant Proc 2010;42(6):2062-2064.

270. Matsumoto S, Noguchi H, Takita M, Shimoda M, Tamura Y, Olsen G et al. Super-high-dose islet transplantation is associated with high SUITO index and prolonged insulin independence: a case report. Transplant Proc 2010;42(6):2156-2158.

271. Matsumoto S, Rigley TH, Reems JA, Kuroda Y, Stevens RB. Improved islet yields from Macaca nemestrina and marginal human pancreata after two-layer method preservation and endogenous trypsin inhibition. Am J Transplant 2003;3(1):53-63.

272. Matsumoto S, Takita M, Chaussabel D, Noguchi H, Shimoda M, Sugimoto K 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 Transplant 2011.

273. Matsumoto S, Yamada Y, Okitsu T, Iwanaga Y, Noguchi H, Nagata H et al. Simple evaluation of engraftment by secretory unit of islet transplant objects for living donor and cadaveric donor fresh or cultured islet transplantation. Transplant Proc 2005;37(8):3435-3437.

274. 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.

275. McCall M, Shapiro AM. Update on islet transplantation. Cold Spring Harb Perspect Med 2012;2(7):a007823.

276. McCall MD, Toso C, Baetge EE, Shapiro AM. Are stem cells a cure for diabetes? Clin Sci (Lond) 2010;118(2):87-97.

277. Meier JJ, Bhushan A, Butler AE, Rizza RA, Butler PC. Sustained beta cell apoptosis in patients with long-standing type 1 diabetes: indirect evidence for islet regeneration? Diabetologia 2005;48(11):2221-2228.

278. Meloche RM. Transplantation for the treatment of type 1 diabetes. World J Gastroenterol 2007;13(47):6347-6355.

279. Melov S, Schneider JA, Day BJ, Hinerfeld D, Coskun P, Mirra SS et al. A novel neurological phenotype in mice lacking mitochondrial manganese superoxide dismutase. Nat Genet 1998;18(2):159-163.

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

281. Merani S, Toso C, Emamaullee J, Shapiro AM. Optimal implantation site for pancreatic islet transplantation. Br J Surg 2008;95(12):1449-1461.

282. 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.

283. Metalidis C, Lerut E, Naesens M, Kuypers DR. Expression of CYP3A5 and P-glycoprotein in renal allografts with histological signs of calcineurin inhibitor nephrotoxicity. Transplantation 2011;91(10):1098-1102.

284. Meyer AA, Manktelow A, Johnson M, deSerres S, Herzog S, Peterson HD. Antibody response to xenogeneic proteins in burned patients receiving cultured keratinocyte grafts. J Trauma 1988;28(7):1054-1059.

285. Miki A, Ricordi C, Yamamoto T, Mita A, Barker S, Khan A et al. Effect of human islet rescue gradient purification on islet yield and fractional Beta cell viability. Transplant Proc 2008;40(2):360-361.

286. Minor T, Tolba R, Akbar S, Dombrowski T, Muller A. The suboptimal donor: reduction of ischemic injury in fatty livers by gaseous oxygen persufflation. Transplant Proc 2000;32(1):10.

287. Moberg L, Olsson A, Berne C, Felldin M, Foss A, Kallen R et al. Nicotinamide inhibits tissue factor expression in isolated human pancreatic islets: implications for clinical islet transplantation. Transplantation 2003;76(9):1285-1288.

288. Mohacsi P, Rieben R, Sigurdsson G, Tschanz H, Schaffner T, Nydegger UE et al. Successful management of a B-type cardiac allograft into an O-type man with 3(1/2)-year clinical follow-up. Transplantation 2001;72(7):1328-1330.

289. Mojibian M, Harder B, Hurlburt A, Bruin JE, Asadi A, Kieffer TJ. Implanted islets in the anterior chamber of the eye are prone to autoimmune attack in a mouse model of diabetes. Diabetologia 2013;56(10):2213-2221.

290. Mottershead M, Neuberger J. Daclizumab. Expert Opin Biol Ther 2007;7(10):1583-1596.

291. Murdoch TB, McGhee-Wilson D, Shapiro AM, Lakey JR. Methods of human islet culture for transplantation. Cell Transplant 2004;13(6):605-617.

292. Muthusamy AS, Mumford L, Hudson A, Fuggle SV, Friend PJ. Pancreas transplantation from donors after circulatory death from the United Kingdom. Am J Transplant 2013;13(3):824.

293. Muthusamy AS, Vaidya AC, Sinha S, Roy D, Elker DE, Friend PJ. Alemtuzumab induction and steroid-free maintenance immunosuppression in pancreas transplantation. Am J Transplant 2008;8(10):2126-2131.

294. Naesens M, Kuypers D, Sarwal MM. The bumpy road of genomic medicine in transplantation: lessons from studies on calcineurin inhibitor nephrotoxicity. Transplantation 2012;93(6):578-579.

295. Naesens M, Kuypers DR, Sarwal M. Calcineurin inhibitor nephrotoxicity. Clin J Am Soc Nephrol 2009;4(2):481-508.

296. Nagai K, Yagi S, Afify M, Bleilevens C, Uemoto S, Tolba RH. Impact of venous-systemic oxygen persufflation with nitric oxide gas on steatotic grafts after partial orthotopic liver transplantation in rats. Transplantation 2013;95(1):78-84.

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

298. Narang AS, Mahato RI. Biological and biomaterial approaches for improved islet transplantation. Pharmacol Rev 2006;58(2):194-243.

299. Nashan B, Spetzler V, Schemmer P, Kirste G, Rahmel A. Regarding "Compared Efficacy of Preservation Solutions in Liver Transplantation: A Long-Term Graft Outcome Study From the European Liver Transplant Registry". Am J Transplant 2015;15(12):3272-3273.

300. 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.

301. Nir T, Melton DA, Dor Y. Recovery from diabetes in mice by beta cell regeneration. J Clin Invest 2007;117(9):2553-2561.

302. Nishimura R, Goto M, Sekiguchi S, Fujimori K, Ushiyama A, Satomi S. Assessment for revascularization of transplanted pancreatic islets at subcutaneous site in mice with a highly sensitive imaging system. Transplant Proc 2011;43(9):3239-3240.

303. Nishimura R, Nishioka S, Fujisawa I, Shiku H, Shimada M, Sekiguchi S et al. Tacrolimus inhibits the revascularization of isolated pancreatic islets. PloS One 2013;8(4):e56799.

304. Niu YJ, Shen ZY, Xu C, Li CF, Liu XJ, Teng YQ et al. Establishment of tacrolimus-induced diabetes in rat model and assessment of clinical treatments for post-transplant diabetes mellitus in liver transplant recipients. Clin Lab 2013;59(7-8):869-874.

305. Noguchi H. Pancreas procurement and preservation for islet transplantation: personal considerations. J Transplant 2011;2011:783168.

306. Noguchi H, Naziruddin B, Jackson A, Shimoda M, Fujita Y, Chujo D et al. Comparison of ulinastatin, gabexate mesilate, and nafamostat mesilate in preservation solution for islet isolation. Cell Transplant 2012;21(2-3):509-516.

307. Noguchi H, Naziruddin B, Onaca N, Jackson A, Shimoda M, Ikemoto T et al. Comparison of modified Celsior solution and M-kyoto solution for pancreas preservation in human islet isolation. Cell Transplant 2010;19(6):751-758.

308. Noguchi H, Ueda M, Nakai Y, Iwanaga Y, Okitsu T, Nagata H et al. Modified two-layer preservation method (M-Kyoto/PFC) improves islet yields in islet isolation. Am J Transplant 2006;6(3):496-504.

309. Nostro MC, Keller G. Generation of beta cells from human pluripotent stem cells: Potential for regenerative medicine. Semin Cell Dev Biol 2012;23(6):701-710.

310. Nostro MC, Sarangi F, Yang C, Holland A, Elefanty AG, Stanley EG et al. Efficient generation of NKX6-1+ pancreatic progenitors from multiple human pluripotent stem cell lines. Stem cell reports 2015;4(4):591-604.

311. Nyqvist D, Kohler M, Wahlstedt H, Berggren PO. Donor islet endothelial cells participate in formation of functional vessels within pancreatic islet grafts. Diabetes 2005;54(8):2287-2293.

312. Oberholzer J, Mathe Z, Bucher P, Triponez F, Bosco D, Fournier B et al. Islet autotransplantation after left pancreatectomy for non-enucleable insulinoma. Am J Transplant 2003;3(10):1302-1307.

313. Oetjen E, Baun D, Beimesche S, Krause D, Cierny I, Blume R et al. Inhibition of human insulin gene transcription by the immunosuppressive drugs cyclosporin A and tacrolimus in primary, mature islets of transgenic mice. Mol Pharmacol 2003;63(6):1289-1295.

314. O'Gorman D, Kin T, Imes S, Pawlick R, Senior P, Shapiro AM. Comparison of human islet isolation outcomes using a new mammalian tissue-free enzyme versus collagenase NB-1. Transplantation 2010;90(3):255-259.

315. O'Gorman D, Kin T, Murdoch T, Richer B, McGhee-Wilson D, Ryan E et al. The standardization of pancreatic donors for islet isolation. Transplant Proc 2005;37(2):1309-1310.

316. Olsson R, Olerud J, Pettersson U, Carlsson PO. Increased numbers of low-oxygenated pancreatic islets after intraportal islet transplantation. Diabetes 2011;60(9):2350-2353.

317. Otten HG, Verhaar MC, Borst HP, Hene RJ, van Zuilen AD. Pretransplant donor-specific HLA class-I and -II antibodies are associated with an increased risk for kidney graft failure. Am J Transplant 2012;12(6):1618-1623.

318. Owen RJ, Ryan EA, O'Kelly K, Lakey JR, McCarthy MC, Paty BW et al. Percutaneous transhepatic pancreatic islet Cell Transplant in type 1 diabetes mellitus: radiologic aspects. Radiology 2003;229(1):165-170.

319. 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.

320. Page EK, Dar WA, Knechtle SJ. Biologics in organ transplantation. Transpl Int 2012;25(7):707-719.

321. Pagliuca FW, Millman JR, Gurtler M, Segel M, Van Dervort A, Ryu JH et al. Generation of functional human pancreatic beta cells in vitro. Cell 2014;159(2):428-439.

322. Pak CY, Eun HM, McArthur RG, Yoon JW. Association of cytomegalovirus infection with autoimmune type 1 diabetes. Lancet 1988;2(8601):1-4.

323. Papas KK, Avgoustiniatos ES, Tempelman LA, Weir GC, Colton CK, Pisania A et al. High-density culture of human islets on top of silicone rubber membranes. Transplant Proc 2005;37(8):3412-3414.

324. Papas KK, Colton CK, Nelson RA, Rozak PR, Avgoustiniatos ES, Scott WE, 3rd et al. Human islet oxygen consumption rate and DNA measurements predict diabetes reversal in nude mice. Am J Transplant 2007;7(3):707-713.

325. 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.

326. Park IC, Park MJ, Choe TB, Jang JJ, Hong SI, Lee SH. TNF-alpha induces apoptosis mediated by AEBSF-sensitive serine protease(s) that may involve upstream caspase-3/CPP32 protease activation in a human gastric cancer cell line. Int J Oncol 2000;16(6):1243-1248.

327. Paty BW KA, Senior P. Pancreas and Islet Transplantation. Can J Diabetes 2013;37:S94 - S96.

328. Pawlick R, Gala-Lopez B, Pepper AR, McCall M, Ziff O, Shapiro AM. The combination of anti-NKG2D and CTLA-4 Ig therapy prolongs islet allograft survival in a murine model. Am J Transplant 2014;14(10):2367-2374.

329. Paya C, Humar A, Dominguez E, Washburn K, Blumberg E, Alexander B et al. Efficacy and safety of valganciclovir vs. oral ganciclovir for prevention of cytomegalovirus disease in solid organ transplant recipients. Am J Transplant 2004;4(4):611-620.

330. Pepper A, Gala-Lopez BL, Kin T. Advances in Clinical Islet Isolation. In: S. I, (ed). Islets ofLangerhans. Dordrecht: Springer, 2014: 1165-1187.

331. 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.

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

333. 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.

334. Pepper AR, Pawlick R, Gala-Lopez B, MacGillivary A, Mazzuca DM, White DJ et al. Diabetes Is Reversed in a Murine Model by Marginal Mass Syngeneic Islet Transplantation Using a Subcutaneous Cell Pouch Device. Transplantation 2015;99(11):2294-2300.

335. Philosophe B, Farney AC, Schweitzer EJ, Colonna JO, Jarrell BE, Krishnamurthi V et al. Superiority of portal venous drainage over systemic venous drainage in pancreas transplantation: a retrospective study. Annals of surgery 2001;234(5):689-696.

336. Piganelli JD, Flores SC, Cruz C, Koepp J, Batinic-Haberle I, Crapo J 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.

337. Pigeau GM, Kolic J, Ball BJ, Hoppa MB, Wang YW, Ruckle T et al. Insulin granule recruitment and exocytosis is dependent on p110gamma in insulinoma and human beta-cells. Diabetes 2009;58(9):2084-2092.

338. Pileggi A, Molano RD, Ricordi C, Zahr E, Collins J, Valdes R et al. Reversal of diabetes by pancreatic islet transplantation into a subcutaneous, neovascularized device. Transplantation 2006;81(9):1318-1324.

339. Pileggi A, Ricordi C, Alessiani M, Inverardi L. Factors influencing Islet of Langerhans graft function and monitoring. Clin Chim Acta 2001;310(1):3-16.

340. 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.

341. 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.

342. Ponte GM, Pileggi A, Messinger S, Alejandro A, Ichii H, Baidal DA et al. Toward maximizing the success rates of human islet isolation: influence of donor and isolation factors. Cell Transplant 2007;16(6):595-607.

343. Porcheray F, Wong W, Saidman SL, De Vito J, Girouard TC, Chittenden M et al. B-cell immunity in the context of T-cell tolerance after combined kidney and bone marrow transplantation in humans. Am J Transplant 2009;9(9):2126-2135.

344. Posselt AM, Szot GL, Frassetto LA, Masharani U, Tavakol M, Amin R et al. Islet transplantation in type 1 diabetic patients using calcineurin inhibitor-free immunosuppressive protocols based on T-cell adhesion or costimulation blockade. Transplantation 2010;90(12):1595-1601.

345. Potter KJ, Abedini A, Marek P, Klimek AM, Butterworth S, Driscoll M et al. Islet amyloid deposition limits the viability of human islet grafts but not porcine islet grafts. Proc Natl Acad Sci U S A 2010;107(9):4305-4310.

346. Pozzilli P, Strollo R. Immunotherapy for Type 1 diabetes: getting beyond a negative first impression. Immunotherapy 2012;4(7):655-658.

347. Pugliese A, Reijonen HK, Nepom J, Burke GW, 3rd. Recurrence of autoimmunity in pancreas transplant patients: research update. Diabetes management 2011;1(2):229-238.

348. Rafael E, Tibell A, Rydén M, Lundgren T, Sävendahl L, Borgström B et al. Intramuscular Autotransplantation of Pancreatic Islets in a 7-Year-Old Child: A 2-Year Follow-Up. Am J Transplant 2008;8(2):458-462.

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

350. Ranuncoli A, Cautero N, Ricordi C, Masetti M, Molano RD, Inverardi L et al. Islet Cell Transplant: in vivo and in vitro functional assessment of nonhuman primate pancreatic islets. Cell Transplant 2000;9(3):409-414.

351. Rao PS, Schaubel DE, Guidinger MK, Andreoni KA, Wolfe RA, Merion RM et al. A comprehensive risk quantification score for deceased donor kidneys: the kidney donor risk index. Transplantation 2009;88(2):231-236.

352. Registry CIT. Annual Report. 2008 November 27, 2008.

353. Reichert JM. Metrics for antibody therapeutics development. MAbs 2010;2(6):695-700.

354. Reichert JM. Next generation and biosimilar monoclonal antibodies: essential considerations towards regulatory acceptance in Europe. February 3-4, 2011, Freiburg, Germany. MAbs 2011;3(3):223-240.

355. Reichert JM. Which are the antibodies to watch in 2012? MAbs 2012;4(1):1-3.

356. Reichert JM. Which are the antibodies to watch in 2013? MAbs 2013;5(1):1-4.

357. Report CITR. Sixth Annual Report. www.citregistry.org/ 2009; Available from:

358. Report CORRA. Treatment of End-Stage Organ Failure in Canada, 2001 to 2010. In: Information CIfH, (ed). Ottawa, ON, 2011.

359. Rewers M, Gottlieb P. Immunotherapy for the prevention and treatment of type 1 diabetes: human trials and a look into the future. Diabetes care 2009;32(10):1769-1782.

360. Rezania A, Bruin JE, Arora P, Rubin A, Batushansky I, Asadi A et al. Reversal of diabetes with insulin-producing cells derived in vitro from human pluripotent stem cells. Nat Biotechnol 2014;32(11):1121-1133.

361. Rezania A, Bruin JE, Riedel MJ, Mojibian M, Asadi A, Xu J et al. Maturation of human embryonic stem cell-derived pancreatic progenitors into functional islets capable of treating pre-existing diabetes in mice. Diabetes 2012;61(8):2016-2029.

362. Ricordi C, Gray DW, Hering BJ, Kaufman DB, Warnock GL, Kneteman NM et al. Islet isolation assessment in man and large animals. Acta Diabetologica Lat 1990;27(3):185-195.

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

364. Ricordi C, Lacy PE, Scharp DW. Automated islet isolation from human pancreas. Diabetes 1989;38 Suppl 1:140-142.

365. Ricordi C, Strom TB. Clinical islet transplantation: advances and immunological challenges. Nat Rev Immunol 2004;4(4):259-268.

366. Ricordi C, Tzakis AG, Carroll PB, Zeng YJ, Rilo HL, Alejandro R et al. Human islet isolation and allotransplantation in 22 consecutive cases. Transplantation 1992;53(2):407-414.

367. Ris F, Niclauss N, Morel P, Demuylder-Mischler S, Muller Y, Meier R et al. Islet autotransplantation after extended pancreatectomy for focal benign disease of the pancreas. Transplantation 2011;91(8):895-901.

368. Robertson RP, Davis C, Larsen J, Stratta R, Sutherland DE. Pancreas and islet transplantation in type 1 diabetes. Diabetes care 2006;29(4):935.

369. Rodriguez-Rodriguez AE, Trinanes J, Velazquez-Garcia S, Porrini E, Vega Prieto MJ, Diez Fuentes ML et al. The higher diabetogenic risk of tacrolimus depends on pre-existing insulin resistance. A study in obese and lean Zucker rats. Am J Transplant 2013;13(7):1665-1675.

370. Roep BO, Hiemstra HS, Schloot NC, De Vries RR, Chaudhuri A, Behan PO et al. Molecular mimicry in type 1 diabetes: immune cross-reactivity between islet autoantigen and human cytomegalovirus but not Coxsackie virus. Ann N Y Acad Sci 2002;958:163-165.

371. Rostambeigi N, Lanza IR, Dzeja PP, Deeds MC, Irving BA, Reddi HV et al. Unique cellular and mitochondrial defects mediate FK506-induced islet beta-cell dysfunction. Transplantation 2011;91(6):615-623.

372. Russ HA, Parent AV, Ringler JJ, Hennings TG, Nair GG, Shveygert M et al. Controlled induction of human pancreatic progenitors produces functional beta-like cells in vitro. The EMBO journal 2015;34(13):1759-1772.

373. Ryan EA, Bigam D, Shapiro AM. Current indications for pancreas or islet transplant. Diabetes Obes Metab 2006;8(1):1-7.

374. Ryan EA, Lakey JR, Paty BW, Imes S, Korbutt GS, Kneteman NM et al. Successful islet transplantation: continued insulin reserve provides long-term glycemic control. Diabetes 2002;51(7):2148-2157.

375. 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.

376. Ryan EA, Shandro T, Green K, Paty BW, Senior PA, Bigam D et al. Assessment of the severity of hypoglycemia and glycemic lability in type 1 diabetic subjects undergoing islet transplantation. Diabetes 2004;53(4):955-962.

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

378. Saito T, Ohashi K, Utoh R, Shimizu H, Ise K, Suzuki H et al. Reversal of diabetes by the creation of neo-islet tissues into a subcutaneous site using islet cell sheets. Transplantation 2011;92(11):1231-1236.

379. Sakata N, Aoki T, Yoshimatsu G, Tsuchiya H, Hata T, Katayose Y et al. Strategy for clinical setting in intramuscular and subcutaneous islet transplantation. Diabetes Metab Res Rev 2013.

380. Sakata N, Sumi S, Yoshimatsu G, Goto M, Egawa S, Unno M. Encapsulated islets transplantation: Past, present and future. World J Gastrointest Pathophysiol 2012;3(1):19-26.

381. Sarantopoulos S, Stevenson KE, Kim HT, Cutler CS, Bhuiya NS, Schowalter M et al. Altered B-cell homeostasis and excess BAFF in human chronic graft-versus-host disease. Blood 2009;113(16):3865-3874.

382. Scharfmann R, Xiao X, Heimberg H, Mallet J, Ravassard P. Beta cells within single human islets originate from multiple progenitors. PloS One 2008;3(10):e3559.

383. Scharp DW, Lacy PE, Santiago JV, McCullough CS, Weide LG, Falqui L et al. Insulin independence after islet transplantation into type I diabetic patient. Diabetes 1990;39(4):515-518.

384. Scharp DW KC, Knight MJ, Ballinger WF, Lacy P. The use of Ficoll in the preparations of viable islet of Langerhans from the rat pancreas. Transplantation 1973;16(6):686.

385. Scharp DWL, P. E.; McLear, M.; Longwith, J.; Olack, B. The bioburden of 590 consecutive human pancreata for islet transplant research. Transplant Proc 1992;24:974–975.

386. Schneeberger S, Biebl M, Steurer W, Hesse UJ, Troisi R, Langrehr JM et al. A prospective randomized multicenter trial comparing histidine-tryptophane-ketoglutarate versus University of Wisconsin perfusion solution in clinical pancreas transplantation. Transpl Int 2009;22(2):217-224.

387. Schuit FC, In't Veld PA, Pipeleers DG. Glucose stimulates proinsulin biosynthesis by a dose-dependent recruitment of pancreatic beta cells. Proc Natl Acad Sci U S A 1988;85(11):3865-3869.

388. Schwab N, Ulzheimer JC, Fox RJ, Schneider-Hohendorf T, Kieseier BC, Monoranu CM et al. Fatal PML associated with efalizumab therapy: insights into integrin alphaLbeta2 in JC virus control. Neurology 2012;78(7):458-467; discussion 465.

389. Scott WE, 3rd, O'Brien TD, Ferrer-Fabrega J, Avgoustiniatos ES, Weegman BP, Anazawa T et al. Persufflation improves pancreas preservation when compared with the two-layer method. Transplant Proc 2010;42(6):2016-2019.

390. 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. Can J Diabetes 2012;36:32-37.

391. 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.

392. 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.

393. Shapiro AM. Strategies toward single-donor islets of Langerhans transplantation. Curr Opin Organ Transplant 2011;16(6):627-631.

394. Shapiro AM. State of the art of clinical islet transplantation and novel protocols of immunosuppression. Curr Diab Rep 2011;11(5):345-354.

395. Shapiro AM. Immune antibody monitoring predicts outcome in islet transplantation. Diabetes 2013;62(5):1377-1378.

396. Shapiro AM, Gallant HL, Hao EG, Lakey JR, McCready T, Rajotte RV et al. The portal immunosuppressive storm: relevance to islet transplantation? Ther Drug Monit 2005;27(1):35-37.

397. Shapiro AM, Lakey JR, Paty BW, Senior PA, Bigam DL, Ryan EA. Strategic opportunities in clinical islet transplantation. Transplantation 2005;79(10):1304-1307.

398. 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.

399. Shapiro AM, Nanji SA, Lakey JR. Clinical islet transplant: current and future directions towards tolerance. Immunol Rev 2003;196:219-236.

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

401. Shapiro AM, Ricordi C. islet Cell Transplant - Indications, Risks and Long-Term Outcomes. In: Kirk AD, Knechtle SJ, Larsen CP, Madsen JC, Pearson TC, Webber SA, (eds). Textbook of Organ Transplantation: Wiley-Blackwell, 2014: 1-1904.

402. Shapiro AM, Ricordi C, Hering BJ, Auchincloss H, Lindblad R, Robertson RP et al. International trial of the Edmonton protocol for islet transplantation. N Engl J Med 2006;355(13):1318-1330.

403. Shapiro AMJ. A historical perspective on experimental and clinical islet transplantation. In: A.M. James Shapiro JAMS, (ed). Islet Transplantation and beta cell replacement therapy New York. New York: Informa helthcare, 2007.

404. Sharkawy AA, Klitzman B, Truskey GA, Reichert WM. Engineering the tissue which encapsulates subcutaneous implants. I. Diffusion properties. J Biomed Mater Res 1997;37(3):401-412.

405. Sharkawy AA, Klitzman B, Truskey GA, Reichert WM. Engineering the tissue which encapsulates subcutaneous implants. III. Effective tissue response times. J Biomed Mater Res 1998;40(4):598-605.

406. Sharkawy AA, Klitzman B, Truskey GA, Reichert WM. Engineering the tissue which encapsulates subcutaneous implants. II. Plasma-tissue exchange properties. J Biomed Mater Res 1998;40(4):586-597.

407. Simeonovic CJ, Dhall DP, Wilson JD, Lafferty KJ. A comparative study of transplant sites for endocrine tissue transplantation in the pig. Aust J Exp Biol Med Sci 1986;64 (Pt 1):37-41.

408. Simon MC, Keith B. The role of oxygen availability in embryonic development and stem cell function. Nat Rev Mol Cell Biol 2008;9(4):285-296.

409. Sklavos MM, Bertera S, Tse HM, Bottino R, He J, Beilke JN et al. Redox modulation protects islets from transplant-related injury. Diabetes 2010;59(7):1731-1738.

410. Slegtenhorst BR, Dor FJ, Elkhal A, Rodriguez H, Yang X, Edtinger K et al. Mechanisms and consequences of injury and repair in older organ transplants. Transplantation 2014;97(11):1091-1099.

411. Socarras TO, Vasconcelos AC, Campos PP, Pereira NB, Souza JP, Andrade SP. Foreign body response to subcutaneous implants in diabetic rats. PloS One 2014;9(11):e110945.

412. Soedamah-Muthu SS, Fuller JH, Mulnier HE, Raleigh VS, Lawrenson RA, Colhoun HM. High risk of cardiovascular disease in patients with type 1 diabetes in the U.K.: a cohort study using the general practice research database. Diabetes care 2006;29(4):798-804.

413. Spees JL, Gregory CA, Singh H, Tucker HA, Peister A, Lynch PJ et al. Internalized antigens must be removed to prepare hypoimmunogenic mesenchymal stem cells for cell and gene therapy. Mol Ther 2004;9(5):747-756.

414. Speier S, Nyqvist D, Cabrera O, Yu J, Molano RD, Pileggi A et al. Noninvasive in vivo imaging of pancreatic islet cell biology. Nat Med 2008;14(5):574-578.

415. Squifflet JP, Gruessner RW, Sutherland DE. The history of pancreas transplantation: past, present and future. Acta Chir Belg 2008;108(3):367-378.

416. Srinivasan PK, Yagi S, Doorschodt B, Nagai K, Afify M, Uemoto S et al. Impact of venous systemic oxygen persufflation supplemented with nitric oxide gas on cold-stored, warm ischemia-damaged experimental liver grafts. Liver Transpl 2012;18(2):219-225.

417. Stegall MD, Kim DY, Prieto M, Cohen AJ, Griffin MD, Schwab TR et al. Thymoglobulin induction decreases rejection in solitary pancreas transplantation. Transplantation 2001;72(10):1671-1675.

418. Stewart ZA, Cameron AM, Singer AL, Dagher NN, Montgomery RA, Segev DL. Histidine-tryptophan-ketoglutarate (HTK) is associated with reduced graft survival in pancreas transplantation. Am J Transplant 2009;9(1):217-221.

419. Streit F, Armstrong VW, Oellerich M. Rapid liquid chromatography-tandem mass spectrometry routine method for simultaneous determination of sirolimus, everolimus, tacrolimus, and cyclosporin A in whole blood. Clin Chem 2002;48(6 Pt 1):955-958.

420. Suarez-Pinzon W, Korbutt GS, Power R, Hooton J, Rajotte RV, Rabinovitch A. Testicular sertoli cells protect islet beta-cells from autoimmune destruction in NOD mice by a transforming growth factor-beta1-dependent mechanism. Diabetes 2000;49(11):1810-1818.

421. Sung RS, Christensen LL, Leichtman AB, Greenstein SM, Distant DA, Wynn JJ et al. Determinants of discard of expanded criteria donor kidneys: impact of biopsy and machine perfusion. Am J Transplant 2008;8(4):783-792.

422. Suszynski TM, Rizzari MD, Scott WE, Eckman PM, Fonger JD, John R et al. Persufflation (gaseous oxygen perfusion) as a method of heart preservation. J Cardiothorac Surg 2013;8:105.

423. Suszynski TM, Rizzari MD, Scott WE, 3rd, Tempelman LA, Taylor MJ, Papas KK. Persufflation (or gaseous oxygen perfusion) as a method of organ preservation. Cryobiology 2012;64(3):125-143.

424. Sutherland DE. Current status of beta-cell replacement therapy (pancreas and islet transplantation) for treatment of diabetes mellitus. Transplant Proc 2003;35(5):1625-1627.

425. Sutherland DE, Gruessner A, Hering BJ. Beta-cell replacement therapy (pancreas and islet transplantation) for treatment of diabetes mellitus: an integrated approach. Endocrinol Metab Clin North Am 2004;33(1):135-148, x.

426. Sutherland DE, Gruessner AC, Carlson AM, Blondet JJ, Balamurugan AN, Reigstad KF et al. Islet autotransplant outcomes after total pancreatectomy: a contrast to islet allograft outcomes. Transplantation 2008;86(12):1799-1802.

427. Sutherland DE, Gruessner RW, Gruessner AC. Pancreas transplantation for treatment of diabetes mellitus. World J Surg 2001;25(4):487-496.

428. Sutherland DE, Radosevich DM, Bellin MD, Hering BJ, Beilman GJ, Dunn TB et al. Total pancreatectomy and islet autotransplantation for chronic pancreatitis. J Am Coll Surg 2012;214(4):409-424; discussion 424-406.

429. Sweet IR, Gilbert M, Jensen R, Sabek O, Fraga DW, Gaber AO et al. Glucose stimulation of cytochrome C reduction and oxygen consumption as assessment of human islet quality. Transplantation 2005;80(8):1003-1011.

430. Sweet IR, Gilbert M, Scott S, Todorov I, Jensen R, Nair I et al. Glucose-stimulated increment in oxygen consumption rate as a standardized test of human islet quality. Am J Transplant 2008;8(1):183-192.

431. Sweet IR, Khalil G, Wallen AR, Steedman M, Schenkman KA, Reems JA et al. Continuous measurement of oxygen consumption by pancreatic islets. Diabetes Technol Ther 2002;4(5):661-672.

432. T. K. Islet isolation for clinical transplantation. In: Shahidul. M, (ed). The islets of Langerhans. New York: Springer, 2010.

433. Tabrizi MA, Roskos LK. Preclinical and clinical safety of monoclonal antibodies. Drug Discov Today 2007;12(13-14):540-547.

434. Takita M, Matsumoto S, Noguchi H, Shimoda M, Chujo D, Itoh T et al. Secretory unit of islet transplant objects (SUITO) index can predict outcome of intravenous glucose tolerance test. Transplant Proc 2010;42(6):2065-2067.

435. Takita M, Matsumoto S, Qin H, Noguchi H, Shimoda M, Chujo D et al. Secretory unit of islet transplant objects (SUITO) index can predict severity of hypoglycemic episodes in clinical islet Cell Transplant. Cell Transplant 2012;21(1):91-98.

436. Tamarat R, Silvestre JS, Le Ricousse-Roussanne S, Barateau V, Lecomte-Raclet L, Clergue M et al. Impairment in ischemia-induced neovascularization in diabetes: bone marrow mononuclear cell dysfunction and therapeutic potential of placenta growth factor treatment. The Am J Pathol 2004;164(2):457-466.

437. Tan J, Yang S, Cai J, Guo J, Huang L, Wu Z et al. Simultaneous islet and kidney transplantation in seven patients with type 1 diabetes and end-stage renal disease using a glucocorticoid-free immunosuppressive regimen with alemtuzumab induction. Diabetes 2008;57(10):2666-2671.

438. Taylor C, Craven RA, Harnden P, Selby PJ, Banks RE. Determination of the consequences of VHL mutations on VHL transcripts in renal cell carcinoma. Int J Oncol 2012.

439. Taylor G, Kirkland T, Lakey J, Rajotte R, Warnock G. Bacteremia due to transplantation of contaminated cryopreserved pancreatic islets. Cell Transplant 1994;3.

440. Terasaki PI. A personal perspective: 100-year history of the humoral theory of transplantation. Transplantation 2012;93(8):751-756.

441. Therasse A, Wallia A, Molitch ME. Management of post-transplant diabetes. Curr Diab Rep 2013;13(1):121-129.

442. 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.

443. Tolba RH, Schildberg FA, Schnurr C, Glatzel U, Decker D, Minor T. Reduced liver apoptosis after venous systemic oxygen persufflation in non-heart-beating donors. J Invest Surg 2006;19(4):219-227.

444. Toso C, Isse K, Demetris AJ, Dinyari P, Koh A, Imes S et al. Histologic graft assessment after clinical islet transplantation. Transplantation 2009;88(11):1286-1293.

445. 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.

446. Turgeon NA, Avila JG, Cano JA, Hutchinson JJ, Badell IR, Page AJ et al. Experience with a novel efalizumab-based immunosuppressive regimen to facilitate single donor islet Cell Transplant. Am J Transplant 2010;10(9):2082-2091.

447. Tzakis AG, Ricordi C, Alejandro R, Zeng Y, Fung JJ, Todo S et al. Pancreatic islet transplantation after upper abdominal exenteration and liver replacement. Lancet 1990;336(8712):402-405.

448. Uchizono Y, Iwase M, Nakamura U, Sasaki N, Goto D, Iida M. Tacrolimus impairment of insulin secretion in isolated rat islets occurs at multiple distal sites in stimulus-secretion coupling. Endocrinology 2004;145(5):2264-2272.

449. Uy B MS, 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.

450. van Amerongen MJ, Molema G, Plantinga J, Moorlag H, van Luyn MJ. Neovascularization and vascular markers in a foreign body reaction to subcutaneously implanted degradable biomaterial in mice. Angiogenesis 2002;5(3):173-180.

451. Van Belle T, von Herrath M. Immunosuppression in islet transplantation. J Clin Invest 2008;118(5):1625-1628.

452. Van Deijnen JH, Van Suylichem PT, Wolters GH, Van Schilfgaarde R. Distribution of collagens type I, type III and type V in the pancreas of rat, dog, pig and man. Cell Tissue Res 1994;277(1):115-121.

453. van der Windt DJ, Bottino R, Kumar G, Wijkstrom M, Hara H, Ezzelarab M et al. Clinical islet xenotransplantation: how close are we? Diabetes 2012;61(12):3046-3055.

454. 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.

455. Vardanyan M, Parkin E, Gruessner C, Rodriguez Rilo HL. Pancreas vs. islet transplantation: a call on the future. Curr Opin Organ Transplant 2010;15(1):124-130.

456. Vazeou A. Continuous blood glucose monitoring in diabetes treatment. Diabetes Res Clin Pract 2011;93 Suppl 1:S125-130.

457. Veriter S, Gianello P, Dufrane D. Bioengineered sites for islet Cell Transplant. Curr Diab Rep 2013;13(5):745-755.

458. Villiger P, Ryan EA, Owen R, O'Kelly K, Oberholzer J, Al Saif F et al. Prevention of bleeding after islet transplantation: lessons learned from a multivariate analysis of 132 cases at a single institution. Am J Transplant 2005;5(12):2992-2998.

459. Vincenti F. What's in the pipeline? New immunosuppressive drugs in transplantation. Am J Transplant 2002;2(10):898-903.

460. Vivek K, Mustafa MM, Rodriguez E, Redfield RR, 3rd, Parsons RF, Rostami S et al. Strategies for B-lymphocyte repertoire remodeling in transplantation tolerance. Immunol Res 2011;51(1):1-4.

461. Vrochides D, Paraskevas S, Papanikolaou V. Transplantation for type 1 diabetes mellitus. Whole organ or islets? Hippokratia 2009;13(1):6-8.

462. Wadhwa M, Bird C, Dilger P, Gaines-Das R, Thorpe R. Strategies for detection, measurement and characterization of unwanted antibodies induced by therapeutic biologicals. J Immunol Methods 2003;278(1-2):1-17.

463. Waki K, Terasaki PI. Kidney graft and patient survival with and without a simultaneous pancreas utilizing contralateral kidneys from the same donor. Diabetes care 2006;29(7):1670-1672.

464. Walsh RM, Saavedra JR, Lentz G, Guerron AD, Scheman J, Stevens T et al. Improved quality of life following total pancreatectomy and auto-islet transplantation for chronic pancreatitis. J Gastrointest Surg 2012;16(8):1469-1477.

465. Warnock GL, Kneteman NM, Ryan EA, Evans MG, Seelis RE, Halloran PF et al. Continued function of pancreatic islets after transplantation in type I diabetes. Lancet 1989;2(8662):570-572.

466. Watson-Williams P. Notes on diabetes treated with extract and by grafts of sheep's pancreas. Br Med J 1894(2):1303.

467. Webb MA, Illouz SC, Pollard CA, Gregory R, Mayberry JF, Tordoff SG et al. Islet auto transplantation following total pancreatectomy: a long-term assessment of graft function. Pancreas 2008;37(3):282-287.

468. Webster AC, Pankhurst T, Rinaldi F, Chapman JR, Craig JC. Monoclonal and polyclonal antibody therapy for treating acute rejection in kidney transplant recipients: a systematic review of randomized trial data. Transplantation 2006;81(7):953-965.

469. Weclawiak H, Kamar N, Ould-Mohamed A, Cardeau-Desangles I, Rostaing L. Rationale for using belatacept in combination with sirolimus. Transplant Proc 2010;42(9 Suppl):S29-31.

470. Weclawiak H, Kamar N, Ould-Mohamed A, Cardeau-Desangles I, Rostaing L. Biological agents in kidney transplantation: belatacept is entering the field. Expert Opin Biol Ther 2010;10(10):1501-1508.

471. Weir AB. Hazard identification and risk assessment for biologics targeting the immune system. J Immunotoxicol 2008;5(1):3-10.

472. Weir GC, Bonner-Weir S. Islets of Langerhans: the puzzle of intraislet interactions and their relevance to diabetes. J Clin Invest 1990;85(4):983-987.

473. Weisman MH, Durez P, Hallegua D, Aranda R, Becker JC, Nuamah I et al. Reduction of inflammatory biomarker response by abatacept in treatment of rheumatoid arthritis. J Rheumatol 2006;33(11):2162-2166.

474. Weng LC, Chiang YJ, Lin MH, Hsieh CY, Lin SC, Wei TY et al. Association between use of FK506 and prevalence of post-transplantation diabetes mellitus in kidney transplant patients. Transplant Proc 2014;46(2):529-531.

475. Wenzlau JM, Juhl K, Yu L, Moua O, Sarkar SA, Gottlieb P et al. The cation efflux transporter ZnT8 (Slc30A8) is a major autoantigen in human type 1 diabetes. Proc Natl Acad Sci U S A 2007;104(43):17040-17045.

476. Wertheim JA, Leventhal JR. Clinical implications of basic science discoveries: induced pluripotent stem cell therapy in transplantation--a potential role for immunologic tolerance. Am J Transplant 2015;15(4):887-890.

477. Will RG, Ironside JW, Zeidler M, Cousens SN, Estibeiro K, Alperovitch A et al. A new variant of Creutzfeldt-Jakob disease in the UK. Lancet 1996;347(9006):921-925.

478. Witkowski P, Liu Z, Cernea S, Guo Q, Poumian-Ruiz E, Herold K 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.

479. Wolters GH, Vos-Scheperkeuter GH, Lin HC, van Schilfgaarde R. Different roles of class I and class II Clostridium histolyticum collagenase in rat pancreatic islet isolation. Diabetes 1995;44(2):227-233.

480. 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.

481. Wu H, Wen D, Mahato RI. Third-party mesenchymal stem cells improved human islet transplantation in a humanized diabetic mouse model. Mol Ther 2013;21(9):1778-1786.

482. Xenos ES, Farney AC, Widmer MB, Casanova D, Stevens RB, Blazar BR et al. Effect of tumor necrosis factor alpha and of the soluble tumor necrosis factor receptor on insulin secretion of isolated islets of Langerhans. Transplant Proc 1992;24(6):2863-2864.

483. Xu H, He X, Liu Q, Shi D, Chen Y, Zhu Y et al. Abnormal high expression of B-cell activating factor belonging to the TNF superfamily (BAFF) associated with long-term outcome in kidney transplant recipients. Transplant Proc 2009;41(5):1552-1556.

484. Xu H, He X, Sun J, Shi D, Zhu Y, Zhang X. The expression of B-cell activating factor belonging to tumor necrosis factor superfamily (BAFF) significantly correlated with C4D in kidney allograft rejection. Transplant Proc 2009;41(1):112-116.

485. Xu X, Hou Y, Yin X, Bao L, Tang A, Song L et al. Single-cell exome sequencing reveals single-nucleotide mutation characteristics of a kidney tumor. Cell 2012;148(5):886-895.

486. Yakubovich N, Fung MA, Thompson DM. Three cases of cytomegalovirus infection following pancreatic islet transplantation. Transplant Proc 2007;39(5):1599-1603.

487. Yang YH, Manning Fox JE, Zhang KL, MacDonald PE, Johnson JD. Intraislet SLIT-ROBO signaling is required for beta-cell survival and potentiates insulin secretion. Proc Natl Acad Sci U S A 2013;110(41):16480-16485.

488. Yates CJ, Cohney SJ. Prediction and Diagnosis of Post Transplant Diabetes. Curr Diabetes Rev 2015.

489. Young BY, Gill J, Huang E, Takemoto SK, Anastasi B, Shah T et al. Living donor kidney versus simultaneous pancreas-kidney transplant in type I diabetics: an analysis of the OPTN/UNOS database. Clin J Am Soc Nephrol 2009;4(4):845-852.

490. Yuan X, Theruvath AJ, Ge X, Floerchinger B, Jurisch A, Garcia-Cardena G et al. Machine perfusion or cold storage in organ transplantation: indication, mechanisms, and future perspectives. Transpl Int 2010;23(6):561-570.

491. Zagozda M, Sarnecka A, Durlik M. Microchimerism after pancreas and kidney transplantation--a review. Ann Transplant 2011;16(4):134-137.

492. Zanone MM, Favaro E, Quadri R, Miceli I, Giaretta F, Romagnoli R et al. Association of cytomegalovirus infections with recurrence of humoral and cellular autoimmunity to islet autoantigens and of type 1 diabetes in a pancreas transplanted patient. Transpl Int 2010;23(3):333-337.

493. Zarkhin V, Li L, Sarwal MM. BAFF may modulate the rate of B-cell repopulation after rituximab therapy for acute renal transplant rejection. Transplantation 2009;88(10):1229-1230.

494. 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.

495. Zhang HF, Maslov K, Stoica G, Wang LV. Functional photoacoustic microscopy for high-resolution and noninvasive in vivo imaging. Nat Biotechnol 2006;24(7):848-851.

496. Zhang L, Cao Z, Bai T, Carr L, Ella-Menye JR, Irvin C et al. Zwitterionic hydrogels implanted in mice resist the foreign-body reaction. Nat Biotechnol 2013;31(6):553-556.

497. Zhang ZY, Zhou ZQ, Song KB, Kim SC, Zhou GW. Hepatic stellate cells induce immunotolerance of islet allografts. Transplant Proc 2014;46(5):1594-1600.

APPENDIX A.

NEW IMMUNOSUPPRESSION THERAPIES

A. - New immunosuppression therapies

BOOK CHAPTER

Book title: Islets: Biology, Immunology, and Clinical Transplantation**.**

Chapter title: New immunosuppression therapies

Authors: Boris L. Gala-Lopez. MD, MSc.
Immunobiology & Islet Transplantation Research
Clinical Islet Transplant Program
Alberta Diabetes Institute, University of Alberta

Andrew R. Pepper. PhD
Immunobiology & Islet Transplantation Research
Clinical Islet Transplant Program
Alberta Diabetes Institute, University of Alberta

A.M. James Shapiro, MD, PhD, FRCS(Eng), FRCSC, MSM, FRSC
Professor of Surgery, Medicine and Surgical Oncology
Director Clinical Islet and Living Donor Liver Transplant Programs
Clinical Senior Scholar Alberta Innovates - Healthcare Solutions (AIHS)
University of Alberta.

Note: A version of this section will be published as book chapter in *Islets: Biology, Immunology, and Clinical Transplantation***.** Kandeel FR. (ed). April 2016.

A.1. – INTRODUCTION

Type 1 diabetes is a chronic, progressive autoimmune disease that results from the immune-mediated destruction of the insulin-producing β-cells within the pancreatic islets. Vascular complications remain a major source of morbidity and mortality in patients with diabetes, with data suggesting that major decline in total mortality and renal failure rates reflect better management, and that complication are delayed rather than prevented (1, 2). Despite advances in monitoring and therapeutics, morbidity and mortality remain increased in patients with type 1 diabetes (T1DM) compared with non-diabetic populations (3).

Significant progress has occurred in the outcomes of clinical islet transplantation, reflecting improvements in immunosuppression (IS) and preparation of sufficient quantities of highly viable islets for transplantation (4). Solitary islet transplantation (IT) has become an accepted modality to stabilize frequent hypoglycemia or severe glycemic lability in selected subjects with poor diabetic control, resistant to standard, intensive or insulin-pump based therapies (5, 6). Sustained C-peptide production and high rates of insulin independence after pancreatic islet transplant in T1DM was reported 13 years ago by the Edmonton group (4). This reality became possible with the use of newer, more potent IS agents, the avoidance of corticosteroids, and high-quality islet preparations, although typically two islet infusions were required to attain insulin independence.

Follow-up observations in these initial Edmonton islet transplant recipients and elsewhere indicated that insulin independence was not durable long-term, and most returning to modest amounts of insulin without risk of recurrent hypoglycemia by the third to fifth year. Additionally, approximately 25% required additional late islet infusions during the second or third year post-

transplant (7, 8). The reasons for the chronic failure of a portion of the islet transplants are currently under investigation, but are likely associated with immune rejection, recurrence of autoimmunity or chronic exposure to diabetogenic IS agents (8).

This chapter reviews the most important events occurring around the immune response and IS therapies for pancreatic islets and potential mechanisms interfering with successful survival. Historical vignettes are provided to demonstrate the remarkable international team effort that has led to the process in use today, as well as the principles of current treatments. Details of the most important aspects of islet immunology, including different phases of engraftment, emphasizing the hurdles that limit long-term islet survival. Finally, current clinical protocols are discussed together with different ongoing research projects, which could potentially improve engraftment efficiency, long-term survival and the final results of clinical islet transplant.

A.2. - HISTORICAL PERSPECTIVE

Diabetes affects millions of people all over the world and is responsible for multiple complications including heart diseases and strokes, high blood pressure, renal failure and ketoacidosis, which make diabetes rank as a major leading cause of death in northern America and Europe. Type II diabetes accounts for approximately 90% of adult cases and is generally associated with hyperglycemia and insulin resistance, eventually leading to defects in insulin secretion (9). T1DM, in contrast, results from autoimmune destruction of the insulin-secreting cells (β-cells) contained in the pancreatic islets. It has received extensive clinical attention and experimental work given the inexorable chronic and degenerative complications suffered by the majority of sufferers over time.

Enticing approaches have been developed in an attempt to revert or delay the autoimmune process leading to T1DM and restore β-cell mass. Multiple agents, including IS, vaccines and anti-inflammatory agents, have been used in autoimmune NOD mice and in clinical trials to slow down disease progression, but only a few had a measurable impact on the course of T1DM in patients. Most studies require the use of cocktail therapies of two or more different drugs, to act synergistically to intervene in this devastating disease (10, 11).

The alternative approach has been transplanting new β-cells from a donor in form of whole pancreas or isolated islets to restore euglycemia. The first attempt to cure T1DM by pancreas transplantation was carried out at the University of Minnesota, in Minneapolis, on December 17, 1966, followed by a series of whole pancreas transplants (12). Numerous technical modifications improved the overall results of pancreas transplant but certainly, refinements in IS (beginning with the introduction of cyclosporine and continuing with use of multiple more potent drug schemes) was one of the most important elements to make possible the current long-term result. The history of islet transplant is also filled with hurdles, sacrifices and battled milestones beginning with early efforts in the 19th Century with rudimentary experimental treatments, followed by the implementation of the concept of islet isolation and purification. They have allowed a major advance in the field allowing the clinical islet transplant to become a reality (8). Using these methodologies, in 1989, Lacy and collaborators at Washington University in St. Louis demonstrated the possibility of reversing diabetes with temporary insulin independence after transplantation of human islets. However, the islet-cell transplant failed at one month after insulin independence was achieved, most likely due to inadequate recipient immunosuppression (9). In 1990, the first successful series of human islet allografts was reported by the Pittsburgh group. Prolonged insulin independence was achieved with a steroid-free IS regimen based on the

then recently introduced agent FK506. This was the first unequivocal evidence of long-term insulin independence after human islet allotransplantation, with insulin independence lasting up to five years (9). These results were in the unique setting of total pancreatectomy and cluster abdominal transplantation, and importantly in the absence of background autoimmunity. These unprecedented results led to great enthusiasm in the field, and several centers, including those in Milan, Miami, Edmonton, St. Louis and Minneapolis, began or resumed testing of clinical islet allotransplantation protocols (9).

A definitive milestone in clinical islet transplantation was the experience published by the Clinical Islet Program at University of Alberta. They considered patients for islet-alone transplantation based on the presence of T1DM for more than 5 years, complicated by either reduced awareness of hypoglycemia, metabolic lability or instability, or evidence of progressive but early secondary complications that persist despite best efforts of optimal glycemic control. A protocol was developed in Edmonton to treat such patients with islet-alone grafts, now known as the "Edmonton Protocol." Grafts were non-Human Leukocyte Antigen (HLA) matched, patients were not sensitized (negative panel reactive antibody [PRA] pre-transplant), islets were ABO compatible, and sequential transplants were used to deliver an adequate islet infusion mass by a percutaneous portal venous access route. Immunosuppression was tailored to avoid steroids and minimize calcineurin inhibitors to prevent diabetogenicity, with the combination of sirolimus, low-dose tacrolimus, and the daclizumab induction (13).

The remarkable long-term results (100% insulin-independence at 1 year post-transplant) obtained in this cohort of patients became a beacon for the other programs and a starting point for new strategies to improve the success of this treatment.

A.3. - ISLET IMMUNOBIOLOGY AND ENGRAFTMENT

A.3.1. - Immunobiology of the Islets of Langerhans

The islets of Langerhans within the pancreas can be thought as 'micro-organs' occupying approximately 1-2% of the pancreas; however it is estimated suggested they receive between 5-15% of the pancreatic blood supply and are responsible for the gland's endocrine function (14-16). It is has been suggested that the human pancreas contains over 1 million islets of approximately 2,500 cells each, although the individual size varies substantially (14). The anatomy of the different cells within the islet has clear homeostatic benefit. Each islet contains alpha (α), beta (β), delta (δ), PP and epsilon cells that synthesize and release glucagon, insulin, somatostatin, pancreatic polypeptide and ghrelin, respectively, typically in a nutrient-dependent fashion (17).

Pancreatic β-cells form the bulk of the endocrine cellular content, (approximately 60%) within the pancreas and secrete insulin, a 51-aminoacid peptide with strong hypoglycemic action. The β-cell also co-secretes Islet Associated Polypeptide (IAPP), also called amylin, a 37-aminoacid peptide related to 'Calcitonin Gene Related Peptide' (18). Under pathological conditions, IAPP molecules may polymerize and form large intra-islet amyloid deposits that are often characteristic for type II diabetes and insulinoma. Furthermore, in the field of islet transplantation it has recently been described that islet amyloid-induced inflammation may contribute to β-cell dysfunction in islet transplant recipients (19). It has also been demonstrated that several membrane proteins of the insulin granule may induce the humoral autoimmune response associated with T1DM, such as the zinc transporter (20), insulinoma-associated protein

2 (IA-2; ICA-512) (21), and glutamic acid decarboxylase (22). Interestingly, the β-cells in the human pancreas show marked variation in granulation, cell, and nuclear size, which may reflect heterogeneity in glucose responsiveness and biosynthetic activity (23). It is plausible that due to this heterogeneity, certain β-cells might be more susceptible to the autoimmune destruction (14). The anatomy of the islet may well have important glucose homeostatic benefits; for instances having insulin producing β-cells and glucagon producing α-cells in close proximity allow their hormones to be secreted directly into the portal system optimizing their effects on glucose control. Although predominately comprised of endocrine cells, non-endocrine cells make up a portion of the islet structure (15). Vascular endothelial cells, located between the islet cell trabeculae, account for the majority of non-endocrine cells within the islet (15, 24). Other cells such as nerve fibers, pericytes, macrophages, and dendritic cells are also islet residents (25). It has been proposed that the latter two cell types, which express major histocompatibility complex (MHC) class II molecules on their cell surfaces, may play an important role in islet allograft rejection and possibly the initiation of the autoimmune response in the type 1 diabetic (24). The current understanding of the etiology of the disease onset is due predominately to an autoimmune response consisting of CD-8 positive T-lymphocytes, against pancreatic β-cell autoantigens in genetically susceptible patients, which in the healthy population are normally kept under control through peripheral tolerance and regulatory mechanisms (26-29). It has been revealed that the presence of islet autoantibodies in conjunction with HLA-DQ genotype is predictive of T1DM risk (30, 31). The highest-risk genotypes, HLA-DR3 or HLA-DR4 class II alleles, can be responsible for the development of anti-islet autoimmunity via production of autoantibodies to insulin; to glutamic acid decarboxylase, an enzyme produced primarily by pancreatic islet cells; and to the transmembrane protein tyrosine kinase IA-2, which regulates

vesicle number and insulin secretion (29, 32). However, science has yet to yield the exact mechanism leading to either this inflammatory response or the specific auto-antigens responsible. Furthermore, the balance between β-cell replications and their autoimmune attack can be considered a factor in determining the clinical onset and outcome of the disease (31). The progression of the disease may be influenced by β-cell regeneration as evident in studies where β-cell apoptosis was observed in chronic diabetic patients, suggesting that β-cells replications continues long after disease onset; however the exact mechanism and significance is still unknown (33-35).

Despite strong evidence illustrating that diabetes has a prominent autoimmune etiology, evidence of a partial environmental influence cannot be ignored. It is likely that an environmental event like a viral infection such as in the case when Coxsackie B4 enterovirus was isolated from early onset type 1 diabetic patients characterized by a non-destructive islet inflammation consisting of natural killer cells (27, 36); or lymphopenia needs to occur to precipitate disease (37, 38). Unraveling the complete etiology of T1DM will not only prove essential for the prevention and management of the disease but also may be critical in achieving long-term islet graft survival.

A.3.2. - Islet Engraftment

Following the isolation of pancreatic islets from cadaveric donors, the islets preparation in suspension is loaded into a gas-permeable infusion bag to facilitate the gravity infusion into the patient's liver. The transplantation procedure generally begins with the minimally invasive percutaneous transhepatic puncture and catheterization of the portal vein or less routinely via laparotomy. The radiologist then places a catheter into the portal vein using a fluoroscopy and

contrast dye. Once the catheter is in place, the islet suspension is infused, concluding with the tract being sealed with a fibrin-based sealant. Upon infusion, the islets travel through the portal vasculature until they are finally become trapped and embolize within the smaller portal capillary networks (39-41). During this process, islets are exposed to platelets, initiating the coagulation cascade and activating complement proteins in the "instant blood mediated inflammatory reaction" (IBMIR), and ultimately a loss of beta cell mass (42). Unfortunately, the islets that do become engrafted are initially avascular, stressed and vulnerable to apoptosis and necrosis elicited by multiple mechanisms (**Figure A.1**).

Once the islet graft becomes neovascularized (approximately 2-4 weeks post-transplant) it must contend with both underling autoimmune and alloimmune attack. The alloresponse is largely a T-cell-mediated response to the MHCs on the surface of donor tissues. Antigen presenting cells (APCs), such as dendritic cells (DCs) derived from the donor or recipient, process and present donor peptides and molecules through MHCs to recipient T-cells, transduced through the CD3 complex. When in the context of both recipient APCs and T-cells this is referred to as the "indirect" pathway. Additionally, recipient T-cells can recognize donor antigen directly on the surface of infiltrating donor-derived APCs through what has been described as the "direct" pathway (26, 28, 32, 43). Both the direct and indirect pathways collimate in the interaction of the T-cell receptors (TCR/CD3) on the surface of the recipient T-cells recognizing the peptide-MHC complex, initiating the first signaling cascades of lymphocyte proliferation and activation. In addition to this primary signal, the APCs provide additional interactions through co-stimulatory molecules, providing a second signal, through the communication of CD80 and CD86 on the APC with CD28 on the T-cells. Combined, these signals active three primary signal transduction

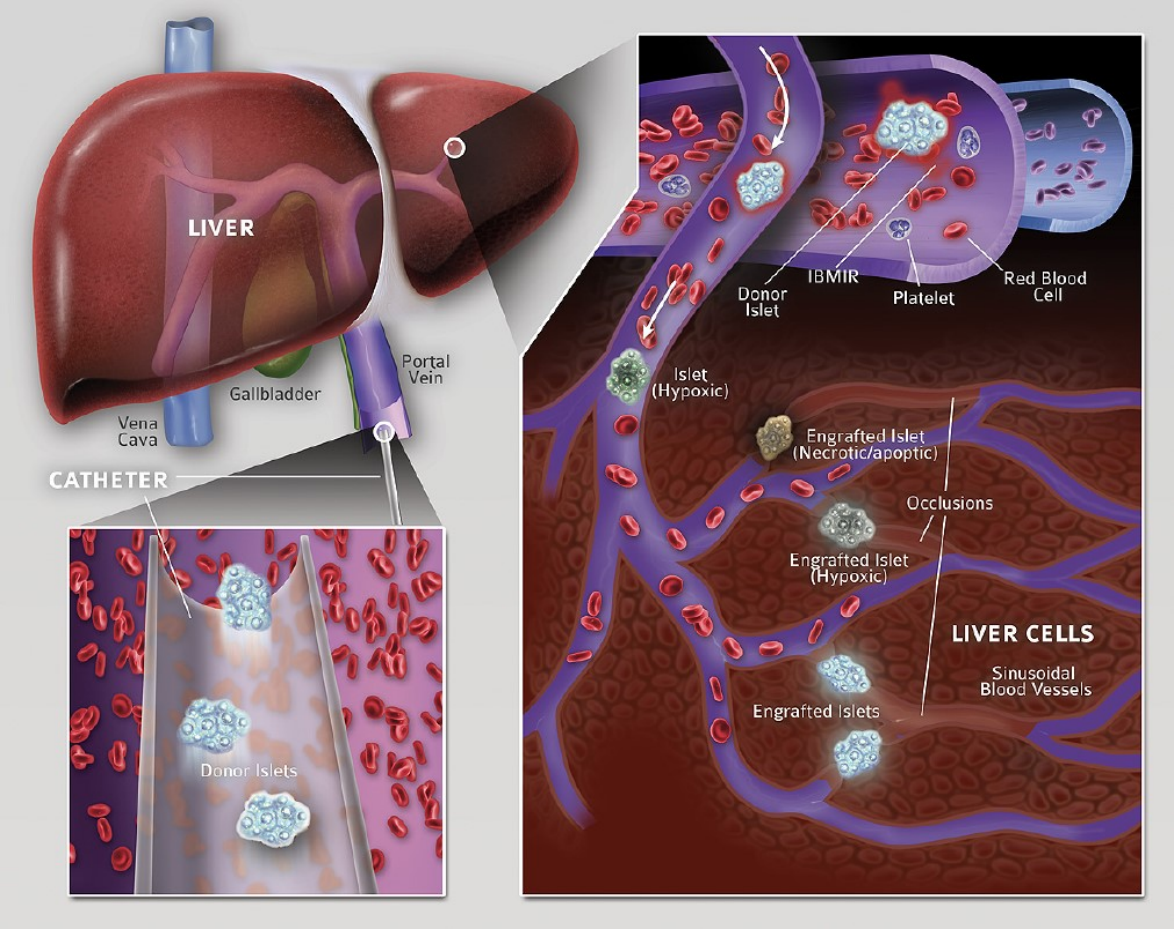


Figure A.1 Islet transplantation: Infusion to engraftment. Freshly, isolated pancreatic islets are infused into the diabetic recipient's liver via a minimally-invasive percutaneous transhepatic puncture and catheterization of the portal vein. The islets migrate through the portal vasculature and eventually embolized into the hepatic sinusoids. Since the islets are in direct contact with blood, they are immediately exposed to innate inflammatory process called "instant blood mediated inflammatory reaction" (IBMIR). The avascular islets are initially hypoxic and devoid of appropriate nutrient/waste exchange until re-vascularized (weeks post-transplant), making them vulnerable to apoptosis and necrosis elicited by multiple mechanisms. Artwork by Hayato Tanaka.

pathways: the calcium-calcineurin pathway, the RAS-mitogen activation protein kinase pathway, and the nuclear factor-κB pathway (26, 43, 44). The resulting activated transcription factors trigger the expression of multiple molecules including CD25, CD154 and interleukin-2 (IL-2). An additional signal required for activation involves the "target of rapamycin" pathway which is triggered by cytokines such as IL-2, ultimately eliciting cell proliferation, which in turn requires nucleotide synthesis. Upon activation, proliferation and differentiation, the T-cells promote a series of proinflammatory events and initiate the activation of other cell types resulting in recruitment of leukocytes and humoral factors to the islet graft. The effector response includes the production of the cytokines IFN-γ and IL-2 by type 1 helper (Th1) CD4+ T cells, the cytotoxic factors granzyme and perforin by CD8+ T cells (26, 27, 32, 43). In parallel, B cells are activated through their antigen-receptor complexes in the lymphoid follicles or spleen result in the production of alloantibodies (Abs) to donor HLA antigens (45). Thus, within days of the islets embolizing into the liver, in addition to the islet autoimmune response, the alloresponse triggers priming of allograft rejection including effector T-cells and alloantibodies (43) (**Figure A.2**).

To establish long-term islet graft function and potential for induced tolerance, a fragile balance between immunity and rejection must be achieved. The process of immune recognition and immune destruction of transplanted islet cells (or any transplanted organ) has been well described thus providing the opportunity for therapeutic immune intervention, targeting the pathways known to be involved, including: (1) inflammation; (2) maturation of dendritic cells (DCs) and migration to draining lymph nodes; (3) T cell activation by DCs, resulting in expansion of anti-donor T cells; and (4) migration of T cells to the graft where they mediate

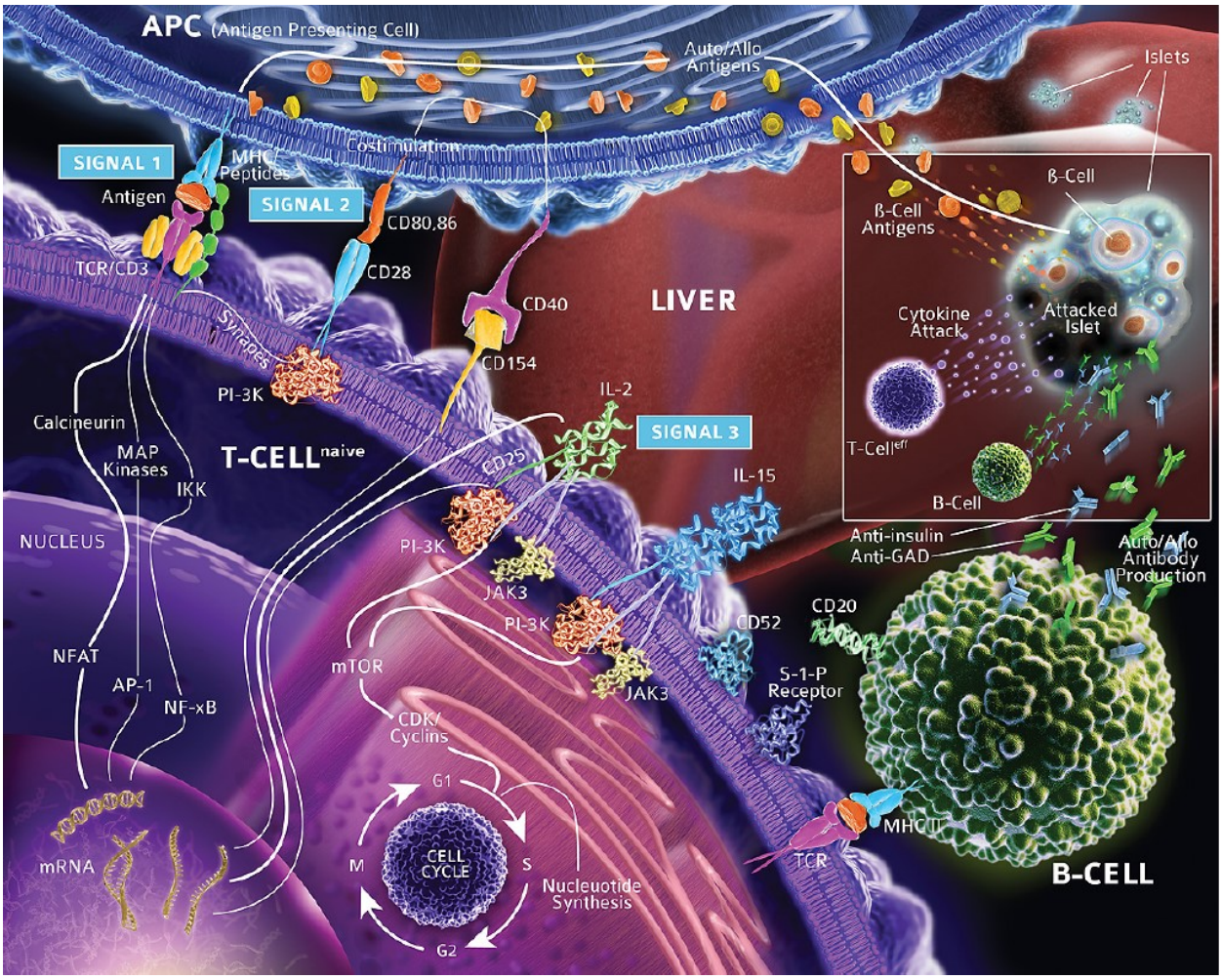


Figure A.2 Cellular pathways mediating the T-Cell induced auto- and alloimmune response. Antigen-presenting cells (APC) of host or donor origin migrate to T-cell areas of secondary lymphoid organs. Here, APCs present donor and auto-antigens to naïve and central memory T cells. As a result T cells are activated and undergo clonal expansion and differentiation to express effector functions (T-cell $_{\text{Effector}}$). These T-cells become activated as a result of three signaling pathways: signal-transduction pathways — the calcium–calcineurin pathway, the mitogen-activated protein (MAP) kinase pathway, and the protein kinase C–nuclear factor*k*B (NF*k*B) pathway. As a result, proliferation cytokines are expressed and the cell cycle pathways are initiated. Antigen stimulated T- cells and B-cell home to and infiltrate the islet graft and engage through the release of perforin, granzymes and auto- and alloreactive antibodies, resulting in islet graft rejection. Artwork by Hayato Tanaka.

cytotoxicity (28). The following sections describe therapeutic targets and emerging strategies to abrogation one or multiple steps in immune recognition and immune destruction of the transplanted islets. If proven effective, these approaches have the potential increase the long-term efficacy of IT, increase the prevalence of single-donor insulin independence and eliminate the use of non-specific immunosuppression.

A.4. - CURRENT IMMUNOSUPPRESSION PROTOCOLS

The two major impediments to the clinical success of IT are the immune destruction of transplanted islets and the limited supply of islet tissue. Immunological challenges to islet survival, engraftment, and function post-transplantation are 2-fold: alloimmune destruction and autoimmune rejection. Although the former is common to all organ and tissue transplantation situations, T1DM offers additional challenges due to the autoimmunity, a primed immune response which is harder to overcome (46).

Among the biological strategies used to overcome immune rejection are the use of novel IS agents and regimens, and donor-specific induction of immune tolerance in the host. Immunosuppressive agents are typically delivered systemically to address the underlying autoimmunity as well as the allo-immune responses to transplanted islets. These IS are permanently needed following transplantation and have multiple unavoidable side effects, such as an increased vulnerability to infection for the patient and cytotoxicity to the transplanted islets (many are diabetogenic) (4, 47).

Generalized IS of the transplant recipient is the standard protocol today to prevent graft rejection by the host immune system. The first generation drugs that were applied to this end include azathioprine, glucocorticoids, and antilymphocyte serum. Although highly effective, these drugs have significant toxicity, especially nephrotoxicity, which is prevalent in up to 75% of patients over time (28). Additional side effects include hypertension, hepatotoxicity, neurotoxicity, hirsutism, gingival hyperplasia, gastrointestinal toxicity, ulcers, hyperglycemia, osteoporosis, and increased risk of infection and neoplasms (48).

Second-generation drugs had higher potency and larger therapeutic window and were rapidly added to the drug cocktail in IT. These include cyclosporine and tacrolimus. However, many of these agents are diabetogenic and toxic to the islets (47). The use of IS agents that do not challenge the islet graft is thus warranted. Although newer agents are constantly being developed, e.g., FTY720 and lisofylline, improvements have also been reported with novel combinations of existing agents (28).

One of the requirements of IS protocols in IT is the effectiveness in preventing any cytodestructive host immune response. This is critical taking into account that the marginal mass of engrafted islets has virtually no excess capacity to tolerate β-cell injury without reaching the tipping point causing metabolic dysfunction (47).

The Edmonton approach reported a high success rate of islet allotransplantation by sequential IT, 2 to 10 weeks apart using two or more pancreases to achieve adequate mass of engrafted islets and by using a glucocorticoid-free immunosuppressive regimen that includes IL-2 receptor antibody (daclizumab), sirolimus, and low-dose tacrolimus (4). Such an approach resulted in 100% insulin independence with a mean follow-up of 1 year and represented a major

improvement compared to previous reports limited to only 11% of insulin independence at 1 year (8).

Although IS therapy has improved over time, with the identification of new drugs and combinations, targeted modulation of the innate and adaptive immune response to transplanted islets may provide a pathway to reduce or eliminate systemic IS. Immune tolerance to an allogeneic or xenogenic islet transplant can be achieved at various stages in immune system development. These approaches target the graft, the graft donor, or the host. A multicenter clinical trial to coordinate the implementation of the Edmonton protocol, called the Immune Tolerance Network, was initiated by the National Institutes of Health together with Juvenile Diabetes Foundation International with seven centers in the United States and Canada, and three in Europe(6). Results of this trial were mixed, with variable rates of insulin independence reflecting different site experience with both islet isolation and clinical dosing of sirolimus IS. The Immune Tolerance Network sponsors investigator-initiated research in targeted prevention of immune-mediated transplant rejection by blocking immune signals at three different levels: T-cell recognition of antigen/MHC complex on APCs, costimulation to augment T-cell proliferative response to antigenic stimuli, and targeting clonal activation/deletion.

Despite these encouraging results, the long-term graft function was challenging to sustain in many cases. Only 40 – 50% remains insulin-free after 3 years of transplant and only 10% at 5 years (49). However, being insulin-dependent again does not necessarily equate with complete loss of graft function and/or poor glycemic control, as 80% of these patients were C-peptide positive after 5 years (**Figure A.3**). Potential reasons for graft loss remain unknown but alloimmunity could be a major factor. A significant number of patients will become panel reactive antibody (PRA) positive following transplantation, and around half of those with high

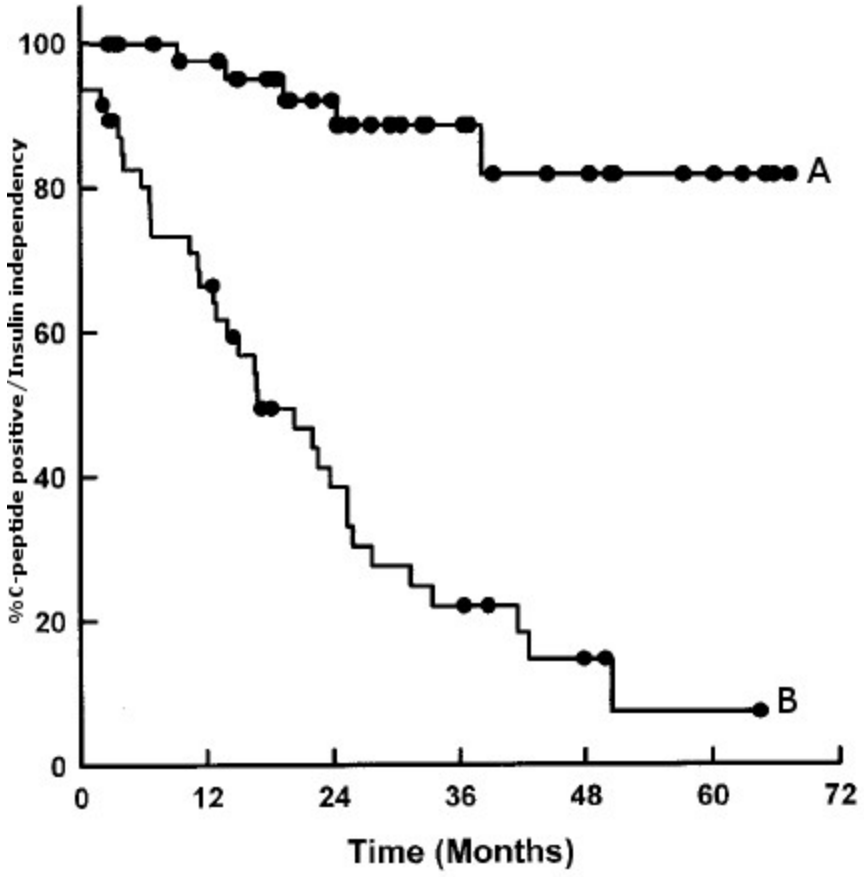


Figure A.3 Survival analysis for C-peptide secretion (**A**) and insulin dependence (**B**) over time post-transplantation at University of Alberta. (Adapted with permission from Shapiro A.M.J *et al*.(4))

PRA may lose graft function (50). Autoimmunity is another potential factor where patients who develop autoantibodies may exhibit a decrease in graft function (49).

A variety of experimental studies is now focused to further improve results in islet transplant by introducing tolerance-inducing medication. Some new promising agents include humanized anti-CD154, anti CD28 LEA29Y (belatacept) and anti CD52 (alemtuzumab) (51-53).

A.5. - PATIENT CONSIDERATIONS

A.5.1. - Combined Islet-Kidney Transplant

After the establishment of transplantation of isolated islets of Langerhans as an accepted treatment option for patients with T1DM, the procedure also emerged as an alternative to whole-organ pancreas transplantation. Considering the much lower incidence of complication after islets transplantation, several kidney-pancreas centers initiated programs of simultaneous islet-kidney transplantation (SIK) as an alternative for these patients.

The goal of islet in combination with kidney transplantation is not necessarily to arrive at the insulin-independence but the achievement of a good glycemic control by a single IT (54). A single infusion of functioning islets can reduce long-term levels of HbA1c and consequently prevent the occurrence of severe asymptomatic episodes of hypoglycemia and delay diabetes-related complications (55).

Various centers have reported encouraging results that match those of islet alone transplantation. The group of Zurich reported their experience comparing long- term outcomes of T1DM subjects with end-stage renal failure to kidney-islets transplantation and treated with IT using the

Edmonton approach compared to kidney-pancreas transplant treated with conventional IS (56). The study demonstrated a similar kidney function and survival and an improvement of the blood glucose control in both groups (56). Today, different protocols of IS are used in combined kidney-islet transplantation. All of these protocols are steroid free and schedule different combinations using daclizumab or etanercept during the induction period plus mycophenolate mofetil or sirolimus and low- dose of tacrolimus or cyclosporine A. Since the number of the islets transplantation performed worldwide is low, a long period of time will be necessary in order to define which IS protocol is the most favorable. So far they all seem equally effective as long as they are steroids free (54).

Considerations in islet-after-kidney (IAK) transplantation are more straightforward, as patients are already subjected to the chronic risks of IS. In IAK transplantation, patients must have a well-functioning prior kidney transplant and be able to tolerate standard maintenance IS. Corticosteroids are used by some centers for maintenance therapy in kidney transplantation, and although there are concerns regarding both insulin resistance and islet toxicity from prednisone, any negative impact is perceived to be negligible if the dose is ≤ 5mg per day. Interestingly, islet transplant rejection has also been successfully reversed by pulsed steroid therapy (54).

A.5.2. - Risk of Sensitization

Despite the success of IT and increasing worldwide acceptance, more than one donor is often required to achieve insulin independence. The limited pool of donors prevents allocation according to HLA matching. Therefore, successful recipients of IT may have multiple HLA mismatches with their donor(s). Each HLA mismatch will likely expose the recipient to several

mismatched epitopes, which are also present on other HLA antigens. This may result in the development of antibodies to donor and non-donor HLA antigens (57).

Islet transplant patients normally require high levels of IS and hence, are exposed to the multiple side effects of these treatments leading to IS adjustments. Given the likelihood of multiple HLA mismatches and the possibility of insufficient IS, islet transplant recipients are at significant risk of developing *de novo* HLA antibodies, which are proven to precede graft failure in kidney heart and lung transplants (58). Previous reports suggest that this may also be true for islet transplantation (59).

A recent study from Edmonton reported a limited number of recipients that developed *de novo* HLA antibodies on IS (57). These individuals had a lower fasting C-peptide than recipients who did not develop *de novo* HLA antibodies. These findings emphasized the need high-resolution, standardized HLA antibody screening and prospective cross-matching where indicated, before proceeding with transplantation. As well as a tailored IS in these patients, to prevent sensitization in the case of failed IT. Most recent experiences dictate the need to maintain patients on some IS rather than gradual weaning until it is decided whether they are likely to need a future transplant. A number of alternative strategies are also being considered to reduce the risk of sensitization including belatacept, which has been effective in nonhuman primates in reducing allosensitization (57).

A.6. - THERAPEUTIC TARGETS & STRATEGIES

For decades groups have been strategizing methods in which to prevent or reverse autoreactivity to the β-cell in the context of the newly diagnosis type 1 diabetic (27, 29). These methodologies

are relevant to the recipient of an islet transplant in effort to abrogate the immune response to autoantigens. Multiple candidates have been tested in the human trials, generally as monotherapies, but unfortunately, very few had measurable impact on the course of diabetes (27, 29). In light of complex immune interactions associated with any autoimmune disease, compounded by the multitude of cell types and signaling pathways involved in the alloresponse, it is conceivably unlikely that a monotherapeutic approach would be efficacious in preventing long-term islet graft attrition. A major obstacle is to prioritize which combinations appear most promising based on the mode of action, pre-clinical and clinical observations, synergistic and systemic side effects (27). It is important to define the targets of monotherapy strategies to better formulate the appropriate combined intervention protocols (**Figure A.4**).

A.6.1. - Immunosuppressive Targets

A.6.1.1. - Depleting Antibodies

Anti-T-Lymphocyte Globulin (ATG) is a polyclonal antibody produced by immunized horses or rabbits with human lymphoid cells. Since ATG is effectively depleting nearly the entire T-cell population, it is primarily used as an induction agent 3-10 days prior to organ transplantation, producing profound lymphopenia. Interesting, short-term use of ATG has been demonstrated as a treatment option in T1DM, showing remarkable preservation of C-peptide and reduction of insulin requirements by presumably eliminating autoreactive T-cells (26, 27, 29). However, ATG is not without significant side effects including thrombocytopenia, cytokine-release syndrome,

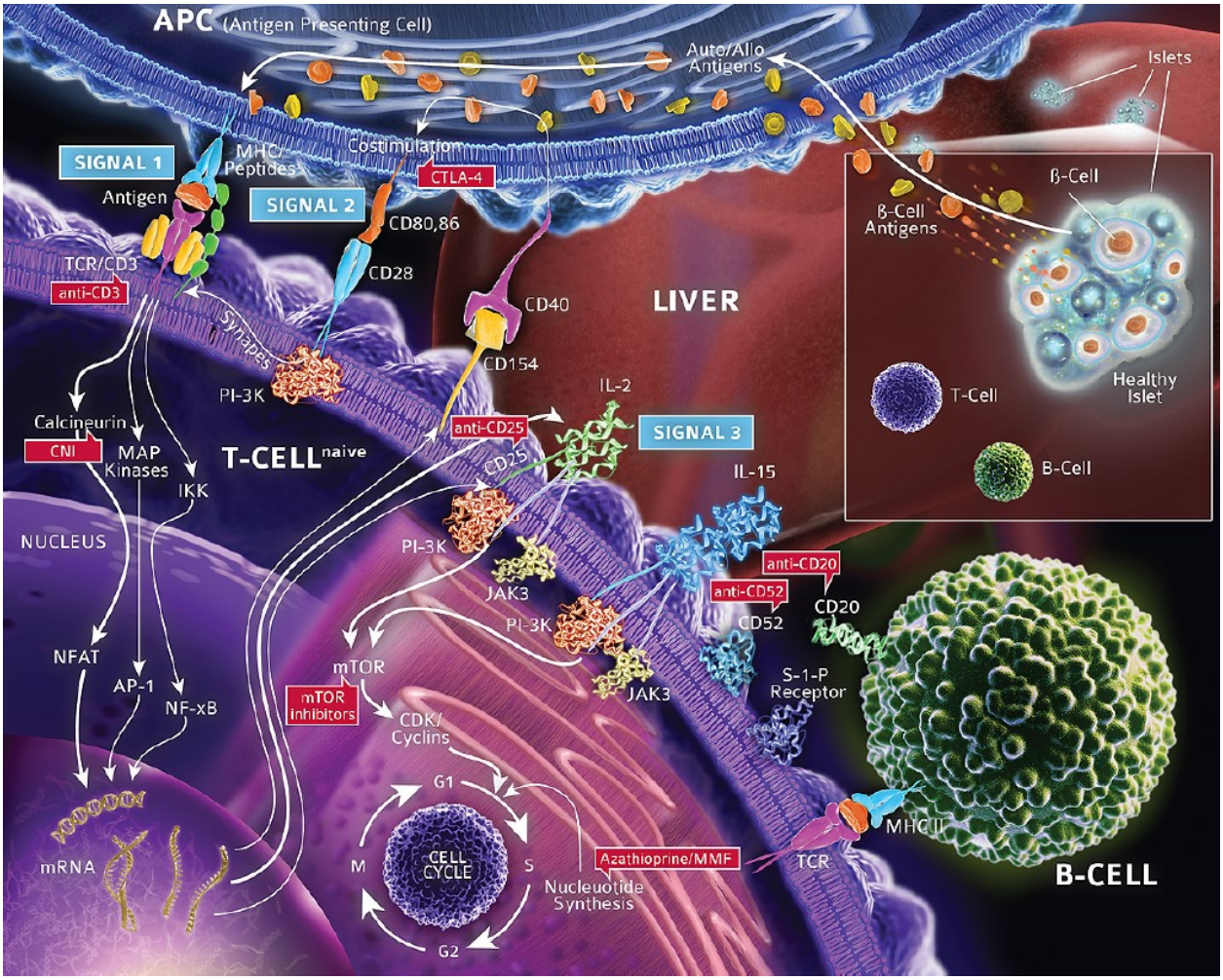


Figure A.4 Cellular targets for immunosuppressive drug intervention. Artwork by Hayato Tanaka.

fever, arthralgia and lymphadenopathy, and is associated with increased long-term risk of post-transplant lymphoproliferative disorder (43).

Similarly, Anti-CD3 antibody (teplizumab) has been demonstrated to preserve C-peptide preservation and reduce insulin requirements when administered to diabetic patients, up to five years post-administration (27). Monoclonal anti-CD3 transiently activates the CD3 receptor, simulates cytokine release and ultimately blocks T-cell proliferation and differentiation (29). Essentially, anti-CD3 disrupts T-cell ability to recognize auto- and alloantigens, blocks IL-2 signaling pathways and induces apoptosis. It has also been postulated that long-term benefits may be as a result of the induction of regulatory T-cells, and therefore the use of humanized CD3 monoclonals, which do not stimulate cytokine release, has been viewed as an attractive treatment modality to reduce the autoreactivity in T1D (26, 27, 29, 43). Side effects such as reactivations of Epstein-Barr virus and flu-like symptoms have been reported; however, taken together anti-CD3 antibody treatment appears to have great promise in potential combination therapies in IT.

The humanized monoclonal anti-body against CD52 (alemtuzumab) has been shown to dramatically deplete lymphocyte populations. Currently, its use has been limited to treating refractory B-cell lymphocytic leukemia however (43); it has not been fully approved for widespread use in transplantation or to treat the autoreactive antibodies of type 1 diabetics. Notwithstanding, some large off-label studies in renal transplantation have demonstrated near tolerance treatment with alemtuzumab indicating it may be a candidate as an effective induction agent and has the potential for eliciting ‘prope’ tolerance when combined with target-of-rapamycin inhibitors and/or calcineurin inhibitors (43). Side effects include first dose rash, nausea, neutropenia and anemia.

The role of B-cells in the pathogenesis of the autoimmune reaction in T1D as well as in islet transplant recipients may have been underestimated since it is widely accepted that both auto-and alloreactive T-cells are the main culprits. However, it has been demonstrate in a human phase II trial, that when B-cells are depleted using an anti-CD20 monoclonal antibody (rituximab) a tentative preservation of C-peptide, insulin requirements and improved HbA1c has been observed in newly diagnosed T1D patients (27, 29). CD20 is unique to B-cells and thus inhibiting B-cells via anti-CD20 monoclonal reduces the presentation of autoantigens to T-cells and possibly preventing B-cell expansion and anti-islet autoantibody production (29, 45). Anti-CD20, rituximab is currently approved for treatment of refractory non-Hodgkin's B-cell lymphomas however, it is off label use in combinations with maintenance immunosuppressive has been used in transplantation and thus a potential candidate for reducing the auto and alloreactive responses to an islet graft.

A.6.1.2. - Non-Depleting Antibodies or Fusion Proteins

The expression of the IL-2 receptor α-chain, CD25, is a consequence of T-cell activation. Therefore, blocking CD25 with monoclonal antibodies (daclizumab and basilixamb) does not reduce the steady state T-cell repertoire; however, it prevents the colony expansion of T-cell when stimulated by allo- and presumably autoantigens (27, 43, 44). In conjunction with other agents, anti-CD25 is widely utilized in organ transplantation, and was a critical component of the Edmonton Protocol (6).

Another attractive therapeutic strategy is to target cell-surface molecules which are important in the immune synapse, secondary to the TCR:MHC interaction. The co-stimulatory signaling

pathways of interest are the interactions between CD80 and CD86 on the APC with CD28 of the T-cell. Cytotoxic-T-lymphocyte-associated antigen 4 (CTLA-4) immunoglobulin (abatacept) has been utilized in blocking these interactions in various clinical trials involving newly diagnosed T1DM patients and transplantation recipients (29). A second generation, high affinity variant, LEA29Y (belatacept) has shown great potential in preventing graft rejection and conceivably will be tested in the near future as a therapeutic monotherapy for diabetic patients (26, 29, 43). The co-stimulatory signaling pathways between CD40:CD154 and ICAM-1:LFA-1 are also an attractive target to prevent autoimmune disease, lymphocyte trafficking and graft rejection. Humanized anti-CD154 (IDEC131) has been implemented in autoimmune clinical trials, and has the potential to be extended to islet transplantation (43). Furthermore, humanized monoclonal antibodies targeting the CD11a chain of LFA-1 (efalizumab) has been successful used in the treatment of psoriasis and in a phase I/II open label trial for renal transplantation (43). Recently, a study implementing efalizumab in the immunosuppression protocol, demonstrated insulin independence from a single donor (28, 60). Unfortunately, patients were withdrawn from efalizumab due to concerns about the development of progressive multifocal leukoencephalopathy (60).

The advantage of the non-depleting immunotherapy approach is that they reduce responsiveness without compromising lymphocyte populations. The low non-immune toxicity can be attributed to specifically targeting proteins only expression on immune cells. Therefore, non-depleting lymphocyte strategies appear attractive when combined with less intense maintenance IS cocktails.

A.6.1.3. - Anti-Inflammatory & Chemokines/Cytokines

Since both T1DM and islet graft rejection are potentiated by inflammatory process leading to insulitis, blocking pro-inflammatory events appears to be an appealing area of therapeutic intervention. A number of agents have proven effective in abrogating autoimmune disease including T1DM. The tumor necrosis factor-α inhibitor (etanercept), has increased C-peptide secretion, lowered HbA1c and exogenous insulin requirements when administered to early onset T1DM patients (29). Etanercept has also been demonstrated to increase the efficacy of IT, and reduce the number of autoreactive T cells (27, 28). Interleukin-1 (IL-1), is another potent pro-inflammatory molecule, and has been reported to cause beta cell dysfunction (26-29). Anakinra, which blocks both isoforms IL-1α and IL-1β through IL1 receptor blockade is currently being explore in clinical trials for both T1DM and IT to ascertain its efficacy in slowing disease progression and preserving beta-cell mass, respectively(49, 55).

In addition, strategies to inhibit PMN infiltration in response to chemokines signaling are also being explored in autoimmune disease and transplantation. For example, FTY720, derived from myriocin, a fungus-derived sphingosine analogue has been demonstrated to alter lymphocyte trafficking, by driving T-cells into lymphoid tissues, subsequently prevent them from leaving these tissue and homing into the inflammatory tissue (43), such as the islet within a T1DM or islet graft. FTY720 development was halted unfortunately after unexpectedly high rates of bradycardia and macular degeneration were encountered in initial clinical trials. Furthermore, the reparixin, a potent and selective inhibitor of CXCL8 is currently being tested in a phase II multicenter, open label trial, to determine its efficacy following a single islet infusion into patients with T1DM (NCT01220856).

Furthermore, approaches to block T-cell expansion by disrupting cytokine signaling are also being explored. Janus kinase 3 (JAK3), a tyrosine kinase associated with cytokine receptor-γ, is involved in multiple cytokine receptors. Currently, JAK3 inhibitors are being developed, as they appear to be powerful and attractive candidates for use in organ transplantation (43).

A.6.1.4. - Calcineurin Inhibitors

Historically, cyclosporine revolutionized organ transplantation and became the foundation of IS for over two decades. Mechanistically, cyclosporine engages cyclophilin, an intracellular protein of the immunophilin family, forming a complex that inhibits calcineurin phosphatase and ultimately T-cell activation (26, 43). Despite, its immunopotency, adverse side effects have been well documented in a dose dependent manner including but not limited to nephrotoxicity, hypertension, hyperlipidemia and post-transplant diabetes mellitus (43). Due to the latter side effects, its use in IT should be cautioned.

Similarly, tacrolimus bind to another immunophilin, FK506-binding protein 12, which also yields a complex that inhibits calcineurin with greater potency compared to cyclosporine. However, it too shares similar side effects to cyclosporine and also has diabetogenic properties (43).

A.6.1.5. - mTOR Inhibitors

An important component of the Edmonton Protocol was the utilization of an inhibitor of the mammalian target-of-rapamycin (mTOR), sirolimus and everolimus engage FKBP12 to

formulate a complex that binds and inhibits mTOR. This amelioration blocks cytokine receptors from triggering the cell cycle and IL2-driven T-cell proliferation (26, 28, 43). Physicians must be aware of added toxicity when mTOR inhibitors are combined with calcineurin inhibitors. It is of note, that the non-immune toxicity of mTOR inhibits include impaired wound healing and anti-angiogenesis (43), which may be of concern in IT as the grafts ability to neovascularize may be compromised. As a result of marked increase in side effects with chronic use of mTOR inhibitors, the Edmonton group has reverted to the routine use of tacrolimus + MMF, with elimination of mTOR altogether. This led to excellent longer-term islet graft function but avoided many of the troublesome side effects associated with his dose sirolimus therapy.

A.6.1.6. - Cell Cycle Arrest

The prodrug azathioprine has a long history in organ transplantation as a potent inhibitor the cell cycle. Azathioprine converts 6-mecraptopurine to an inhibitor of metalloproteinase, which in turn is converted to thioguanine nucleotides that disrupts DNA synthesis (43). Similarly, the prodrug mycophenolate mofetil (MMF) releases mycophenolic acid, which inhibits inosine monophosphate dehydrogenase, which is a critical component of purine synthesis (32, 43). Likewise, pyrimidine synthesis specific inhibitors such as FK778 have been utilized in organ transplantation (43). Ultimately, cell cycle arrest strategies have been used in organ transplantation to prevent the proliferation of T-and B-cells however there targeting is not immune specific. Nevertheless, the combination of MMF and calcineurin inhibitors has led to improved patient and graft survival while reducing early and late allograft rejection across multiple organ transplantation fields, including IT (49, 55).

A.6.2. - Antigen-Specific Vaccines

An emerging concept for immunotherapy for T1DM, and in abrogating the autoreactive T-cell population within the recipient of an islet transplant, is the idea of vaccination to establish antigen-specific regulatory T-cells with the goal of inducing tolerance to autoantigens. Currently, several vaccination strategies are being conducted implementing the immunization of T1DM patients against insulin and GAD65 (27, 29). It is conceivably possible that this methodology if proven efficacy in the nearly onset T1DM patient, could be translated to islet transplantation.

A.6.3. - Combination Strategies

In the context of the multiple pathways known to be involved in β-cell dysfunction as well as the alloresponse to foreign antigens, it is unlikely that a monotherapy will further optimize clinical IT and lead single-donor recipients. Indeed, strategies towards single-donor IT have begun, facing the challenges of inducing immunological tolerance, preventing islet destruction both from alloimmunity and the recurrence of T1DM in addition to avoiding the potential side effects associated with IS therapies. Combining anti-inflammatory strategies to maintenance IS have led to improved single-donor success rates at the University of Minnesota (61, 62). Peritransplant insulin and heparin administration greatly increase the success rate of single-donor islet transplants from 10 to 40% (39). Blockade of tumor necrosis factor-alpha (TNF-α) through the use of etanercept also has improved single-donor islet transplant outcomes (39, 62-65). In preclinical models anti-inflammatory agents anakinra, an interleukin-1 receptor antagonist (IL1-Ra) and etanercept significantly increased islet engraftment in marginal mass studies (63, 66).

Furthermore, anti-apoptosis and growth stimulation (glucagon-like peptide 1(GLP-1)) have also demonstrated favorable results in preclinical studies and clinical studies demonstrated that short acting GLP-1 analogue exenatide increased single-donor islet engraftment success (67-69). Finally, changes to classical IS strategies appear to facilitate single-donor islet engraftment. The use of T-cell depletion induction methods such as alemtuzumab in conjunction with TAC/MMF have resulted in substantial improvements in long-term insulin-independence (>5years) (49). In addition, a current example of the extraordinary progress that has been made when combine IS strategies are implemented, is the remarkable success that has been achieved when co-stimulation blockage using belatacept (inhibiting CD80-CD86 interactions) in conjunction with T-cell depletion induction and in the absence of calcineurin inhibitors led to insulin independence with islets from a single donor and prolonged allograft survival (60).

A.7. – SUMMARY

This chapter has reviewed the current status of immunotherapy in islet transplantation and the new exciting studies with promising agents to improve long-term results. Islet transplantation offers the potential to restore euglycemia, completely protect against hypoglycemia and lability in a way that exogenous insulin is unable to do, and with far less risk than whole pancreas transplantation. Yet, immune response continues to be a key player in the characterization of medium and long-term results, along with other engraftment hurdles.

Remarkable strides have occurred since the Edmonton Protocol was introduced in 2000, and currently over 1000 patients have safely undergoing islet transplant in up to 40 international centers. New and more potent drugs are continuously tested to achieve better results keeping the

same principle of '*islet-friendly*' medications without beta-cell toxicity, but still with sufficient protection from auto- and allo-immunity. They all take us a step closer to a definitive cure for type 1 diabetes.

A.8. – REFERENCES

1. Cryer PE. The barrier of hypoglycemia in diabetes. Diabetes 2008;57(12):3169-3176.

2. Vazeou A. Continuous blood glucose monitoring in diabetes treatment. Diabetes Res Clin Pract 2011;93 Suppl 1:S125-130.

3. Soedamah-Muthu SS, Fuller JH, Mulnier HE, Raleigh VS, Lawrenson RA, Colhoun HM. High risk of cardiovascular disease in patients with type 1 diabetes in the U.K.: a cohort study using the general practice research database. Diabetes care 2006;29(4):798-804.

4. 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.

5. Ryan EA, Bigam D, Shapiro AM. Current indications for pancreas or islet transplant. Diabetes Obes Metab 2006;8(1):1-7.

6. Shapiro AM, Ricordi C, Hering BJ, Auchincloss H, Lindblad R, Robertson RP et al. International trial of the Edmonton protocol for islet transplantation. N Engl J Med 2006;355(13):1318-1330.

7. Jahansouz C, Kumer SC, Brayman KL. Evolution of beta-Cell Replacement Therapy in Diabetes Mellitus: Islet Cell Transplant. J Transplant 2011;2011:247959.

8. 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.

9. Ricordi C, Strom TB. Clinical islet transplantation: advances and immunological challenges. Nat Rev Immunol 2004;4(4):259-268.

10. Chentoufi AA, Geenen V, Giannokakis N, Amrani A. Type 1 diabetes immunological tolerance and immunotherapy. Clin Dev Immunol 2011;2011:103738.

11. Pozzilli P, Strollo R. Immunotherapy for Type 1 diabetes: getting beyond a negative first impression. Immunotherapy 2012;4(7):655-658.

12. Squifflet JP, Gruessner RW, Sutherland DE. The history of pancreas transplantation: past, present and future. Acta Chir Belg 2008;108(3):367-378.

13. 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.

14. Weir GC, Bonner-Weir S. Islets of Langerhans: the puzzle of intraislet interactions and their relevance to diabetes. J Clin Invest 1990;85(4):983-987.

15. Ballian N, Brunicardi FC. Islet vasculature as a regulator of endocrine pancreas function. World J Surg 2007;31(4):705-714.

16. Lifson N, Kramlinger KG, Mayrand RR, Lender EJ. Blood flow to the rabbit pancreas with special reference to the islets of Langerhans. Gastroenterology 1980;79(3):466-473.

17. Scharfmann R, Xiao X, Heimberg H, Mallet J, Ravassard P. Beta cells within single human islets originate from multiple progenitors. PloS One 2008;3(10):e3559.

18. Johnson KH, O'Brien TD, Hayden DW, Jordan K, Ghobrial HK, Mahoney WC et al. Immunolocalization of islet amyloid polypeptide (IAPP) in pancreatic beta cells by means of peroxidase-antiperoxidase (PAP) and protein A-gold techniques. The Am J Pathol 1988;130(1):1-8.

19. Potter KJ, Abedini A, Marek P, Klimek AM, Butterworth S, Driscoll M et al. Islet amyloid deposition limits the viability of human islet grafts but not porcine islet grafts. Proc Natl Acad Sci U S A 2010;107(9):4305-4310.

20. Wenzlau JM, Juhl K, Yu L, Moua O, Sarkar SA, Gottlieb P et al. The cation efflux transporter ZnT8 (Slc30A8) is a major autoantigen in human type 1 diabetes. Proc Natl Acad Sci U S A 2007;104(43):17040-17045.

21. Lan MS, Wasserfall C, Maclaren NK, Notkins AL. IA-2, a transmembrane protein of the protein tyrosine phosphatase family, is a major autoantigen in insulin-dependent diabetes mellitus. Proc Natl Acad Sci U S A 1996;93(13):6367-6370.

22. Baekkeskov S, Aanstoot HJ, Christgau S, Reetz A, Solimena M, Cascalho M 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.

23. Schuit FC, In't Veld PA, Pipeleers DG. Glucose stimulates proinsulin biosynthesis by a dose-dependent recruitment of pancreatic beta cells. Proc Natl Acad Sci U S A 1988;85(11):3865-3869.

24. In't Veld P, Marichal M. Microscopic anatomy of the human islet of Langerhans. In: Islam MS, (ed). *The Islets of Langerhans*, Advances in Experimental Medicine and Biology: Springer Science, 2010: 1-19.

25. de Koning EJ, van den Brand JJ, Mott VL, Charge SB, Hansen BC, Bodkin NL et al. Macrophages and pancreatic islet amyloidosis. Amyloid 1998;5(4):247-254.

26. Bhatt S, Fung JJ, Lu L, Qian S. Tolerance-inducing strategies in islet transplantation. Int J Endocrinol 2012;2012:396524.

27. Boettler T, von Herrath M. Immunotherapy of type 1 diabetes--how to rationally prioritize combination therapies in T1D. Int Immunopharmacol 2010;10(12):1491-1495.

28. 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.

29. Rewers M, Gottlieb P. Immunotherapy for the prevention and treatment of type 1 diabetes: human trials and a look into the future. Diabetes care 2009;32(10):1769-1782.

30. Bingley PJ, Bonifacio E, Gale EA. Can we really predict IDDM? Diabetes 1993;42(2):213-220.

31. In't Veld P, Lievens D, De Grijse J, Ling Z, Van der Auwera B, Pipeleers-Marichal M et al. Screening for insulitis in adult autoantibody-positive organ donors. Diabetes 2007;56(9):2400-2404.

32. Van Belle T, von Herrath M. Immunosuppression in islet transplantation. J Clin Invest 2008;118(5):1625-1628.

33. Butler AE, Galasso R, Meier JJ, Basu R, Rizza RA, Butler PC. Modestly increased beta cell apoptosis but no increased beta cell replication in recent-onset type 1 diabetic patients who died of diabetic ketoacidosis. Diabetologia 2007;50(11):2323-2331.

34. Butler PC, Meier JJ, Butler AE, Bhushan A. The replication of beta cells in normal physiology, in disease and for therapy. Nat Clin Pract Endocrinol Metab 2007;3(11):758-768.

35. Meier JJ, Bhushan A, Butler AE, Rizza RA, Butler PC. Sustained beta cell apoptosis in patients with long-standing type 1 diabetes: indirect evidence for islet regeneration? Diabetologia 2005;48(11):2221-2228.

36. Dotta F, Censini S, van Halteren AG, Marselli L, Masini M, Dionisi S et al. Coxsackie B4 virus infection of beta cells and natural killer cell insulitis in recent-onset type 1 diabetic patients. Proc Natl Acad Sci U S A 2007;104(12):5115-5120.

37. King KM, Rubin G. A history of diabetes: from antiquity to discovering insulin. Br J Nurs 2003;12(18):1091-1095.

38. Adverse events and their association with treatment regimens in the diabetes control and complications trial. Diabetes care 1995;18(11):1415-1427.

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

40. Meloche RM. Transplantation for the treatment of type 1 diabetes. World J Gastroenterol 2007;13(47):6347-6355.

41. 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.

42. 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.

43. Halloran PF. Immunosuppressive drugs for kidney transplantation. N Engl J Med 2004;351(26):2715-2729.

44. Vincenti F. What's in the pipeline? New immunosuppressive drugs in transplantation. Am J Transplant 2002;2(10):898-903.

45. Kirk AD, Turgeon NA, Iwakoshi NN. B cells and transplantation tolerance. Nat Rev Nephrol 2010;6(10):584-593.

46. Narang AS, Mahato RI. Biological and biomaterial approaches for improved islet transplantation. Pharmacol Rev 2006;58(2):194-243.

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

48. Terasaki PI. A personal perspective: 100-year history of the humoral theory of transplantation. Transplantation 2012;93(8):751-756.

49. Shapiro AM. Strategies toward single-donor islets of Langerhans transplantation. Curr Opin Organ Transplant 2011;16(6):627-631.

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

51. Adams AB, Shirasugi N, Jones TR, Durham MM, Strobert EA, Cowan S et al. Development of a chimeric anti-CD40 monoclonal antibody that synergizes with LEA29Y to prolong islet allograft survival. J Immunol 2005;174(1):542-550.

52. Calne R, Moffatt SD, Friend PJ, Jamieson NV, Bradley JA, Hale G et al. Campath IH allows low-dose cyclosporine monotherapy in 31 cadaveric renal allograft recipients. Transplantation 1999;68(10):1613-1616.

53. Kenyon NS, Fernandez LA, Lehmann R, Masetti M, Ranuncoli A, Chatzipetrou M et al. Long-term survival and function of intrahepatic islet allografts in baboons treated with humanized anti-CD154. Diabetes 1999;48(7):1473-1481.

54. Lehmann R, Weber M, Berthold P, Zullig R, Pfammatter T, Moritz W et al. Successful simultaneous islet-kidney transplantation using a steroid-free immunosuppression: two-year follow-up. Am J Transplant 2004;4(7):1117-1123.

55. Shapiro AM. State of the art of clinical islet transplantation and novel protocols of immunosuppression. Curr Diab Rep 2011;11(5):345-354.

56. Gerber PA, Pavlicek V, Demartines N, Zuellig R, Pfammatter T, Wuthrich R et al. Simultaneous islet-kidney vs pancreas-kidney transplantation in type 1 diabetes mellitus: a 5 year single centre follow-up. Diabetologia 2008;51(1):110-119.

57. Campbell PM, Senior PA, Salam A, Labranche K, Bigam DL, Kneteman NM et al. High risk of sensitization after failed islet transplantation. Am J Transplant 2007;7(10):2311-2317.

58. Otten HG, Verhaar MC, Borst HP, Hene RJ, van Zuilen AD. Pretransplant donor-specific HLA class-I and -II antibodies are associated with an increased risk for kidney graft failure. Am J Transplant 2012;12(6):1618-1623.

59. Campbell PM, Salam A, Ryan EA, Senior P, Paty BW, Bigam D et al. Pretransplant HLA antibodies are associated with reduced graft survival after clinical islet transplantation. Am J Transplant 2007;7(5):1242-1248.

60. Posselt AM, Szot GL, Frassetto LA, Masharani U, Tavakol M, Amin R et al. Islet transplantation in type 1 diabetic patients using calcineurin inhibitor-free immunosuppressive protocols based on T-cell adhesion or costimulation blockade. Transplantation 2010;90(12):1595-1601.

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

62. Hering BJ, Kandaswamy R, Ansite JD, Eckman PM, Nakano M, Sawada T et al. Single-donor, marginal-dose islet transplantation in patients with type 1 diabetes. JAMA 2005;293(7):830-835.

63. Matsumoto S, Takita M, Chaussabel D, Noguchi H, Shimoda M, Sugimoto K 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 Transplant 2011.

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

65. Xenos ES, Farney AC, Widmer MB, Casanova D, Stevens RB, Blazar BR et al. Effect of tumor necrosis factor alpha and of the soluble tumor necrosis factor receptor on insulin secretion of isolated islets of Langerhans. Transplant Proc 1992;24(6):2863-2864.

66. 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.

67. Cechin SR, Perez-Alvarez I, Fenjves E, Molano RD, Pileggi A, Berggren PO et al. Anti-Inflammatory Properties of Exenatide in Human Pancreatic Islets. Cell Transplant 2011.

68. Faradji RN, Froud T, Messinger S, Monroy K, Pileggi A, Mineo D et al. Long-term metabolic and hormonal effects of exenatide on islet transplant recipients with allograft dysfunction. Cell Transplant 2009;18(10):1247-1259.

69. Ghofaili KA, Fung M, Ao Z, Meloche M, Shapiro RJ, Warnock GL et al. Effect of exenatide on beta cell function after islet transplantation in type 1 diabetes. Transplantation 2007;83(1):24-28.

APPENDIX B.

BIOLOGICAL AGENTS IN ISLET TRANSPLANTATION

B. – Biological Agents in Islet Transplantation

Curr Diab Rep
DOI 10.1007/s11892-013-0414-8

TRANSPLANTATION (A PILEGGI, SECTION EDITOR)

Biologic Agents in Islet Transplantation

Boris Gala-Lopez · Andrew R. Pepper · A. M. James Shapiro

CURRENT DIABETES REPORTS

© Springer Science+Business Media New York 2013

Abstract Islet transplantation is today an accepted modality for treating selected patients with frequent hypoglycemic events or severe glycemic lability. Despite tremendous progress in islet isolation, culture, and preservation, clinical use is still restricted to a limited subset, and lifelong immunosuppression is required. Issues surrounding limited islet revascularization and immune destruction remain. One of the major challenges is to prevent alloreactivity and recurrence of autoimmunity against β-cells. These two hurdles can be effectively reduced by immunosuppressive therapy combining induction and maintenance treatments. The introduction of highly potent and selective biologic agents has significantly reduced the frequency of acute rejection and has prolonged graft survival, while minimizing the complications of this therapeutic scheme. This review will address the most important biological agents used in islet transplantation. We provide a historical perspective of their introduction into clinical practice and their role in current clinical protocols, aiming at improved engraftment efficiency, increased long-term survival, and better overall results of clinical islet transplantation.

Keywords Islet transplantation · Immunosuppression · Biologic agents · Antibodies · Induction strategies · Maintenance schemes · Immune response · Acute rejection · Graft survival

B. Gala-Lopez · A. R. Pepper · A. M. J. Shapiro
Clinical Islet Transplant Program and Department of Surgery, University of Alberta, Edmonton, AB, Canada

A. M. J. Shapiro (✉)
Medicine and Surgical Oncology, University of Alberta, 2000 College Plaza, 8215 112th St, Edmonton, AB, Canada T6G 2C8
e-mail: amjs@islet.ca

Published online: 07 August 2013

Abbreviation list

IT	Islet transplant
IS	Immunosuppression
ICAM-1	Intercellular Adhesion Molecule-1
IL	Interleukin
T1DM	Type 1 diabetes mellitus
CNI	Calcineurin inhibitor
MMF	mycophenolate mofetil
mTOR	mammalian target of rapamycin
ATG	Anti-thymocyte globulin
IgG1	Immunoglobulin G1
CTLA-4	Cytotoxic T-lymphocyte antigen 4
LFA	Leukocyte Function Antigen
mAb	Monoclonal antibody
TCR	T-cell receptor
BAFF-R	BAAF receptor
GLP-1	Glucagon-like peptide 1

Introduction

Significant progress has occurred in the outcomes of clinical islet transplantation (IT), reflecting improvements in immunosuppression (IS) and preparation of sufficient quantities of highly viable islets for transplantation [1]. The foremost challenge in any transplant is to prevent alloreactivity, as well as recurrence of autoimmunity against β-cells. Recurrent autoimmunity and alloreactivity can be effectively reduced by IS induction therapy in combination with maintenance IS [2]. The introduction of highly potent and selective biologic agents for the initiation and maintenance of IS has reduced the frequency of acute rejection and has prolonged

Note: A version of this section was published as Review Manuscript in *Curr Diab Rep*. 2013 Oct; 13(5): 713 – 22.

REVIEW ARTICLE

Title: Biologic agents in islet transplantation

Authors: Boris Gala-Lopez[1], Andrew R. Pepper[1], AM. James Shapiro[1,2,3].

Affiliation:

1. Clinical Islet Transplant Program, University of Alberta. Edmonton, Alberta, Canada
2. Department of Surgery. University of Alberta.
3. Corresponding author.

Corresponding author:

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

Professor of Surgery, Medicine and Surgical Oncology

Director Clinical Islet and Living Donor Liver Transplant Programs

Clinical Senior Scholar Alberta Innovates - Healthcare Solutions (AIHS)

University of Alberta.

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

Phone 1 (780) 407- 7330, Fax 1 (780) 407- 8259

amjs@islet.ca

B.1. – ABSTRACT

Islet transplantation is today an accepted modality to treat selected patients with frequent hypoglycemic events or severe glycemic lability. Despite tremendous progress in islet isolation, culture, and preservation, clinical use is still restricted to a limited subset, and lifelong immunosuppression is required. Issues surrounding limited islet revascularization and immune destruction remain. One of the major challenges is to prevent alloreactivity and recurrence of autoimmunity against β-cells. These two hurdles can be effectively reduced by immunosuppressive therapy combining induction and maintenance treatments. The introduction of highly potent and selective biologic agents has significantly reduced the frequency of acute rejection and has prolonged the graft survival while minimizing the complications of this therapeutic scheme. This review will address the most important biological agents used in islet transplantation. We provide a historical perspective of their introduction in clinical practice and their role in current clinical protocols, aiming at improved engraftment efficiency, increased long-term survival and better overall results of clinical islet transplantation.

B.2. - INTRODUCTION

Significant progress has occurred in the outcomes of clinical islet transplantation (IT), reflecting improvements in immunosuppression (IS) and preparation of sufficient quantities of highly viable islets for transplantation (1). The foremost challenge in any transplant is to prevent alloreactivity as well as recurrence of autoimmunity against β-cells. Recurrent autoimmunity and alloreactivity can be effectively reduced by immunosuppressive induction therapy in

combination with maintenance immune suppression (2). The introduction of highly potent and selective biologic agents for the initiation and maintenance of immunosuppression has reduced the frequency of acute rejection and has prolonged the graft survival, while minimizing the complications of these therapeutic schemes (3, 4).

Sustained C-peptide production and high rates of insulin independence after pancreatic islet transplant in type 1 diabetes mellitus (T1DM) was reported 13 years ago by the Edmonton group (1). This reality became possible with the use of newer, more potent IS agents, the avoidance of corticosteroids, and high-quality islet preparations, known as the "Edmonton Protocol" (1, 3, 5). This immunosuppression scheme was tailored to avoid steroids and minimize calcineurin inhibitors to prevent diabetogenicity, with the combination of sirolimus, low-dose tacrolimus, and the daclizumab induction (6). However, insulin independence was not durable long-term and most patient returned to modest amounts of insulin without risk of recurrent hypoglycemia by the third to fifth year. Additionally, approximately 25% required additional late islet infusions during the second or third year post-transplant (3, 7). The reasons for the chronic failure of a portion of the islet transplants are likely associated with immune rejection, recurrence of autoimmunity or chronic exposure to diabetogenic IS agents (3). Now the focus is shifting towards the development of new and more effective biologicals, antibodies and fusion proteins which more precisely target the immune system providing adequate immunosuppression to prevent acute rejection without the non-immune adverse effects encountered in a calcineurin inhibitors (CNI)-based regimen (8).

We herein review a series of biological agents used in IT. We provide a historical perspective of their introduction in clinical practice and current clinical protocols are discussed together with

different ongoing research projects, which could potentially improve engraftment efficiency, long-term survival and the final results of clinical islet transplant.

B.3. - HISTORICAL PERSPECTIVE OF BIOLOGICS IN TRANSPLANTATION

The origins of biologics development can be traced back to the late 1800's; with von Behring and Kitasato published their finding anti-toxins for both tetanus and diphtheria (9, 10). This pioneering work translated to the widespread use and experimentation of biologics in medicine leading to therapies such as antitoxins, insulin, antibiotics (i.e. penicillin), and a multitude of vaccines that transformed the treatment and prevention of a multitude of diseases (11, 12). A milestone in the field of biologics was the production of the first mouse monoclonal antibody (mAb) by Kohler and Milstein in the 1970s (13). Monoclonal antibody development and refinement continues today and as of the late 1990s, the European Union has approved on average at least one antibody per year, highlighting the enthusiasm and promise of this therapeutic strategy.

The assimilation of both chemical and biological IS agents, including but not limited to CNI (i.e. cyclosporine and tacrolimus), antiproliferative agents such as mycophenolate mofetil (MMF) together with antibody induction (e.g. anti-thymocyte globulin) has revolutionized organ transplantation and has led to vast improvements in acute rejection and both short- and long-term graft survival (9, 14). The use of therapeutic agents derived from biological sources such as microbes, proteins, antibodies, cells and tissues, termed 'biologics' has demonstrated enticing promises for the field of islet and whole organ transplantation (9). Within the last few decades antibody developments has exploded leading to the approval of approximately 28 by the

European Union and US Food and Drug Administration by 2010 alone, while over 600 have entered into clinical studies (15-17). The majority of these agents used clinically target immunosuppressive induction and maintenance, however, the possibility of avoiding the chronic side effects associated with prolonged CNI and steroid administration has fueled further development of agents targeting specific molecules and immunologic pathways (9). Due to the specificity of biologics, compared to broad spectrum IS, the field of transplantation has begun to adopt agents from oncology, dermatology and rheumatology.

Despite the appealing target specificity that biologics possess, considerations that should not be ignored are the cost, stability, immunogenicity, dosages and toxicity (11, 13, 17-22). In additional when testing novel biological agents' efficacy and safety profiles, relevant animal models must be considered to avoid potential species-specific differences in biological activity.

B.4. - CURRENT EXPERIENCE WITH BIOLOGICAL AGENTS IN THE FIELD OF TRANSPLANT

After the successful establishment of the basic immunosuppression in the 1980s and 1990s a major revolution was seen with the introduction of new agents, including tacrolimus, MMF and mTOR inhibitors (**Table B.1**). They all provided new opportunities to optimize therapeutic regimes while reducing the occurrence of adverse events associated with their widespread use. Despite these potent immunosuppressants transplant clinicians faced new challenges; some patients develop chronic allograft dysfunction that can be attributable to immunological factors, such as donor-specific alloantibodies, and/or non-immunological factors, such as the potential

Table B.1 Biological agents currently being examined in transplantation.

Classification	Biologic Agent	Drug Example	Target(s)	Action	Area of Use
i. Induction	Antithymocyte globulin	Thymoglobulin, Atgam	Lymphocytes	T-cell destruction, clearance via complement dependent lysis.	Induction agent for whole organ and IT
	Anti-CD52	Alemtuzumab	T-cells, B-cells, macrophages, NK cells, granulocytes	Antibody-mediated lysis of CD52 expressing cells	Induction agent for whole organ and IT; B-cell chronic lymphocytic leukemia
ii. Maintenance	Anti-CD3	Muromonab, Teplizumab	T-cells	Blocks T-cell differentiation and proliferation and induction of T-regulatory cells	Prevention of autoimmune disease and alloreactivity in organ transplantation
	Anti-CD25	Daclizumab, Basiliximab	T-cells surface molecule, interleukin-2 receptor alpha-chain	Suppresses T-cell activation	Prevention of alloreactivity in IT
	Anti-LFA-1	Efalizumab	LFA-1 (CD11a and CD18) on T-cells	Impedes lymphocyte diapedesis and adhesion; inhibits LFA-1 :ICAM-1 binding	Promote kidney and islet allograft survival
	Anti-CD20	Rituximab	B-cell surface molecules	B-cell depletion	Non-Hodgkin's B-cell lymphomas, maintenance immunosuppression in organ transplantation
iii. Costimulatory	CTLA-4 Ig	Belatacept, Abatacept	T-cell activation, binding to CD80 and CD86	T-cell costimulatory signal blockade, nondepleting, abrogates signaling response	Prevention of alloreactive response to kidney and islet grafts
	Anti-CD154	IDEC131	T-cell surface maker CD40 ligand (CD154)	T-cell costimulatory signal blockade, nondepleting, abrogates signaling response	Autoimmune clinical trials; however, risk of thrombophilic events
	Anti-CD40	Lucatumumab	Antigen presenting cells surface marker CD40	T-cell costimulatory signal blockade, nondepleting, abrogates signaling response	Prevent acute rejection and prolonged survival of kidney and islet allografts (preclinical)
	Anti-BAFF; BAFF Ig	Belimumab; Atacicept	B-cell activating factor on T-cells	B-cell costimulatory signal blockage	Lupus and kidney transplantation
iv. Complement regulatory	Anti-C5	Eculizumab	Complement protein C5. Inhibits cleavage of C5 to C5a	Blocks complement immune mediate inflammatory, anaphylatoxin and chemotaxin response	Approved for paroxysmal nocturnal hemoglobinuria and atypical hemolytic uremic syndrome. Used in kidney-pancreas transplant
	C1 esterase inhibitor	Berinert	Binds to C1r and C1s protease in C1 complex of complement	Prevents the proteolytic cleaved of downstream complement component and inflammation	Ischemia reperfusion and heart transplant
v. Antiinflammatory	TNF-α inhibitor	Etanercept, Infliximab	mAb or fusion protein blocking TNF-α binding to cell surfaces	Blocking proinflammatory cytokine response	Early onset T1DM patient to improve glycemic control and reduce autoreactive T-cell in IT
	IL-1 receptor blockade	Anakinra	IL-1 receptor on surfaces of host cells	IL-1 receptor antagonist, thus blocking IL-1α and IL-β induced inflammation	Early onset T1DM patient to improve glycemic control and reduce autoreactive T-cell in IT
vi. Hormone stimulation	GLP-1 agonist	Exenatide	GLP-1 receptor on pancreatic beta cells	Stimulation of insulin secretion from pancreatic beta cells	IT

Note. IT, islet transplant; LFA, leukocyte function antigen; ICAM, intercellular adhesion molecule; T1DM, type 1 diabetes mellitus; GLP, glucagon-like peptide

nephrotoxicity of calcineurin-inhibitor drugs, hypertension, diabetes, dyslipidemia and *de novo* cancers (23, 24)

B.4.1. - Antithymocyte Globulins

Antithymocyte globulin (ATG) preparations are prepared by immunizing either horses or rabbits with human-derived splenic or other lymphoid tissue and then harvesting and stabilizing the resultant immune serums (4). These agents have been successful in reducing recurrent autoimmunity and alloreactivity when used in immunosuppressive induction therapy, in combination with maintenance immune suppression (25).

Polyclonal rabbit antithymocyte globulin induces IS in vivo by promoting T-cell clearance from the circulation and modulation of T-cell activation, homing, and cytotoxic activities (4). T-cell destruction by ATG (equine) occurs through complement-dependent lysis after the antibody preparation binds to a variety of cell-surface markers, including CD2, CD3, CD4, CD8, CD11a, and CD18. Destruction of lymphocytes occurs systemically and within the thymus and spleen (26).

These widely used agents are commonly used as rejection therapy for steroid-resistant rejection episodes, and several reports have confirmed that the addition of ATGs allowed the avoidance of steroid exposure, an area of great current interest (4). In the settings of IT some reports highlight the potential prolonged glycemic benefits in selected recipients when potent induction immunosuppression, including ATGs is used (27). This benefit may in part be mediated by improved islet engraftment and mitigation of autoreactive T-cell responses (27).

B.4.2. - Anti-CD3

Muromonab CD3, a murine monoclonal antibody with activity against the CD3 surface antigen of T cells, was first introduced into clinical practice in 1986. It has been extensively used for the treatment of acute cellular rejection in renal transplantation (28). This anti-CD3 antibody was the first biological agent used in clinical medicine and has been associated with significant adverse effects, which lead to a significant decrease in its use (4).

In searching for an improved treatment for T1DM anti-CD3 was rescued and used to suppress the autoimmune response in both, the prevention of T1DM and graft loss after IT (29). This treatment is presumed to induce tolerance by induction of adaptive regulatory T cells, which is a more acceptable, less problematic immune modulation than chronic immune suppression itself (29).

Islet transplantation in combination with anti-CD3 antibody treatment has shown to be effective in reversing diabetes in a limited clinical application (30). It remains the most promising immune therapy for reversing recent-onset T1DM. However, current clinical trials have revealed their major drawback, namely the narrow therapeutic window in which low doses are ineffective and higher doses that preserve functional beta cell mass cause side effects (31).

B.4.3. - Anti-CD25

Monoclonal antibodies directed against specific T-cell surface molecules have been developed for clinical use as immunosuppressants. One of these is anti-CD25 (daclizumab and basiliximab), a humanized IgG1 monoclonal antibody directed against the low-affinity interleukin-2 receptor

α-chain (32). Both are products of recombinant DNA technology; basiliximab is considered a chimeric antibody, because it consists of approximately 70% human and 30% murine proteins (33). Daclizumab on the other hand is humanized and consists of 90% human and 10% murine components. The effectiveness of daclizumab is comparable to that of basiliximab, with a very low adverse-effect profile comparable to that seen with placebo.

These antibodies target activated T-cells, and their use in a clinical setting has significantly increased over the past decades (2, 4, 34). Similar immunosuppressive properties for both ATG and anti-CD25 have been reported in terms of preventing alloreactivity (31, 35). However, it is universally recognized that anti-CD25 therapies are considerably less potent than T-depletional antibodies in terms of prophylaxis against rejection events. They have also been used similarly to the polyclonal antisera, to attempt to develop regimens that avoid the use of drugs, such as corticosteroids and CNIs, whose toxicity is now perceived as being undesirable.

Anti-CD25 have been extensively used in IT since it early stages, in combination with other drugs. In fact, daclizumab was one of the critical components of the most successful immunotherapy approach, termed the 'Edmonton protocol' based upon the pioneering experience reported from the University of Alberta in 2000 (1).

B.4.4. - Alemtuzumab

Alemtuzumab is an anti-CD52, humanized, monoclonal antibody used in the treatment of B-cell chronic lymphocytic leukemia. CD52 is present on virtually all B cells and T cells, as well as macrophages, NK cells, and some granulocytes (35, 36). When the alemtuzumab antibody binds to CD52, it triggers an antibody-dependent lysis of these cells.

Alemtuzumab produces a significant immune depletion associated with immune reconstitution. Its use as an induction agent has become an increasingly popular due to its overwhelming efficacy as a depleting antibody. At this time, very few randomized controlled trials have evaluated its efficacy and safety, although some studies report no obvious differences in rates of graft and patient survival, biopsy-proven acute rejection episodes, and treatment failure after alemtuzumab induction with standard immunosuppression maintenance (4).

The effect of alemtuzumab on multiple inflammatory cell types, for example, macrophages, may prevent the production of pro-inflammatory mediators by intrahepatic macrophages and endothelial cells, thus reducing early islet losses secondary to the deleterious effects of cytokines at the time of islet infusion (37, 38). Hence, induction with alemtuzumab may improve short and long-term results as demonstrated by recent reports (37, 39). Yet, more definitive and complete results on the benefits of this agent in IT will be coming out of the recently finished phase 2 clinical trial performed at the University of Alberta (NCT00175253).

B.4.5. – Belatacept

Belatacept is a selective co-stimulatory (signal 2) blockers. It is a more potent second-generation molecule of its parent compound abatacept, which was the first molecule developed by fusion of extracellular domain of cytotoxic T-lymphocyte antigen 4 (CTLA4) with Fc fragment of human immunoglobulin G1 (IgG1) (40, 41). Belatacept is a fully human fusion protein of extracellular domain of CTLA4 with fragment of the Fc domain of human IgG1 (8). It differs from abatacept by engineered point mutations resulting in two amino acid substitutions (lucine 104 - glutamate and alanine 29- tyrosine). This resulted in a fourfold increase in binding affinity to CD86 and a

twofold increase in binding affinity to CD80 in comparison with abatacept, and a 10-fold increase in co-stimulation blockade in T-cell activation. This dramatic increase directly correlated with prolonged graft survival and decreased production of anti-donor antibodies in non-human primate renal transplant model (42).

Following the promising results of phase 3 trials in kidney transplantation (43), regulatory approval has been achieved for belatacept, which has become a new, non-depleting, metabolically well-tolerated agent that is clearly attractive for use in clinical islet transplantation. A recent study has reported a significant increase in islet allograft survival with the combination of belatacept and sirolimus (44). Moreover, Posselt and collaborators also demonstrated the potential benefits of belatacept in permitting long-term islet allograft survival, as one of the first reports of CNI, steroid-free regimes for clinical islet transplant (45).

Finally, encouraging results of these initial studies prompted for a larger clinical trial under the Clinical Islet Transplantation consortium determining safety and efficacy of IT when combined with an immunosuppressive medication regimen containing belatacept.

B.4.6. - Efalizumab

Efalizumab is a CD11a-specific humanized mAb that targets the Leukocyte Function Antigen (LFA-1) pathway. LFA-1 is comprised of two subunits, CD11a and CD18, and binds Intercellular Adhesion Molecule (ICAM)-1 (46). Efalizumab impedes LFA-1 to ICAM-1 binding and in doing so prevents lymphocyte diapedesis and disrupts adhesion events necessary for optimal T-cell function. Preclinical murine and primate studies have demonstrated that LFA-1-specific antibodies prolong the survival of islet and other organ allografts (45, 46), and phase I/II

studies in renal transplantation suggest that efalizumab has efficacy in preventing human allograft rejection. However, it was recently withdrawn from clinical use due to concerns about the development of progressive multifocal myeloencephalopathy in three patients out of more than 46,000 who received the drug as treatment for psoriasis for more than 3 years (47, 48).

B.5. - FUTURE BIOLOGIC PROSPECTS FOR ISLET TRANSPLANTATION

Immunological challenges to islet survival, engraftment, and function post-transplantation are 2-fold: alloimmune destruction and autoimmune rejection. Although the former is common to all organ and tissue transplantation situations, T1DM offers additional challenges due to the autoimmunity (32). A variety of experimental studies are now focused to further improve results in islet transplant by introducing tolerance-inducing medication. Summarized below are some of the new promising biological agents.

B.5.1. - Induction Agents: Depleting Antibodies

Anti-CD3 antibody (teplizumab) has been demonstrated to preserve C-peptide preservation and reduce insulin requirements when administered to diabetic patients, up to five years post-administration (34). Monoclonal anti-CD3 transiently activates the CD3 receptor, simulates cytokine release and ultimately blocks T-cell proliferation and differentiation (49). Essentially, anti-CD3 disrupts T-cell ability to recognize auto- and alloantigens, blocks IL-2 signaling pathways and induces apoptosis. It has also been postulated that long-term benefits may be as a

result of the induction of regulatory T-cells, and therefore the use of humanized CD3-monoclonals, which do not stimulate cytokine release, has been viewed as an attractive treatment modality to reduce the autoreactivity in T1DM (34, 49-51). Side effects such as reactivations of Epstein-Barr virus and flu-like symptoms have been reported; however, taken together anti-CD3 antibody treatment appears to have great promise in potential combination therapies in IT, including a well-tolerated substitute for muromonab, A1-CD3, developed in Prague (52).

B.5.2. - B-Cell Therapeutics

B.5.2.1. - Anti-CD20

The influence of B-cells in the autoimmune pathogenesis of T1DM and an islet graft may have been underestimated since it is widely accepted that both auto-and alloreactive T-cells are the main culprits. However, a human phase 2 trial elucidated that when B-cells are depleted using an anti-CD20 monoclonal antibody (Rituximab) a tentative preservation of C-peptide, insulin requirements and improved HbA1c was observed in newly diagnosed T1DM patients (34, 49). CD20 is unique to B-cells and thus inhibiting B-cells via anti-CD20 monoclonals reduces the presentation of autoantigens to T-cells and possibly preventing B-cell expansion and anti-islet autoantibody production (49, 53). Anti-CD20, Rituximab is currently approved for treatment of refractory non-Hodgkin's B-cell lymphomas however, it is off label use in combinations with maintenance IS has been used in transplantation since the early 1990s (54, 55) and thus a potential candidate for reducing the auto and alloreactive responses to an islet allograft.

B.5.2.2. - B-Cell Activating Factor (BAFF) Blockade

Similar to the necessary costimulation required for T-cell activation, B-cells are governed by similar costimulation in order to survive, proliferate and mature. BAFF, expressed predominately on T-cells and dendritic cells, is a member of the tumor necrosis factor cytokine family (9). BAFFs which are also known as B Lymphocyte Stimulator (BlyS), TALL-1, THANK, and zTNF4 binds to B-cell maturation antigen, transmembrane activator and BAFF receptor (BAFF-R) ultimately stimulating B-Cell activation. Originally tested in autoimmune diseases, BAFF blockade has recently been translated to the field of transplantation and has been described in chronic graft-versus-host disease (56-60). Belimumab, approved by the FDA in 2011 for systemic lupus, is a human recombinant IgG1 monoclonal antibody to BAFF. A similar anti-BAFF mAb depleted follicular and alloreactive B-cells in a murine cardiac allograft model (61). This group has recently enrolled patients in a phase 2 clinical trial of desensitization with belimumab in sensitized patients awaiting kidney transplantation. This may be a potential future attractive strategy for islet recipients especially in sensitized patients. A BAFF neutralizing recombinant Fc fusion protein, Atacicept, is currently in a phase 2/3 clinical trial for lupus, which may warrant further evaluation in clinical transplantation (9).

B.5.2.3. - Complement

Complement proteins have been used as hallmarks for diagnosing antibody-mediated rejection (i.e. C4d deposition) and acute renal allograft rejection (i.e. urine C5a and plasma C1rsC1-inhibitor complexes levels). Eculizumab a recombinant humanized IgG2/mAb to C5 (FDA

approved for paroxysmal nocturnal hemoglobinuria and atypical hemolytic uremic syndrome) has demonstrated effective rescue from complement activation and antibody-mediated rejection in case studies of an ABO-incompatible kidney-pancreas transplant and re-transplanted kidney recipient (62, 63). The C1 esterase inhibitor Berinert, has demonstrated successful results in ischemia-reperfusion injury (64) and in a case study of a O-type recipient transplanted with a B-type heart (65). Complement inhibition in the field of islet transplantation may have a therapeutic benefit considering the islets a typically infused into the portal circulation prior to embolizing into the sinusoids of the liver.

B.5.2.4. - Costimulatory Blockade: Fusion Proteins

Another attractive therapeutic strategies is to target cell-surface molecules which are important in the immune synapse, secondary to the TCR:MHC interaction. The benefits of blocking the co-stimulatory signaling pathways between CD80 and CD86 on the APC with CD28 of the T-cell have been well established. Tolerance can be achieved by interfering with costimulatory interactions, inhibiting the secondary signal required for full T-cell activation (50). The B7-CD28 pathway is a key pathway in T cell activation, survival, and function. In rodent models, B7-CD28 blockade through administration of inhibitory CTLA4-Ig led to prolonged allograft survival and tolerance (50). The co-stimulatory signaling pathway between CD40:CD154 is also an attractive target to prevent autoimmune disease, lymphocyte trafficking and graft rejection. Ligation of the CD40 receptor on antigen-presenting cells with CD40 ligand (CD154) on T cells enhances TCR signaling and effector responses (66). Humanized anti-CD154 (IDEC131) has been implemented in autoimmune clinical trials, and had potential to be extended to islet

transplantation (51). However, high rates of unexpected fatal thrombophilic events in early clinical trials have rendered anti-CD154 therapies too risky for future clinical application. Recently, a humanized non-depleting anti-CD40 has been demonstrated to prevent acute rejection and prolonged graft survival in both nonhuman primate models of kidney and islet allografts, but without the associated risk of thrombophilia (67, 68). Whether true tolerance can be achieved with anti-CD154 mAb alone or in combination with bone marrow transplantation, donor-specific transfusion or conventional immunosuppression is debatable. Indeed, tolerance induction is readily achieved in juvenile mouse models of a simplified immune system without high burden of immunological memory, but translating these findings to the more complex, diverse and memory-laden human system has been much more challenging. Anti-CD154 mAb treatment has been shown to result in "indefinite" survival in islet, bone marrow, and cardiac allograft models in animal models (50). Anti-CD40 mAb such as lucatumumab, Chi220, ASKP1240, PG102 and PRO64553 appear to be attractive and viable immunosuppressive strategies for islet and whole organ transplantation (9). The advantage of the non-depleting immunotherapy approach is that they reduce responsiveness without compromising lymphocyte populations. Therefore, non-depleting lymphocyte strategies appear attractive when combined with less intense maintenance IS cocktails.

B.5.2.5. - Anti-Inflammatory & Chemokines/Cytokines

Since both T1DM and islet graft rejection are characterized by inflammatory process leading to insulitis, blocking pro-inflammatory events appears to be an appealing area of therapeutic intervention. A number of agents have proven effective in abrogating autoimmune disease

including T1DM. The tumor necrosis factor-α inhibitor (etanercept), has increased C-peptide secretion, lowered HbA1c and exogenous insulin requirements when administered to early onset T1DM patients (49). Etanercept has also been demonstrated to increase the efficacy of IT, and reduce the number of autoreactive T-cells (34, 69). Interleukin-1 (IL-1), is another potent pro-inflammatory molecule, and has been reported to cause beta cell dysfunction (34, 49, 50, 69). Anakinra, which blocks both isoforms IL-1α and IL-1β through IL1 receptor blockade is currently being explore in clinical trials for both T1DM and IT to ascertain its efficacy in slowing disease progression and preserving beta-cell mass (35, 70).

In addition, strategies to inhibit PMN infiltration in response to chemokines signaling are also being explored in autoimmune disease and transplantation. For example, FTY720, derived from myriocin, a fungus-derived sphingosine analogue has been demonstrated to alter lymphocyte trafficking, by driving T-cells into lymphoid tissues, subsequently prevent them from leaving these tissue and homing into the inflammatory tissue (51), such as the islet within a T1DM or islet graft. FTY720 development was halted unfortunately after unexpectedly high rates of bradycardia and macular degeneration were encountered in initial clinical trials.

B.5.3. - Biologic Combination Strategies

In the context of the multiple pathways known to be involved in β-cell dysfunction as well as the alloresponse to foreign antigens, it is unlikely that a monotherapy will further optimize clinical IT and lead single-donor recipients. Indeed, strategies towards single-donor IT have begun by implementing biologics to IS cocktails, which face the challenges of inducing tolerance and graft survival. Combining anti-inflammatory biologics to maintenance IS have led to improved single-

donor success rates at the University of Minnesota (71, 72). Peritransplant insulin and heparin administration greatly increase the success rate of single-donor islet transplants from 10 to 40% (73). Blockade of tumor necrosis factor-alpha (TNF-α) by using etanercept also has improved single-donor islet transplant outcomes (72-76). In preclinical models anti-inflammatory agents anakinra, an interleukin-1 receptor antagonist (IL1-Ra) and etanercept significantly increased islet engraftment in marginal mass studies (74-77). Furthermore, anti-apoptosis and growth stimulation (glucagon-like peptide 1(GLP-1)) have also demonstrated favorable results in preclinical studies and clinical studies demonstrated that short acting GLP-1 analogue exenatide increased single-donor islet engraftment success (78-80). Clonal depletion of alloreactive T cells appears promote a hypo-responsive environment and peripheral mechanisms of anergy, thus driving the shift towards tolerance (50, 66). The use of T-cell depletion induction methods such as alemtuzumab in conjunction with tacrolimus/MMF have resulted in substantial improvements in long-term insulin-independence (>5years) (70). In addition, a current example of the extraordinary progress that has been made when combine IS strategies are implemented, is the remarkable success that has been achieved when co-stimulation blockage using belatacept (inhibiting CD80-CD86 interactions) in conjunction with T-cell depletion induction and in the absence of calcineurin inhibitors led to insulin independence with islets from a single donor and prolonged allograft survival (45).

B.6. – SUMMARY

This paper has reviewed the general use of biological agents in transplantation and their particular application in the field of islet transplant. Islet transplantation provides the potential to restore euglycemia, protecting against hypoglycemia and labiality in a way that exogenous insulin is unable to do, and with far less risk than whole pancreas transplantation. Yet, immune response continues to be a key player in the characterization of medium and long-term results, along with other engraftment hurdles.

The future of islet transplantation depends on the development of tolerance inducing therapies. While temporary immunosuppression can be advantageous, the long-term risks outweigh the benefit. Tolerance suggests freedom from insulin dependency and an improvement in the patient's overall quality of life. A tolerizing regimen that utilizes biologics and techniques that selectively target donor-reactive T-cells while expanding populations of regulatory T cells will undoubtedly result in better outcomes.

New and more potent drugs are continuously tested to achieve better results keeping the same principle of '*islet-friendly*' medications without beta-cell toxicity, but still with sufficient protection from auto- and allo-immunity. They all take us a step closer to a definitive cure for type 1 diabetes.

B.7. – REFERENCES

1. 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.

2. Kaufman DB, Shapiro R, Lucey MR, Cherikh WS, R TB, Dyke DB. Immunosuppression: practice and trends. Am J Transplant 2004;4 Suppl 9:38-53.

3. 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.

4. Gabardi S, Martin ST, Roberts KL, Grafals M. Induction immunosuppressive therapies in renal transplantation. American journal of health-system pharmacy : Am J Health Syst Pharm 2011;68(3):211-218.

5. Shapiro AM, Ricordi C, Hering BJ, Auchincloss H, Lindblad R, Robertson RP et al. International trial of the Edmonton protocol for islet transplantation. N Engl J Med 2006;355(13):1318-1330.

6. 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.

7. Jahansouz C, Kumer SC, Brayman KL. Evolution of beta-Cell Replacement Therapy in Diabetes Mellitus: Islet Cell Transplant. J Transplant 2011;2011:247959.

8. Arora S, Tangirala B, Osadchuk L, Sureshkumar KK. Belatacept : a new biological agent for maintenance immunosuppression in kidney transplantation. Expert Opin Biol Ther 2012;12(7):965-979.

9. Page EK, Dar WA, Knechtle SJ. Biologics in organ transplantation. Transpl Int 2012;25(7):707-719.

10. Kantha SS. A centennial review; the 1890 tetanus antitoxin paper of von Behring and Kitasato and the related developments. Keio J Med 1991;40(1):35-39.

11. Bristow AF. Assignment of quantities to biological medicines: an old problem re-discovered. Philos Trans A Math Phys Eng Sci 2011;369(1953):4004-4013.

12. Bristow AF, Barrowcliffe T, Bangham DR. Standardization of biological medicines: the first hundred years, 1900-2000. Notes Rec R Soc Lond 2006;60(3):271-289.

13. Kohler G, Milstein C. Continuous cultures of fused cells secreting antibody of predefined specificity. Nature 1975;256(5517):495-497.

14. Cooper JE, Wiseman AC. Novel immunosuppressive agents in kidney transplantation. Clin Nephrol 2010;73(5):333-343.

15. Reichert JM. Metrics for antibody therapeutics development. MAbs 2010;2(6):695-700.

16. Reichert JM. Next generation and biosimilar monoclonal antibodies: essential considerations towards regulatory acceptance in Europe. February 3-4, 2011, Freiburg, Germany. MAbs 2011;3(3):223-240.

17. Reichert JM. Which are the antibodies to watch in 2012? MAbs 2012;4(1):1-3.

18. Daugherty AL, Mrsny RJ. Formulation and delivery issues for monoclonal antibody therapeutics. Adv Drug Deliv Rev 2006;58(5-6):686-706.

19. Reichert JM. Which are the antibodies to watch in 2013? MAbs 2013;5(1):1-4.

20. Tabrizi MA, Roskos LK. Preclinical and clinical safety of monoclonal antibodies. Drug Discov Today 2007;12(13-14):540-547.

21. Wadhwa M, Bird C, Dilger P, Gaines-Das R, Thorpe R. Strategies for detection, measurement and characterization of unwanted antibodies induced by therapeutic biologicals. J Immunol Methods 2003;278(1-2):1-17.

22. Weir AB. Hazard identification and risk assessment for biologics targeting the immune system. J Immunotoxicol 2008;5(1):3-10.

23. Weclawiak H, Kamar N, Ould-Mohamed A, Cardeau-Desangles I, Rostaing L. Rationale for using belatacept in combination with sirolimus. Transplant Proc 2010;42(9 Suppl):S29-31.

24. Weclawiak H, Kamar N, Ould-Mohamed A, Cardeau-Desangles I, Rostaing L. Biological agents in kidney transplantation: belatacept is entering the field. Expert Opin Biol Ther 2010;10(10):1501-1508.

25. Huurman VA, Kalpoe JS, van de Linde P, Vaessen N, Ringers J, Kroes AC et al. Choice of antibody immunotherapy influences cytomegalovirus viremia in simultaneous pancreas-kidney transplant recipients. Diabetes Care 2006;29(4):842-847.

26. Beiras-Fernandez A, Thein E, Hammer C. Induction of immunosuppression with polyclonal antithymocyte globulins: an overview. Exp Clin Transplant2003;1(2):79-84.

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

28. Webster AC, Pankhurst T, Rinaldi F, Chapman JR, Craig JC. Monoclonal and polyclonal antibody therapy for treating acute rejection in kidney transplant recipients: a systematic review of randomized trial data. Transplantation 2006;81(7):953-965.

29. Begum S, Chen W, Herold KC, Papaioannou VE. Remission of type 1 diabetes after anti-CD3 antibody treatment and transplantation of embryonic pancreatic precursors. Endocrinology 2009;150(10):4512-4520.

30. Hering BJ, Kandaswamy R, Harmon JV, Ansite JD, Clemmings SM, Sakai T et al. Transplantation of cultured islets from two-layer preserved pancreases in type 1 diabetes with anti-CD3 antibody. Am J Transplant 2004;4(3):390-401.

31. Baeke F, Van Belle TL, Takiishi T, Ding L, Korf H, Laureys J et al. Low doses of anti-CD3, ciclosporin A and the vitamin D analogue, TX527, synergise to delay recurrence of autoimmune diabetes in an islet-transplanted NOD mouse model of diabetes. Diabetologia 2012;55(10):2723-2732.

32. Narang AS, Mahato RI. Biological and biomaterial approaches for improved islet transplantation. Pharmacol Rev 2006;58(2):194-243.

33. Mottershead M, Neuberger J. Daclizumab. Expert Opin Biol Ther 2007;7(10):1583-1596.

34. Boettler T, von Herrath M. Immunotherapy of type 1 diabetes--how to rationally prioritize combination therapies in T1D. Int Immunopharmacol 2010;10(12):1491-1495.

35. Shapiro AM. State of the art of clinical islet transplantation and novel protocols of immunosuppression. Curr Diab Rep 2011;11(5):345-354.

36. Terasaki PI. A personal perspective: 100-year history of the humoral theory of transplantation. Transplantation 2012;93(8):751-756.

37. Froud T, Baidal DA, Faradji R, Cure P, Mineo D, Selvaggi G et al. Islet transplantation with alemtuzumab induction and calcineurin-free maintenance immunosuppression results in improved short- and long-term outcomes. Transplantation 2008;86(12):1695-1701.

38. Tan J, Yang S, Cai J, Guo J, Huang L, Wu Z et al. Simultaneous islet and kidney transplantation in seven patients with type 1 diabetes and end-stage renal disease using a glucocorticoid-free immunosuppressive regimen with alemtuzumab induction. Diabetes 2008;57(10):2666-2671.

39. Shapiro AM, Lakey JR, Paty BW, Senior PA, Bigam DL, Ryan EA. Strategic opportunities in clinical islet transplantation. Transplantation 2005;79(10):1304-1307.

40. Martin ST, Tichy EM, Gabardi S. Belatacept: a novel biologic for maintenance immunosuppression after renal transplantation. Pharmacotherapy 2011;31(4):394-407.

41. Weisman MH, Durez P, Hallegua D, Aranda R, Becker JC, Nuamah I et al. Reduction of inflammatory biomarker response by abatacept in treatment of rheumatoid arthritis. J Rheumatol 2006;33(11):2162-2166.

42. Latek R, Fleener C, Lamian V, Kulbokas E, 3rd, Davis PM, Suchard SJ et al. Assessment of belatacept-mediated costimulation blockade through evaluation of CD80/86-receptor saturation. Transplantation 2009;87(6):926-933.

43. Durrbach A, Pestana JM, Pearson T, Vincenti F, Garcia VD, Campistol J et al. A phase III study of belatacept versus cyclosporine in kidney transplants from extended criteria donors (BENEFIT-EXT study). Am J Transplant 2010;10(3):547-557.

44. Lowe MC, Badell IR, Turner AP, Thompson PW, Leopardi FV, Strobert EA et al. Belatacept and sirolimus prolong nonhuman primate islet allograft survival: adverse consequences of concomitant alefacept therapy. Am J Transplant 2013;13(2):312-319.

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

protocols based on T-cell adhesion or costimulation blockade. Transplantation 2010;90(12):1595-1601.

46. Turgeon NA, Avila JG, Cano JA, Hutchinson JJ, Badell IR, Page AJ et al. Experience with a novel efalizumab-based immunosuppressive regimen to facilitate single donor islet Cell Transplant. Am J Transplant 2010;10(9):2082-2091.

47. Schwab N, Ulzheimer JC, Fox RJ, Schneider-Hohendorf T, Kieseier BC, Monoranu CM et al. Fatal PML associated with efalizumab therapy: insights into integrin alphaLbeta2 in JC virus control. Neurology 2012;78(7):458-467; discussion 465.

48. Kothary N, Diak IL, Brinker A, Bezabeh S, Avigan M, Dal Pan G. Progressive multifocal leukoencephalopathy associated with efalizumab use in psoriasis patients. J Am Acad Dermatol 2011;65(3):546-551.

49. Rewers M, Gottlieb P. Immunotherapy for the prevention and treatment of type 1 diabetes: human trials and a look into the future. Diabetes Care 2009;32(10):1769-1782.

50. Bhatt S, Fung JJ, Lu L, Qian S. Tolerance-inducing strategies in islet transplantation. Int J Endocrinol 2012;2012:396524.

51. Halloran PF. Immunosuppressive drugs for kidney transplantation. N Engl J Med 2004;351(26):2715-2729.

52. Hilgert I, Franek F, Stefanova I, Kaslik J, Jirka J, Kristofova H et al. Therapeutic in vivo use of the A1-CD3 monoclonal antibody. Transplantation 1993;55(2):435-438.

53. Kirk AD, Turgeon NA, Iwakoshi NN. B cells and transplantation tolerance. Nat Rev Nephrol 2010;6(10):584-593.

54. Faye A, Van Den Abeele T, Peuchmaur M, Mathieu-Boue A, Vilmer E. Anti-CD20 monoclonal antibody for post-transplant lymphoproliferative disorders. Lancet 1998;352(9136):1285.

55. Fischer A, Blanche S, Le Bidois J, Bordigoni P, Garnier JL, Niaudet P et al. Anti-B-cell monoclonal antibodies in the treatment of severe B-cell lymphoproliferative syndrome following bone marrow and organ transplantation. N Engl J Med 1991;324(21):1451-1456.

56. Porcheray F, Wong W, Saidman SL, De Vito J, Girouard TC, Chittenden M et al. B-cell immunity in the context of T-cell tolerance after combined kidney and bone marrow transplantation in humans. Am J Transplant 2009;9(9):2126-2135.

57. Sarantopoulos S, Stevenson KE, Kim HT, Cutler CS, Bhuiya NS, Schowalter M et al. Altered B-cell homeostasis and excess BAFF in human chronic graft-versus-host disease. Blood 2009;113(16):3865-3874.

58. Xu H, He X, Liu Q, Shi D, Chen Y, Zhu Y et al. Abnormal high expression of B-cell activating factor belonging to the TNF superfamily (BAFF) associated with long-term outcome in kidney transplant recipients. Transplant Proc 2009;41(5):1552-1556.

59. Xu H, He X, Sun J, Shi D, Zhu Y, Zhang X. The expression of B-cell activating factor belonging to tumor necrosis factor superfamily (BAFF) significantly correlated with C4D in kidney allograft rejection. Transplant Proc 2009;41(1):112-116.

60. Zarkhin V, Li L, Sarwal MM. BAFF may modulate the rate of B-cell repopulation after rituximab therapy for acute renal transplant rejection. Transplantation 2009;88(10):1229-1230.

61. Vivek K, Mustafa MM, Rodriguez E, Redfield RR, 3rd, Parsons RF, Rostami S et al. Strategies for B-lymphocyte repertoire remodeling in transplantation tolerance. Immunol Res 2011;51(1):1-4.

62. Biglarnia AR, Nilsson B, Nilsson T, von Zur-Muhlen B, Wagner M, Berne C et al. Prompt reversal of a severe complement activation by eculizumab in a patient undergoing intentional ABO-incompatible pancreas and kidney transplantation. Transpl Int 2011;24(8):e61-66.

63. Locke JE, Magro CM, Singer AL, Segev DL, Haas M, Hillel AT et al. The use of antibody to complement protein C5 for salvage treatment of severe antibody-mediated rejection. Am J Transplant 2009;9(1):231-235.

64. Kirschfink M. C1-inhibitor and transplantation. Immunobiology 2002;205(4-5):534-541.

65. Mohacsi P, Rieben R, Sigurdsson G, Tschanz H, Schaffner T, Nydegger UE et al. Successful management of a B-type cardiac allograft into an O-type man with 3(1/2)-year clinical follow-up. Transplantation 2001;72(7):1328-1330.

66. Shapiro AM, Nanji SA, Lakey JR. Clinical islet transplant: current and future directions towards tolerance. Immunol Rev 2003;196:219-236.

67. Aoyagi T, Yamashita K, Suzuki T, Uno M, Goto R, Taniguchi M et al. A human anti-CD40 monoclonal antibody, 4D11, for kidney transplantation in cynomolgus monkeys: induction and maintenance therapy. Am J Transplant 2009;9(8):1732-1741.

68. Badell IR, Thompson PW, Turner AP, Russell MC, Avila JG, Cano JA et al. Nondepleting anti-CD40-based therapy prolongs allograft survival in nonhuman primates. Am J Transplant 2012;12(1):126-135.

69. 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.

70. Shapiro AM. Strategies toward single-donor islets of Langerhans transplantation. Curr Opin Organ Transplant 2011;16(6):627-631.

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

72. Hering BJ, Kandaswamy R, Ansite JD, Eckman PM, Nakano M, Sawada T et al. Single-donor, marginal-dose islet transplantation in patients with type 1 diabetes. JAMA 2005;293(7):830-835.

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

74. Matsumoto S, Takita M, Chaussabel D, Noguchi H, Shimoda M, Sugimoto K 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 Transplant 2011.

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

76. Xenos ES, Farney AC, Widmer MB, Casanova D, Stevens RB, Blazar BR et al. Effect of tumor necrosis factor alpha and of the soluble tumor necrosis factor receptor on insulin secretion of isolated islets of Langerhans. Transplant Proc 1992;24(6):2863-2864.

77. 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.

78. Cechin SR, Perez-Alvarez I, Fenjves E, Molano RD, Pileggi A, Berggren PO et al. Anti-Inflammatory Properties of Exenatide in Human Pancreatic Islets. Cell Transplant 2011.

79. Faradji RN, Froud T, Messinger S, Monroy K, Pileggi A, Mineo D et al. Long-term metabolic and hormonal effects of exenatide on islet transplant recipients with allograft dysfunction. Cell Transplant 2009;18(10):1247-1259.

80. Ghofaili KA, Fung M, Ao Z, Meloche M, Shapiro RJ, Warnock GL et al. Effect of exenatide on beta cell function after islet transplantation in type 1 diabetes. Transplantation 2007;83(1):24-28.

APPENDIX C.

AUTOLOGOUS ISLET TRANSPLANTATION AFTER TOTAL PANCREATECTOMY FOR RENAL CELL CARCINOMA METASTASES

C. - Autologous Islet Transplantation after Total Pancreatectomy for Renal Cell Carcinoma Metastases

American Journal of Transplantation

American Journal of Transplantation 2013; XX: 1–5
Wiley Periodicals Inc.

© Copyright 2013 The American Society of Transplantation and the American Society of Transplant Surgeons

doi: 10.1111/ajt.12354

Case Report

Autologous Islet Transplantation After Total Pancreatectomy for Renal Cell Carcinoma Metastases

B. L. Gala-Lopez[1,2], E. Semlacher[3], N. Manouchehri[2], T. Kin[1] and A. M. J. Shapiro[1,2,*]

[1]Clinical Islet Transplant Program, University of Alberta, Alberta, Canada
[2]Department of Surgery, University of Alberta, Alberta, Canada
[3]Department of Medicine, University of Alberta, Alberta, Canada
**Corresponding author: A.M. James Shapiro, amjs@islet.ca*

Pancreatic metastases from renal cell carcinoma (RCC) may have a chronic and highly indolent course, and may be resected for cure after considerable delay following treatment of the primary tumor, in contrast to other more common pancreatic tumors. Surgical resection is the treatment of choice, which may lead to postpancreatectomy diabetes mellitus in the case of extensive resection. We present a 70-year-old patient with multifocal pancreatic metastases from RCC causing obstructive jaundice. A total pancreatectomy was required to excise two distant tumors in the head and tail of the pancreas, together with a segment VI liver resection. An autologous islet transplant (AIT) prepared from the central, uninvolved pancreas was carried out to prevent postpancreatectomy diabetes. The patient was rendered insulin-free and remains so with excellent glycemic control for 1 year of follow-up, and there is no evidence of tumor recurrence. The patient has been treated with adjuvant sunitinib to minimize risk of further recurrence. In conclusion, AIT after pancreatectomy may represent a useful option to treat patients with metastatic RCC. A critical component of this approach was dependent upon elaborate additional testing to exclude contamination of the islet preparation by cancerous cells.

Key words: Islet autotransplantation, renal cell carcinoma, pancreatectomy, pancreatic metastases

Abbreviations: AIT, autologous islet transplantation; CT, computed tomography; IEQ, islet equivalents; RCC, renal cell carcinoma; US, abdominal ultrasound.

Received 25 April 2013, revised 28 May 2013 and accepted 31 May 2013

Introduction

The natural history of renal cell carcinoma (RCC) has a high 5-year survival (up to 95%) when the tumor is limited to the kidney (1,2). In patients with RCC, up to 30% have metastases at presentation, and 40–50% will develop widespread metastatic disease over time (3). The long-term (5-year) survival rate is 10–15% once metastases have spread (1).

Pancreatic metastases are generally uncommon, with an incidence varying from 2% to 5%. However, the pancreas is a target site for metastases from carcinoma of the kidney, and may present typically after a prolonged delay after nephrectomy (1,3,4). Pancreatic metastases from RCC have a high resectability rate compared with other more common pancreatic tumors (3). However, these lesions may be multifocal in 30% of the cases and require more radical resection resulting in ''brittle'' diabetes mellitus, which may be difficult to manage. Autologous islet transplantation (AIT) after near-total or total pancreas resection offers a potential means to preserve endocrine function, provided this does not compromise tumor resection margins (5–8).

Islet autotransplantation was first performed in 1977 at the University of Minnesota, allowing the patient to remain insulin free until his death 6 years later (8,9). Since then, more than 500 AIT have been performed, mainly for patients undergoing total pancreatectomy for chronic pancreatitis (5,9). In such patients, approximately 70% of insulin independence has been achieved at 3 years after AIT if more than 5000 islet equivalents (IEQ) are transplanted (9–11).

Islet autotransplantation has been utilized for a selected series of benign pancreatic diseases, including pseudocysts, cystic neoplasms, insulinomas and neuroendocrine tumors (10,12,13). Balancing the potential benefit of preventing surgical diabetes against the oncologic risk of inadvertently embolizing tumor cells poses an interesting dilemma. We present the case report and metabolic studies of a patient who underwent AIT after total pancreatectomy for multifocal RCC pancreatic metastases.

Note: A version of this section was published as an Original Paper in the *Am J Transplant* 2013 Sept; 13(9): 2487 - 91.

CASE REPORT

Autologous Islet Transplantation after Total Pancreatectomy for Renal Cell Carcinoma Metastases.

Authors: Gala-Lopez, Boris L[1,2], Semlacher, Eric[3], Manouchehri, Namdar[2], Kin, Tatsuya[1] and Shapiro, AM James[1, 2]

Affiliations: [1]Clinical Islet Transplant Program, University of Alberta

[2]Department of Surgery, University of Alberta

[3] Department of Medicine, University of Alberta

Corresponding author contact information:

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

Professor of Surgery, Medicine and Surgical Oncology

Director, Clinical Islet and Living Donor Liver Transplant Programs, University of Alberta

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

tel. (780) 407 7330 fax. (780) 407 8259 Email: amjs@islet.ca

Running title: Islet transplant for pancreatic metastases

C.1. - ABSTRACT

Pancreatic metastases from renal cell carcinoma (RCC) may have a chronic and highly indolent course, and may be resected for cure after considerable delay following treatment of the primary tumor, in contrast to other more common pancreatic tumors. Surgical resection is the treatment of choice, which may lead to post-pancreatectomy diabetes mellitus in the case of extensive resection.

We present a 70-year-old patient with multifocal pancreatic metastases from RCC causing obstructive jaundice. A total pancreatectomy was required to excise two distant tumors in the head and tail of the pancreas, together with a segment VI liver resection. An autologous islet transplant (AIT) prepared from the central, uninvolved pancreas was carried out to prevent post-pancreatectomy diabetes. The patient was rendered insulin-free and remains so with excellent glycemic control for one year of follow-up, and there is no evidence of tumor recurrence. The patient has been treated with adjuvant sunitinib to minimize risk of further recurrence.

In conclusion, AIT after pancreatectomy may represent a useful option to treat patients with metastatic RCC. A critical component of this approach was dependent upon elaborate additional testing to exclude contamination of the islet preparation by cancerous cells.

C.2. - INTRODUCTION

The natural history of renal cell carcinoma (RCC) has a high 5-year survival (up to 95%) when the tumor is limited to the kidney (1, 2). In patients with RCC, up to 30% have metastases at presentation, and 40%-50% will develop widespread metastatic disease over time (3). The long-term (5-year) survival rate is 10%-15% once metastases have spread (1).

Pancreatic metastases are generally uncommon, with an incidence varying from 2 to 5%. However, the pancreas is a target site for metastases from carcinoma of the kidney, and may present typically after a prolonged delay after nephrectomy (1, 3, 4). Pancreatic metastases from RCC have a high resectability rate compared with other more common pancreatic tumors (3). However, these lesions may be multifocal in 30% of the cases and require more radical resection resulting in 'brittle' diabetes mellitus, which may be difficult to manage. Autologous islet transplantation (AIT) after near-total or total pancreas resection offers a potential means to preserve endocrine function, provided this does not compromise tumor resection margins (5-8). Islet autotransplantation was first performed in 1977 at the University of Minnesota, allowing the patient to remain insulin free until his death 6 years later (5, 8). Since then, more than 500 AIT have been performed, mainly for patients undergoing total pancreatectomy for chronic pancreatitis (5). In such patients, approximately 70% of insulin independence has been achieved at 3 years after AIT if more than 5,000 islet equivalents (IEQ) are transplanted (5, 9, 10).

Islet autotransplantation has been utilized for a selected series of benign pancreatic diseases including pseudocysts, cystic neoplasms, insulinomas and neuroendocrine tumors (9, 11, 12). Balancing the potential benefit of preventing surgical diabetes against the oncologic risk of inadvertently embolizing tumor cells poses an interesting dilemma. We present the case report

and metabolic studies of a patient who underwent AIT after total pancreatectomy for multifocal RCC pancreatic metastases.

C.3. - CASE REPORT

A 70-year-old female with history of rheumatoid arthritis had a remote diagnosis of RCC fifteen years prior to admission, treated at that time by left radical nephrectomy. She presented with a two-week history of malaise, pruritus, and obstructive jaundice with elevated transaminase, bilirubin, and alkaline phosphatase. The previous nephrectomy pathology confirmed RCC stage II (T2, N0, M0) resected with negative margins and uninvolved lymph nodes, and no further adjuvant treatment had been given. Physical examination revealed marked jaundice with a palpable Courvoisier's gallbladder. Pancreatic tumor markers were normal (CA 19.9 <1 kU/L and CEA 1.9 ug/L). A pancreatic protocol contrast-enhanced computed tomography (CT, 1.25 mm slice reconstructions) revealed multifocal, hypervascular pancreatic lesions in the uncinate process of the pancreas (32 mm diameter) with distal biliary obstruction (**Figure C.1B**), and in the pancreatic tail (13 mm diameter) (**Figure C.1C**), both with central diminished attenuation. No peri-pancreatic lymphadenopathy was identified. Specifically there was no evidence of tumor or pancreatic ductal dilatation within the central pancreatic body, which appeared to be normal on CT. Within the liver, a 24 mm similar hypervascular lesion was identified within segment 6, highly suspicious for metastatic focus (**Figure C.1A**). The liver lesion was biopsied by fine needle ultrasound-guided approach, and demonstrated clear cells consistent with RCC primary origin. A chest CT revealed no additional metastatic disease, and the pancreatic and liver disease was therefore potentially resectable.

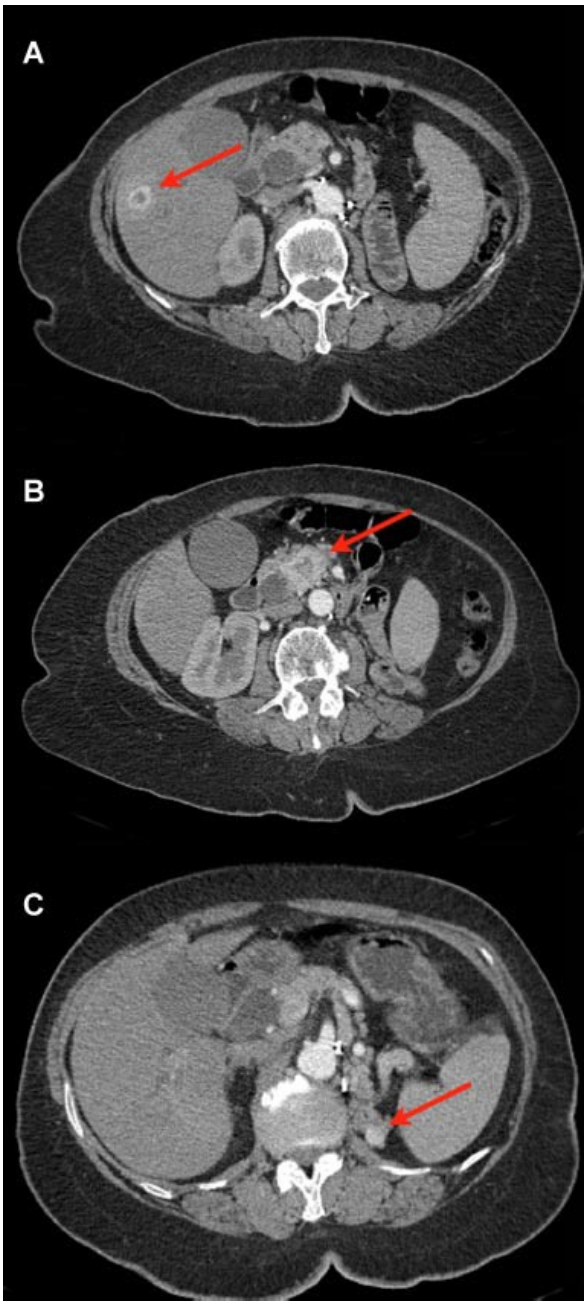


Figure C.1 Computed tomography imaging pre-operatively demonstrating hypervascular metastatic renal cell carcinoma deposits in **A**. Segment 6 liver; **B**. Uncinate process pancreas with biliary obstruction, and **C**. Pancreatic tail.

Laparotomy did not reveal any additional pancreatic, hepatic or peritoneal disease. A high-resolution intra-operative contact ultrasound probe was used to examine the entire pancreas after complete pancreatic mobilization, to rule out occult, additional parenchymal lesions. Based on RCC involvement of the uncinate and tail of the pancreas (**Figure C.2**), with sparing of the central pancreas, we carried out a total pancreatectomy with splenectomy. Intra-operatively, we transected both the pancreatic neck and the distal pancreatic body, and confirmed by frozen-section pathology that both proximal and distal margins on the pancreatic body were uninvolved with RCC. The tumor-free central pancreatic body was then sent for processing for AIT. During this period, we maintained the pancreatic body vasculature intact in order to maximize islet oxygenation and viability (**Figure C.3A**). The central pancreas weighed 38.8g. On the back-table, the pancreatic duct was cannulated (**Figure C.3B**), and the splenic artery was flushed with 1 liter of chilled HTK solution (Custodiol, Methapharm. Brantford, ON).

The islet isolation method was similar to the standard digestion using the Ricordi method as used for allogeneic transplantation (13, 14). Enzymatic digestion used CIzyme Collagenase HA (2,196 Wunsch Units) (VitaCyte, Indianapolis, USA) and CIzyme Thermolysin (828,000 units) (VitaCyte, Indianapolis, USA). After digestion 3.9 g of tissue remained in the chamber.

In this case, islet purification was performed to further minimize the risk of embolization of pancreatic exocrine tissue. It consisted of a combination of Biocoll and University of Wisconsin solution to make a continuous density gradient (15). There were only 1,056 IEQ left in the less pure layer. The high purity (55%) layer yielded 268,195 IEQ (6,192 IEQ/g of pancreas, 4,400 IEQ/kg of recipient's body weight), in a packed cell volume of 2.0 mL. No tissue samples were submitted for pathology analysis.

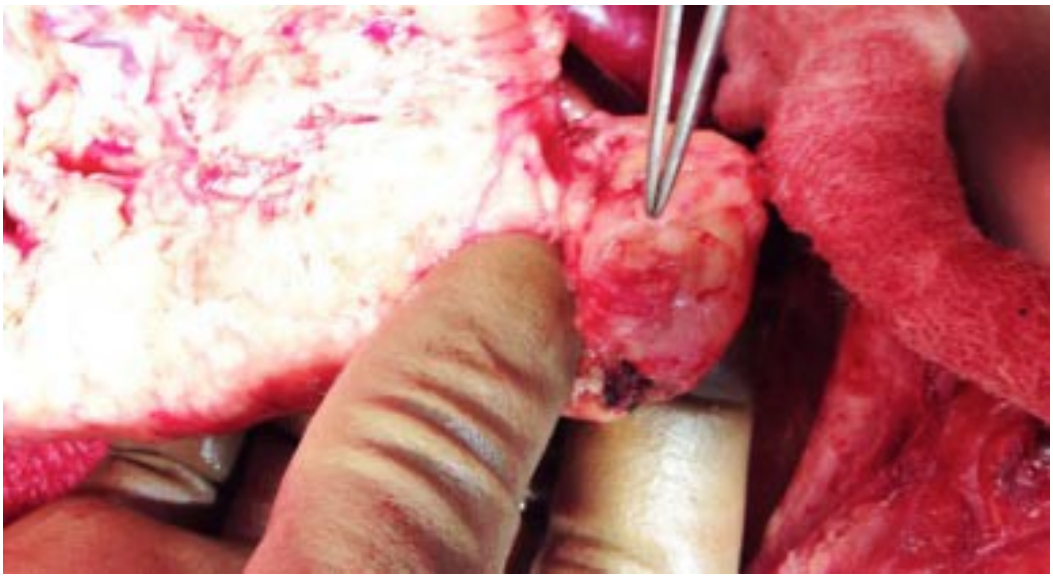


Figure C.2 Intraoperative photograph of renal cell carcinoma in the pancreatic tail.

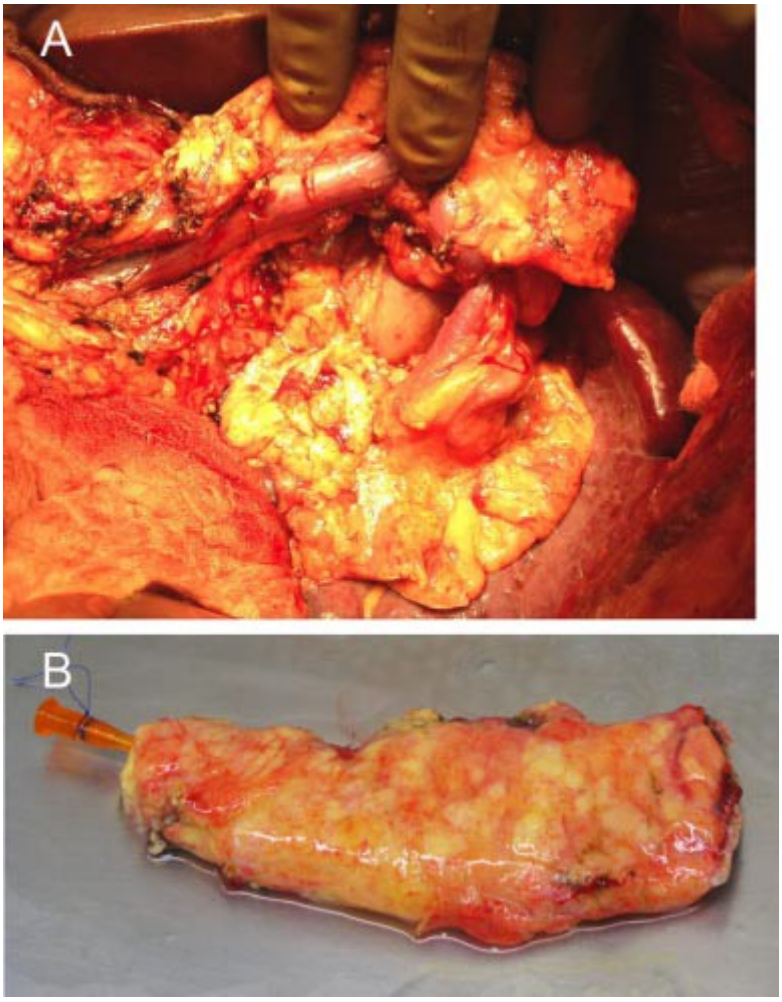


Figure C.3 A. Intraoperative photograph of preserved vascular flow in the central pancreas after both Whipple and distal pancreatic resection, with the goal being to maintain islet oxygenation and viability up till the point of explantation. **B**. Back-table cannulation of the pancreatic duct prior to digestion and islet isolation.

While the islets were being prepared the proximal pancreatectomy was completed with the subsequent reconstruction (hepaticojejunostomy, roux-en-Y pylorus-preserving gastrojejunostomy and entero-enterostomy). The right lobe of the liver was mobilized and an anatomical resection was carried out of the segment 6 lesion. Finally, a 9-French dual lumen catheter (Broviac, Bard Canada Inc., Oakville, ON) was pre-flushed with heparinized saline and secured within the stump of the splenic vein and advanced to the portal confluence to allow infusion of the islet preparation together with simultaneous portal pressure monitoring. The patient tolerated the transplant procedure well and the portal pressure remained within normal range (mean portal pressure 16.6 mmHg) throughout the islet infusion. Heparin (Heparin Sodium Injection, Sandoz Canada. Boucherville, QC) was used systemically (400 U/h IV) and also within the islet infusion bag (70 U/kg). No blood products were required.

Postoperative recovery was entirely uneventful. A Doppler ultrasound on the first postoperative day confirmed portal venous patency. Glycemic control remained within the normal range throughout hospitalization (6-8 mmol/L). The final pathology confirmed RCC metastases with clear cell change within the uncinate, tail and segment 6 liver, all resected with negative margins. The cells were positive for vimentin and CD10, but negative for p53. The patient was discharged on post-operative Day 13 with close follow up every three months. She remains insulin-free with excellent glycemic control (fasting glucose 5.2 mmol/L, c-peptide 0.48 nmol/L, HbA1c 5.3%), and is tumor free at almost one year of follow-up. She has received adjuvant sunitinib (Sutent, Pfizer Canada. Kirkland, QC) chemotherapy to further reduce risk of additional recurrent disease.

C.4. – DISCUSSION

Extended pancreatic resections can provide curative treatment of both primary and metastatic pancreatic tumors. Total pancreatectomy with surgical removal of the entire islet cell mass leads to surgical diabetes that, in the absence of insulin and counter-regulatory glucagon hormonal balance, results in difficult to control diabetes with substantial risk of severe hypoglycemia (5). Although autologous islet transplantation has successfully prevented surgical diabetes in patients with chronic pancreatitis, concern of inadvertent infusion of occult malignant cells in the islet preparation has restricted application of this approach in patients with underlying malignancies (9, 16). A case report of total pancreatectomy with AIT in the setting of pancreatic head adenocarcinoma has been described previously (16).

To our knowledge, the current case represents the first report of AIT after total pancreatectomy for multifocal pancreatic metastases for RCC. The rationale was to preserve quality of life and prevent glycemic lability in a patient with an indolent but malignant metastatic disease, with low risk of inadvertent tumoral embolization to the liver. Since the tumor occurred in two disparate but discrete locations in the uncinate and tail of the pancreas, the option of localized resection was not reasonable. The alternative option of Whipple pancreaticoduodenectomy combined with distal pancreatic resection would have had added risk of pancreatic fistula. Application of AIT allowed preservation of insulin and glucagon secretory reserve and has resulted in prolonged and sustained independence from insulin in a 70-year-old lady that would otherwise have developed 'brittle' diabetes.

The islet isolation performed in this particular case resulted in high islet yield from a non-fibrotic pancreas. Islet yields up to 7,000 IEQs/g are exceptional in autoislet isolation as the main

indication for the procedure is in patients with chronic pancreatitis, but may occur occasionally in islet allotransplantation.

Although all evident foci of metastatic tumoral deposits were radically resected with negative margins at surgery, we cannot rule out with absolute certainty the possibility of malignant cell foci within the final islet preparation. Specific precautions to minimize this risk included a) high resolution pre-operative imaging; b) selection of a metastatic tumor of known biology and very indolent course; c) intraoperative contact high-resolution pancreatic ultrasound to rule out smaller pancreatic lesions in the central pancreas; d) frozen section interpretation of resection margins to be sure the central pancreas was not involved with infiltrative tumor, and e) deliberate application of islet purification steps in the autologous islet preparation process to further minimize islet contamination with exocrine pancreatic elements. Perhaps detection of *VHL* and *PBRM1* mutations within the cell preparation could also have been used to rule out presence of residual RCC cells, but this was not used in this case (17-19).

With one year of follow-up, and combined with adjuvant sunitinib and serial imaging, there has been no evidence of residual or recurrent metastatic disease, and the patient remains insulin independent without risk of hypoglycemia. Since this patient also presented with a liver metastasis, we caution that if she were to develop further liver metastases, we would be unable to discern whether the AIT process would be contributory. We suggest that AIT provides an additional tool in the armamentarium for the pancreatic surgeon dealing with complex but indolent malignant pancreatic lesions. Further cellular screen tools would be critically important to minimize risk of inadvertent embolization of malignant cells.

C.5. – REFERENCES

1. Ballarin R, Spaggiari M, Cautero N, De Ruvo N, Montalti R, Longo C et al. Pancreatic metastases from renal cell carcinoma: the state of the art. World J Gastroenterol 2011;17(43):4747-4756.

2. Cho DC. Therapeutic challenges in advanced renal cell carcinoma. Clin Pract (Lond) 2013;10(1):39-46.

3. Bassi C, Butturini G, Falconi M, Sargenti M, Mantovani W, Pederzoli P. High recurrence rate after atypical resection for pancreatic metastases from renal cell carcinoma. Br J Surg 2003;90(5):555-559.

4. Aguayo-Albasini JL, Garcia-Garcia ML, Martin-Lorenzo JG, Cases-Baldo MJ, Campillo-Soto A, Mengual-Ballester M. Subtotal distal pancreatectomy for metachronous metastatic renal clear cell carcinoma. Rev Esp Enferm Dig 2012;104(11):613-614.

5. Sutherland DE, Radosevich DM, Bellin MD, Hering BJ, Beilman GJ, Dunn TB et al. Total pancreatectomy and islet autotransplantation for chronic pancreatitis. J Am Coll Surg 2012;214(4):409-424; discussion 424-406.

6. Bellin MD, Freeman ML, Schwarzenberg SJ, Dunn TB, Beilman GJ, Vickers SM et al. Quality of life improves for pediatric patients after total pancreatectomy and islet autotransplant for chronic pancreatitis. Clin Gastroenterol Hepatol 2011;9(9):793-799.

7. Brendle TA. Preventing surgically induced diabetes after total pancreatectomy via autologous islet cell reimplantation. AORN J 2010;92(2):169-181; quiz 182-163.

8. Sutherland DE, Gruessner AC, Carlson AM, Blondet JJ, Balamurugan AN, Reigstad KF et al. Islet autotransplant outcomes after total pancreatectomy: a contrast to islet allograft outcomes. Transplantation 2008;86(12):1799-1802.

9. Ris F, Niclauss N, Morel P, Demuylder-Mischler S, Muller Y, Meier R et al. Islet autotransplantation after extended pancreatectomy for focal benign disease of the pancreas. Transplantation 2011;91(8):895-901.

10. Webb MA, Illouz SC, Pollard CA, Gregory R, Mayberry JF, Tordoff SG et al. Islet auto transplantation following total pancreatectomy: a long-term assessment of graft function. Pancreas 2008;37(3):282-287.

11. Dong M, Parsaik AK, Erwin PJ, Farnell MB, Murad MH, Kudva YC. Systematic review and meta-analysis: islet autotransplantation after pancreatectomy for minimizing diabetes. Clin Endocrinol 2011;75(6):771-779.

12. Oberholzer J, Mathe Z, Bucher P, Triponez F, Bosco D, Fournier B et al. Islet autotransplantation after left pancreatectomy for non-enucleable insulinoma. Am J Transplant 2003;3(10):1302-1307.

13. O'Gorman D, Kin T, Imes S, Pawlick R, Senior P, Shapiro AM. Comparison of human islet isolation outcomes using a new mammalian tissue-free enzyme versus collagenase NB-1. Transplantation 2010;90(3):255-259.

14. Kawahara T, Kin T, Shapiro AM. A comparison of islet autotransplantation with allotransplantation and factors elevating acute portal pressure in clinical islet transplantation. J Hepatobiliary Pancreat Sci 2012;19(3):281-288.

15. Barbaro B, Salehi P, Wang Y, Qi M, Gangemi A, Kuechle J et al. Improved human pancreatic islet purification with the refined UIC-UB density gradient. Transplantation 2007;84(9):1200-1203.

16. Forster S, Liu X, Adam U, Schareck WD, Hopt UT. Islet autotransplantation combined with pancreatectomy for treatment of pancreatic adenocarcinoma: a case report. Transplant Proc 2004;36(4):1125-1126.

17. Kapur P, Pena-Llopis S, Christie A, Zhrebker L, Pavia-Jimenez A, Rathmell WK et al. Effects on survival of BAP1 and PBRM1 mutations in sporadic clear-cell renal-cell carcinoma: a retrospective analysis with independent validation. Lancet Oncol 2013;14(2):159-167.

18. Taylor C, Craven RA, Harnden P, Selby PJ, Banks RE. Determination of the consequences of VHL mutations on VHL transcripts in renal cell carcinoma. Int J Oncol 2012.

19. Xu X, Hou Y, Yin X, Bao L, Tang A, Song L et al. Single-cell exome sequencing reveals single-nucleotide mutation characteristics of a kidney tumor. Cell 2012;148(5):886-895.

APPENDIX D.

SUPPORTING EVIDENCE FOR STRUCTURAL AND FUNCTIONAL INTEGRITY OF BMX-010 USED IN CLINICAL TRIALS AT UNIVERSITY OF ALBERTA

D.1. – INTRODUCTION

Manganese (III) meso-tetrakis(N-ethylpyridinium-2-yl)porphyrin (known as MnTE-2-PyP or BMX-010; CASRN 219818-60-7) is a manganese porphyrin drug candidate intended for use as a protective agent for the ex vivo treatment of cells and tissue used for transplantation. As referred in **Chapter 2**, this drug has demonstrated antioxidant capabilities and immunomodulatory effect, when used in islet transplantation (1, 2). To be used in clinical settings, the compound was carefully tested according to Health Canada requirements and an extensive safety assessment was performed by Gad and collaborators (3).

Our experience with BMX-010 in clinical islet transplantation showed no islet toxicity but this trial also failed to show any added benefit in islet *in-vitro* function. In order to guarantee the structural and functional integrity of the specific BMX-010 lot used in our clinical trial, various experimental assessments were performed and are presented here for the reader's consideration.

D.2. – CERTIFICATE OF ANALYSIS

As required by Health Canada and other regulatory agencies, BMX-010 was manufactured in GMP and GLP conditions by a Ricerca Biosciences (Concord, OH, USA), a company subcontracted by BioMimetix for this clinical trial. Upon release of the drug, a quality assurance analysis was performed to demonstrate structural integrity and stability upon exposure to known damaging conditions. **Figure D1.A-C** shows the Certificate of Analysis provided with this specific lot.

Received from Veronica on Apr 15, 2013

CERTIFICATE OF ANALYSIS

(Page 1 of 3)

Mn-TE-2-Pyp, Lot 60220-09-001

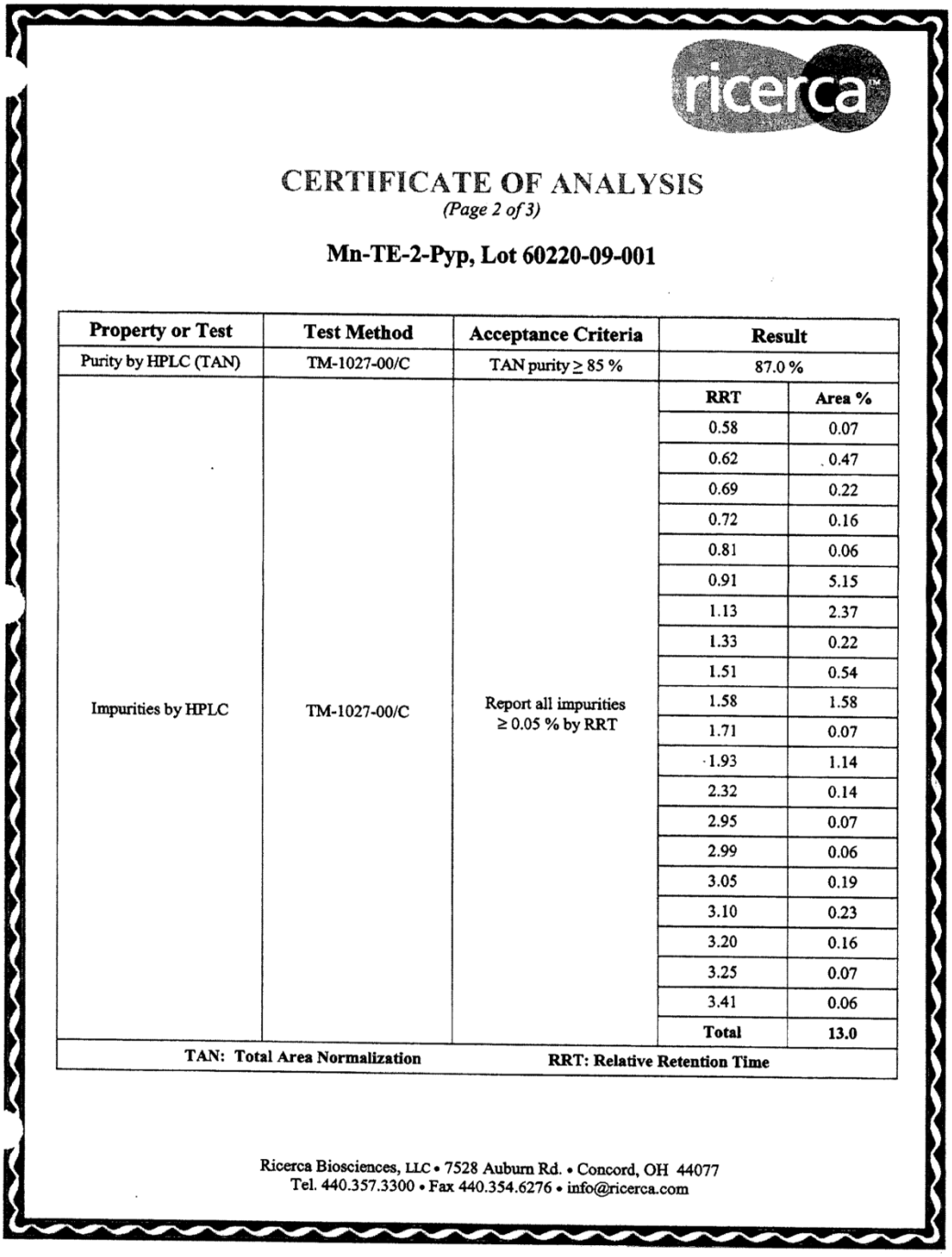
Specification:	S-60220-00	**Production Date:**	17-NOV-2009
Quantity Produced:	129 g	**Release Date:**	05-JAN-2010

Property or Test	Test Method	Acceptance Criteria		Result		
Appearance	TM-0084-04	Report result		Dark brown solid		
Mass spectrum (LC/MS/MS)	TM-0447-02	Consistent with structure		Sample is consistent with structure of the reference material		
Elemental Analysis for % C, H, N	Prevalere Life Sciences	Report results and compare to expected			**Expected [1]**	**Found**
				% C	56.8	52.6
				% H	4.9	5.1
				% N	11.0	10.4
X-ray Diffraction	TM-0355-02 Addendum 78-00/C	Report result		Sample is amorphous		
Identity by FTIR	TM-0346-04 Addendum 375-00/C	Report result		Spectrum in agreement with the reference		
Karl Fischer Moisture	TM-0083-02 Addendum 110-00/C	Report result		4.96 wt %		
Mn by ICP-AES	TM-1063-00/C	Report result		5.29 %		
Residual Solvents by Gas Chromatography	TM-1062-00/C	Solvent	ICH Limit (ppm)	Found (ppm)		
		Acetonitrile	410	< 110		
		Acetone	5000	< 1305		
		2-Propanol	5000	< 1260		
		Methylene chloride	600	ND		
		MTBE	5000	1090		
		DMF	880	ND		
	TM-1199-00/C	Ethanol	5000	< 1300		

[1] Based on empirical formula for pure-form material, corrected for 4.96% moisture.
ND: None Detected

Ricerca Biosciences, LLC • 7528 Auburn Rd. • Concord, OH 44077
Tel. 440.357.3300 • Fax 440.354.6276 • info@ricerca.com

Figure D.1A. Certificate of analysis for BMX-010, lot No. 60220-09-001 provided by Ricerca Biosciences.



CERTIFICATE OF ANALYSIS

(Page 2 of 3)

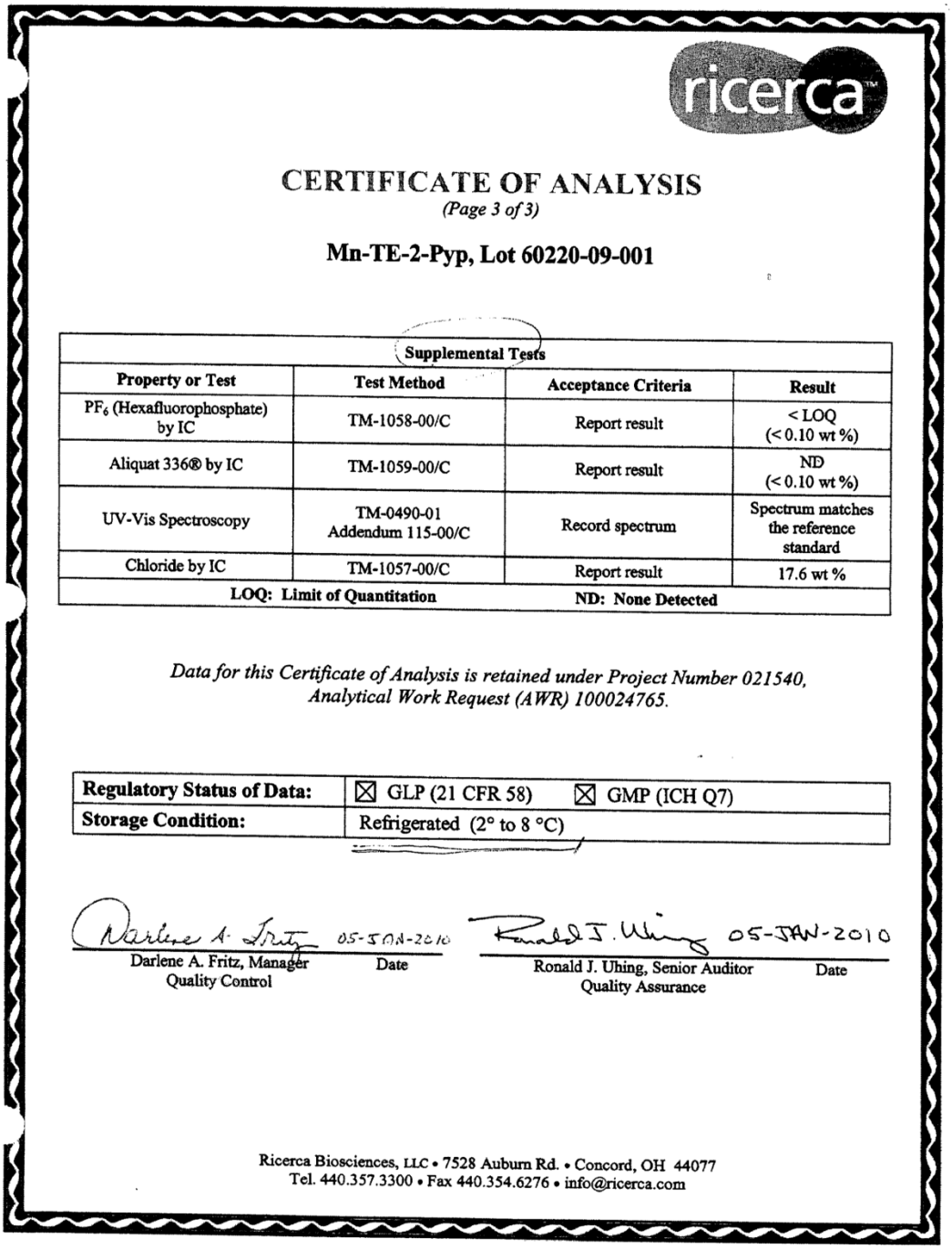
Mn-TE-2-Pyp, Lot 60220-09-001

Property or Test	Test Method	Acceptance Criteria	Result	
Purity by HPLC (TAN)	TM-1027-00/C	TAN purity ≥ 85 %	87.0 %	
Impurities by HPLC	TM-1027-00/C	Report all impurities ≥ 0.05 % by RRT	**RRT**	**Area %**
			0.58	0.07
			0.62	0.47
			0.69	0.22
			0.72	0.16
			0.81	0.06
			0.91	5.15
			1.13	2.37
			1.33	0.22
			1.51	0.54
			1.58	1.58
			1.71	0.07
			1.93	1.14
			2.32	0.14
			2.95	0.07
			2.99	0.06
			3.05	0.19
			3.10	0.23
			3.20	0.16
			3.25	0.07
			3.41	0.06
			Total	**13.0**

TAN: Total Area Normalization **RRT: Relative Retention Time**

Ricerca Biosciences, LLC • 7528 Auburn Rd. • Concord, OH 44077
Tel. 440.357.3300 • Fax 440.354.6276 • info@ricerca.com

Figure D.1B. Certificate of analysis for BMX-010, lot No. 60220-09-001 provided by Ricerca Biosciences. Continued.



CERTIFICATE OF ANALYSIS

(Page 3 of 3)

Mn-TE-2-Pyp, Lot 60220-09-001

Supplemental Tests			
Property or Test	**Test Method**	**Acceptance Criteria**	**Result**
PF_6 (Hexafluorophosphate) by IC	TM-1058-00/C	Report result	< LOQ (< 0.10 wt %)
Aliquat 336® by IC	TM-1059-00/C	Report result	ND (< 0.10 wt %)
UV-Vis Spectroscopy	TM-0490-01 Addendum 115-00/C	Record spectrum	Spectrum matches the reference standard
Chloride by IC	TM-1057-00/C	Report result	17.6 wt %
LOQ: Limit of Quantitation		**ND: None Detected**	

Data for this Certificate of Analysis is retained under Project Number 021540, Analytical Work Request (AWR) 100024765.

Regulatory Status of Data:	☒ GLP (21 CFR 58) ☒ GMP (ICH Q7)
Storage Condition:	Refrigerated (2° to 8 °C)

Darlene A. Fritz, Manager, Quality Control — Date: 05-JAN-2010

Ronald J. Uhing, Senior Auditor, Quality Assurance — Date: 05-JAN-2010

Ricerca Biosciences, LLC • 7528 Auburn Rd. • Concord, OH 44077
Tel. 440.357.3300 • Fax 440.354.6276 • info@ricerca.com

Figure D.1C. Certificate of analysis for BMX-010, lot No. 60220-09-001 provided by Ricerca Biosciences. Continued.

According to this certificate, the product supplied to the Clinical Islet Laboratory at University of Alberta complies with structural and stability standards.

D.3. - ANTIOXIDANT PROPERTIES

To demonstrate functional integrity for this lot of BMX-010, an *in-house* experiment was performed using human islets provided by the Clinical Islet Laboratory from the University of Alberta. The islets were divided in four groups (approximately 100 IEQ each) and cultured for 24h in a CMRL based medium supplemented with or without 34 μmol/L BMX-010 from Lot No. 60220-09-001. Cells were subsequently challenged with 250 μmol/L hydrogen peroxide (H_2O_2) for another 24h followed by measurements of reactive oxygen species in the medium and assessment of cell viability using a membrane integrity stain.

The resulting study groups (in triplicates) were as follow:

1. **Control**: culture medium alone
2. **BMX**: culture medium + BMX-010
3. **H_2O_2**: culture medium + H_2O_2
4. **BMX+H_2O_2**: culture medium + BMX-010 + H_2O_2

Frozen samples from the supernatant of all study groups were assayed for reactive oxygen species released into the culture media, using the Acridan Luminogen PS-3 assay (Amershan ECL Plus kit, Fisher Scientific Inc. Ottawa, ON, Canada) (4). Acridan Luminogen PS-3 is excited by reactive oxygen and nitrogen species in the presence of hydrogen peroxide, producing chemiluminescense at 430 nm. CMRL culture medium alone served as a control, and results were expressed as fold-change increase compared to control.

An increase in the extracellular concentration of ROS was observed in islets exposed to hydrogen peroxide, more significant in the H_2O_2 group compared to Control ($p<0.01$). However, cells pre-treated with BMX-010 released less ROS into the media (**Figure D2.A**)

Cell viability was assessed using a fluorescent membrane integrity assay with counter-stains using SYTO® 13 Green and Ethidium bromide (Life Technologies, Burlington, ON, and Sigma-Aldrich, ON) (5). Control and BMX groups showed a similar viability (70% vs. 67%) after 48h of culture, contrasting with almost no survival in the H_2O_2 group (Control: 70% vs. H2O2: 1.5%, $p<0.0001$). Significant mortality was also observed in islets from the BMX+ H_2O_2 group. However, BMX-010 protected some of these islets against loss of viability, compared to the H_2O_2 group (39.6% vs. 1.5%, $p<0.001$) (**Figure D2.B**).

D.4. - IMMUNOMODULATORY EFFECT

To evaluate the immunomodulatory effect of this BMX-010 lot, Piganelli and collaborators at the Department of Immunology, University of Pittsburgh performed an *in vitro* experiment to evaluate the effects of this lot of BMX-010 on interferon gamma (IFN-γ) expression from pre-stimulated murine spleen cells. Splenocytes from OT II mice (C57BL/6-Tg(TcraTcrb)425Cbn/Crl) (Charles River, Boothwyn, PA, USA) were isolated and cultured at a concentration of $2x10^6$ cells/mL for 24h, with or without BMX-010

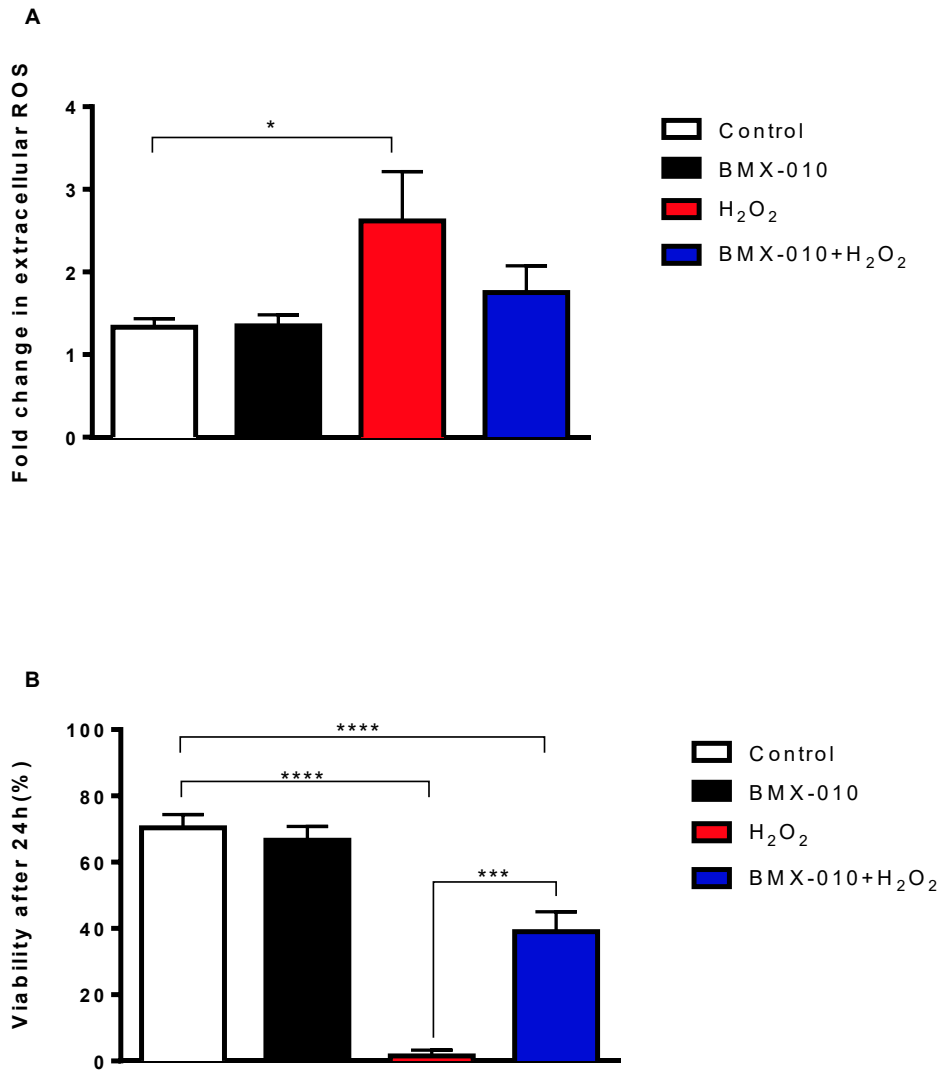


Figure D.2. In vitro assessment of human islets pre-treated with BMX-010 and exposed to hydrogen peroxide (H_2O_2). (**A**) Extracellular concentration of reactive oxygen species (ROS) after exposure to H_2O_2 for 24h. Results are expressed as a fold-change concentration relative to CMRL medium. (**B**). Cell viability after exposure to H_2O_2 for 24h measured by fluorescent membrane integrity assay, using counterstain with Syto Green and Ethidium Bromide. Data represented as means ± s.e.m. from triplicates samples.

(MnP, 34 and 68 μmol/L), and subsequently stimulated with Concanavalin A (ConA, 2.5 μmol/L) or Ovalbumin (Ova, 0.5 μmol/L) (Sigma-Aldrich Corp. St. Louis, MO, USA) for 48h and 72h. After the corresponding stimulation period, the supernatants were harvested and frozen at -80°C. Interferon gamma was measured in triplicate samples using a mouse-specific IFN-gamma ELISA assay (Thermo Fisher Scientific, Waltham, MA, USA) (range: 37 – 3000 pg/mL). Stimulation with ConA resulted in high titers of IFN-γ in the Control group, more evident at 72h of exposure (212.3 ng/mL). A lower concentration of IFN-γ was measured in the media of cells pre-treated with BMX-010, with a 41% (34 μmol/L) and 41.7% (68 μmol/L) relative inhibition at 48h ($p<0.05$); and 45% (34 μmol/L) and 57% (68 μmol/L) relative inhibition at 72h ($p<0.01$). Stimulation with Ova elicited lesser expression of IFN-γ, more evident at 72h (103.7ng/mL). No significant effect of BMX pre-treatment was observed at 48h. However, there was significant inhibition at 72h (65.7% for both BMX concentrations), compared to Control ($p<0.01$) (**Figure D3**).

D.5. - CONCLUSIONS

The experimental findings presented in this Appendix D support the structural and functional integrity of the lot of BMX-010 used in clinical trials at University of Alberta. This evidence is consistent with our hypothesis of lack of substantial benefit in islet isolation due to suboptimal dose.

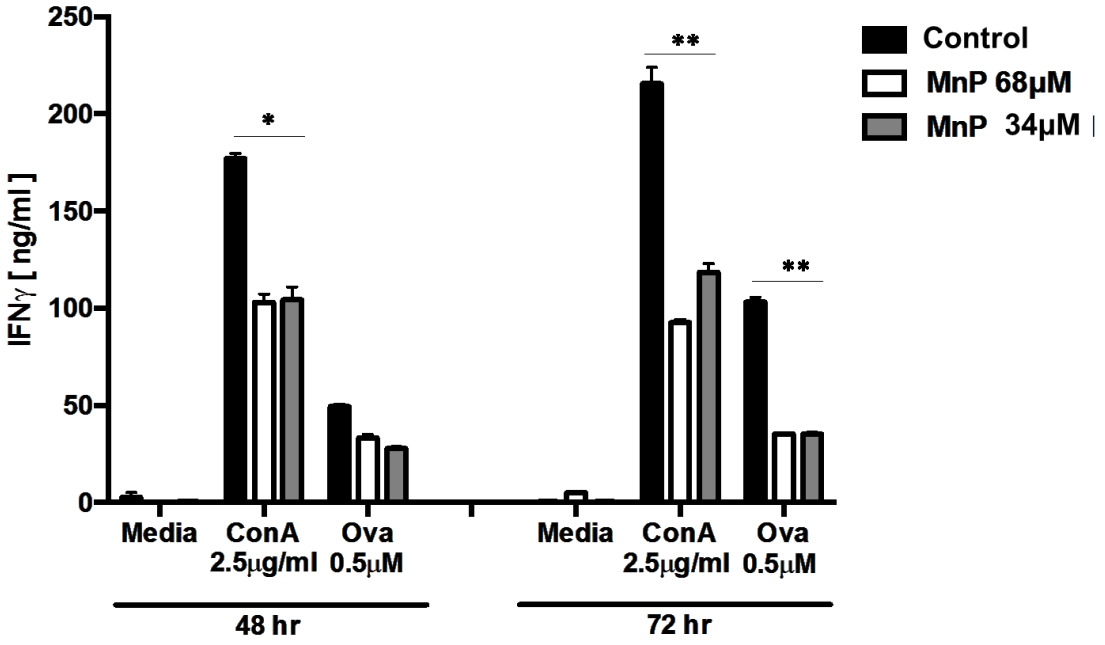


Figure D3. Effects of this lot of BMX-010 on interferon gamma (IFN-γ) expression from pre-stimulated murine spleen cells. Splenocytes from OT II mice were pre-treated with 34 and 68 µmol/L of BMX-010 and subsequently exposed to Concanavalin A (ConA, Ovalbumin (Ova) for 48 and 72h. IFN-γ was measured in the supernatant. Date represented as means ± s.e.m. from triplicates samples.

D.6. - REFERENCES

1. 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.

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

3. Gad SC, Sullivan DW, Jr., Crapo JD, Spainhour CB. A nonclinical safety assessment of MnTE-2-PyP, a manganese porphyrin. Int J toxicol 2013;32(4):274-287.

4. 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.

5. Ranuncoli A, Cautero N, Ricordi C, Masetti M, Molano RD, Inverardi L et al. Islet cell transplantation: in vivo and in vitro functional assessment of nonhuman primate pancreatic islets. Cell Transplant 2000;9(3):409-414.