Clinical and Translational Studies Advancing Clinical Implementation of Stem Cell-Derived Islet Transplantation

by

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Doctor of Philosophy

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Abstract

Twenty-years ago Shapiro et al. revolutionized islet transplantation (ITx), realizing the potential for a cell-based diabetes treatment. Despite improvements, ITx remains restricted by organ donor limitations and immunosuppression. Stem cell (SC)-derived ITx (SC Tx) could eliminate these limitations, with potential for unlimited supply and immunosuppression free ITx. This thesis presents results from several translational and clinical projects aimed at enabling in-human implementation and evaluation of SC ITx.

Chapter 1.1 provides an updated review on ITx including recent advancements, regulations, and future therapies including regulatory T-cells and immune reset. Subsection 1.2 reviews SC ITx as a potential cure for diabetes, outlining key barriers including 1) optimization of SC islet products, 2) scalability, 3) immunologic considerations, and 4) obstacles for clinical trials. The remaining chapters of this thesis approach each of these barriers.

Chapter two focuses on scalability of SC ITx, with subsection 2.1 reviewing potential solutions including automation and three-dimensional (3D) culture systems. We then present a preclinical study comparing 3D and two-dimensional iPSC culture evaluating cell expansion, pluripotency phenotype, and differentiation capacity. Results demonstrate that iPSCs grown in 3D culture achieve 93.8-fold expansion and characterization of cells demonstrates that 3D expanded cells acquire a preferable naïve phenotype. In keeping with this naïve phenotype, transplanted 3D cells produce comparatively more mature teratomas with fewer proliferating graft cells. In summary, 3D culture enables increased iPSC expansion with enhanced *in vitro* and *in vivo* cell quality, resulting in efficient cell production suitable for clinical implementation.

Chapter three focuses on techniques and protocols to efficiently generate SC islets free from off-target populations. Subsection 3.1 provides a review of all current protocols used to direct embryological differentiation of SC islets. This is followed by a preclinical study comparing 32 different protocols to generate and characterize induced pluripotent SC islets. Additionally, this study provides graft evaluation following transplantation of fully differentiated SC islets, rather than pancreatic progenitors, demonstrating that despite further *in vitro* maturation ductal off-target populations persist. Finally, we provide a yield and cost assessment and demonstrate that our optimized protocol can be translated into scalable suspension culture within Vertical-Wheel® bioreactors. This represents the first study to date reporting differentiation within Vertical-Wheel® bioreactors, achieving >10x more islet cells than planar protocols.

Chapter four begins with a review of immunologic considerations for ITx, followed by a preclinical study that evaluate ABH antigen expression of islets and SC islets to assess the potential for ABO-incompatible ITx. In this study, characterization of isolated islets demonstrate that neither ductal tissues or endocrine subpopulations express ABH antigens. Unfortunately, contaminant acinar tissue within islet isolations do express ABH antigens, suggesting that ITx should remain ABO-matched. However, embryonic SC-derived pancreatic progenitors and resultant SC islets do not express ABH antigens, introducing the potential for ABO-incompatible transplantation using SC islets. Such data is of particular relevance to Vertex SC products, which are currently limited to A/AB recipients due to a blood type A starting stem cell product.

Chapter five focuses on current barriers facing SC ITx clinical trials, including uncertainty regarding the optimal transplant site and the need for patient-centered outcome measures. First, this chapter provides a review on humanized mouse models and their utility and limitation for SC ITx immunogenic evaluation. While improved, the unidimensional immune recapitulation of

these models limit their utility, leading us to suggest that evaluation of SC ITx requires in-human trials. Subsection 5.2 presents a clinical study comparing outcomes following intraportal and extrahepatic ITx to appraise the potential for extrahepatic transplant sites to enable in-human SC ITx evaluation. Results demonstrate that despite preclinical success of extrahepatic ITx within the omentum, gastric submucosa, and prevascularized subcutaneous space, in-human implementation achieves negligible islet engraftment compared to the intraportal site. Subsection 5.3 details a second clinical study of patients receiving ITx to define optimal C-peptide, stimulated C-peptide, and BETA-2 cut-offs associated with patient-centered outcomes. This data offers target thresholds to strive for with SC islet products in order to achieve hypoglycemia freedom and insulin independence. Subsection 5.4 provides a review evaluating the potential of expanding SC ITx for patients with type 2 diabetes subtypes, hypothesizing methods to expand SC ITx.

Finally, chapter 6 summarizes findings from this thesis and provides insight into areas for future preclinical and clinical work.

Preface

Dear Reader,

This thesis entitled "Clinical and Translational Studies Advancing Clinical Implementation of Stem Cell-Derived Islet Transplantation" is submitted in partial fulfillment of the requirement for the degree of Doctor of Philosophy in Surgery in the Department of Surgery at the University of Alberta. The work presented herein provides advances to combat barriers to stem cell-derived islet transplantation (SC ITx) including scalability, optimization of SC islet generation, immunogenic considerations, and findings to enable clinical implementation and evaluation. This thesis is divided in chapters containing subsections with preclinical and clinical research, in which the author held a leading role within a collaborative and interdisciplinary team. These chapter subsections are presented as a paper-based format, from manuscripts that are either published (n = 11) or submitted for publication (n = 1).

Chapter 1 is titled "Update on Islet Transplantation and Stem Cell-Derived Islet Transplantation" and provides two subsections including an introduction to current practices, advances, and limitations to deceased donor islet transplantation, and a review of SC ITx, its promise, and barriers that it faces limiting clinical implementation. **Chapter 1 subsection 1** is titled "Update on islet cell transplantation" and reviews the current outcomes from ITx, the impact that anti-inflammatory agents have had on improving outcomes, the potential of immunomodulatory agents like regulatory T-cells, and the current regulatory environment for islets. This review is published in *Current Opinion in Organ Transplantation* (Verhoeff, K; Marfil-Garza, B.A; Shapiro, A.M.J. Update on islet cell transplantation. *Current Opinion in Organ Transplantation*. August 2021, 26(4), 397-404. DOI: 10.1097/MOT.0000000000000891). For this publication, I performed the bibliographical review, prepared the figures and legends,

and wrote the manuscript. AMJS and BMG provided revisions to the final manuscript. **Chapter 1 subsection 2** introduces SC ITx including a comparison of personalized iPSC ITx and allogeneic embryonic SC ITx in a review and highlights the key barriers to SC ITx including scalability, optimization of SC islet generation to eliminate off-target growth, immunogenic considerations, and clinical implementation and evaluation as a frame work for the remaining studies in this thesis. The review has been published in *Cells* and I performed the bibliographical review, prepared the figures and legends, and wrote the manuscript. SJH assisted with figure creation and revisions to the final manuscript, AMJS and BMG provided revisions to the final manuscript. (Verhoeff, K; Henschke, S.J; Marfil-Garza, B.A; Dadheech, N; Shapiro, A.M.J. Inducible Pluripotent Stem Cells as a Potential Cure for Diabetes. *Cells*. January 2021, 10(2), 278. DOI: https://doi.org/10.3390/cells10020278).

Chapter 2 is titled "Optimizing Scalability of Stem Cell-Derived Islet Transplantation" and includes one review manuscript and one preclinical study both focused on improving the scalability of SC ITx. **Chapter 2 subsection 1** provides a review titled "Scaling Stem Cells to cure Millions of Patients with Diabetes: Approaches, Technology, and Future Directions" that reviews current techniques to massively produce and expand stem cells and their ensuing islet product. It reviews the use of artificial intelligence for selecting ideal stem cells, suspension culture and bioreactors to grow and expand stem cells, and the future of engineered approaches to automate and scale such processes. This review is published as a chapter within the book "Handbook of Stem Cells: From Basic to Clinical Sciences" as a first author publication (Verhoeff, K; Shapiro, A.M.J. Scaling Stem Cells to Cure Millions of Patients with Diabetes – Approaches, Technology, and Future Directions. Handbook of Stem Cells: From Basic to Clinical Sciences). I performed the bibliographical review, prepared the figures and legends, and

wrote the manuscript and AMJS provided revisions to the final manuscript. **Chapter 2 subsection 2** provides a preclinical study titled "Suspension culture improves iPSC expansion and pluripotency phenotype", whereby we compare expansion, pluripotency phenotype, and trilineage differentiation capacity of iPSCs cultured two-dimensions (2D) and in three-dimensional (3D) suspension culture using Vertical-Wheel® bioreactors. Results demonstrate that 3D expanded iPSCs have superior naïve pluripotency phenotype and have improved capacity for trilineage differentiation, providing an optimal stem cell starting product for islet generation. This study is presented as a co-first author manuscript is published in *Stem Cell Research and Therapy* (*Cuesta-Gomez, N; *Verhoeff, K; Dadheech, N; Jasra, I.T; Bermudez de Leon, M; Pawlick, R; Marfil-Garza, B; Zapata-Morin, P.A; Jickling, G; Thiesen, A; Shapiro, A.M.J. Suspension culture improves iPSC expansion and pluripotency phenotype. *Stem Cell Research and Therapy*). For this co-first author publication I completed 90% of the immunohistochemistry, all *in vitro* trilineage differentiation, 25% of cell culture, generated all embryoid bodies, and completed 50% of manuscript writing and editing. NCG performed all remaining experiments, 75% of figure creation, experimental methodology, and 50% of manuscript writing and editing, ND, IJ, MBdL RP, BMG, PAZM GJ, AT, provided revisions to the final manuscript. AMJS provided study conceptualization, supervision of work, revisions to the final manuscript.

Chapter 3 is titled "Evaluation of Techniques for Efficient, Safe, and Reliable Stem Cell-Derived Islet Generation" and is composed of two subsections. **Chapter 3 subsection 1** presents a review of current SC islet differentiation protocols. In it, each stage of differentiation is reviewed to evaluate differentiation protocol duration, growth factors, and areas for future study and hypothesizes an optimal islet differentiation protocol. This review is presented as a first

author review manuscript (Verhoeff, K; Cuesta-Gomez, N; Jasra, I; Marfil-Garza, B; Dadheech, N; Shapiro, A.M.J. Optimizing Stem Cell-Derived Islet Cells. Stem Cell Reviews and Reports. May 2022. DOI: 10.1007/s12015-022-10391-3) whereby I performed the bibliographical review, prepared the figures and legends, and wrote the manuscript and remaining authors provided revisions to the final manuscript. **Chapter 3 subsection 2** then provides a preclinical study evaluating 32 different iPSC islet differentiation protocols to generate an optimized six-stage protocol. iPSC islet cells from this protocol characterized in terms of their proteomics and transcriptomics at each stage of differentiation, to enable future protocol optimization and comparisons between currently published protocols. Following Stage 6 iPSC islet generation, we evaluated the *in vitro* glucose stimulated insulin secretion, electrophysiological parameters, and oxygen consumption of the generates islet-like cells. Following 16-weeks of *in vivo* maturation, the iPSC islet grafts are evaluated using immunohistochemistry. This study also reports protocol yield, translating the protocol to suspension culture in Vertical-Wheel® bioreactors to significantly increase the end product yield. This manuscript is under consideration and represents a co-first authorship study (Verhoeff, K*; Cuesta-Gomez, N*; Maghera, J; Dadheech, N; Pawlick, R; Smith, N; O'Gorman, D; Razavy, H; Marfil-Garza, B; Young, LG; MacDonald, PE; Shapiro, AMJ. Cell characterization, graft evaluation, and yield of islet-like cells differentiated from patient-derived iPSCs). My contribution to this work included the original manuscript draft, 50% of study conceptualization, and all *in vitro* work except 25% of transcriptomic assays (completed by NCG), electrophysiology (completed by JM), oxygen consumption assays (completed by NS). NCG completed 50% of study conceptualization and 50% of manuscript editing. RP and BMG completed 15% of *in vivo* work. ND, DO, LGY, PEM, and AMJS contributed to manuscript conception and editing and AMJS supervised the study.

Chapter 4 is titled "Immune Considerations for Pancreatic and Stem Cell-Derived Islet Transplantation" and also includes two subsections. In **Chapter 4 subsection 1**, we provide a review of the immune considerations for SC ITx, including comparing and contrasting alloimmune and autoimmune destruction facing islets after transplant. In the review, we evaluate strategies that may be helpful to combat these immune effects including regulatory T-cell therapies and immune reset. We discuss the potential of those approaches to combat both alloimmune and recurrent autoimmune destruction of SC-islets if applied together. This subsection has been published as a book chapter in Translational Autoimmunity Volume 5 (Verhoeff, K; Shapiro, A.M.J. The Potential of Cellular Transplantation to Harness Autoimmunity and Reverse Clinical Diabetes. Translational Autoimmunity Vol. 5 Challenges for Autoimmune Diseases. Chapter 18. Pages 361-385.). **Chapter 4 subsection 2** then provides a preclinical study evaluating the expression of ABH antigens on human pancreatic tissues, isolated islets, and embryonic SC islets to evaluate the potential of ABO-incompatible ITx. The study demonstrates that within human pancreata, endocrine and ductal tissues do not express ABH antigens, while exocrine tissue does. Unfortunately, isolated islets continued to contain substantial exocrine tissue, leading us to conclude that ITx should remain ABO-matched. However, SC islets did not have contaminant exocrine tissue and did not express ABH antigens before or after transplant into the renal subcapsular space of mice, leading us to conclude that SC ITx could potentially be ABO-incompatible. These results are of particular interest to SC ITx considering that the current Vertex clinical trial product remains limited to those with blood types A and AB, due to the original SC donor being blood type A. The study is published in Transplantation (Verhoeff, K; Cuesta-Gomez, N; Albers, P; Pawlick, R; Marfil-Garza, B.A; Jasra, I; Dadheech, N; O'Gorman, D; Kin, T; Halpin, A; West, LJ; Shapiro, A.M.J. Evaluating

the Potential for ABO-Incompatible Islet Transplantation: Expression of ABH Antigens on Human Pancreata, Isolated Islets, and Embryonic Stem Cell-Derived Islets. *Transplantation*. October 2022. DOI: 10.1097/TP.0000000000004347). My roles including all *in vitro* experimental work and manuscript writing, while PA, RP, completed *in vivo* work, and NCG, BMG, IJ, ND, DO, TK, AH, LJW, AMJS provided study conceptualization and manuscript editing.

Chapter 5 is titled "Strategies for Implementation, Evaluation, and Further Optimization of Stem Cell-Derived Islet Transplantation" and contains four subsections including two clinical studies and two reviews. **Chapter 5 subsection 1** is a review of humanized mouse models to contextualize their utility for SC ITx evaluation. It has been published in Advances in Experimental Medicine and Biology and my role as the first author was to complete the bibliographical review, prepare the figures and legends, and write the manuscript. BMG assisted with figure creation, and BMG, NCG, IJ, ND, and AMJS provided revisions to the final manuscript. (Verhoeff, K; Marfil-Garza, B.A; Cuesta-Gomez, N; Jasra, I; Dadheech, N; Shapiro, A.M.J. Current Status, Barriers, and Future Directions for Humanized Mouse Models to Evaluate Stem Cell Based Islet Cell Transplant. *Advances in Experimental Medicine and Biology: Cell Biology and Translational Medicine*. March 2022. DOI: 10.1007/5584). **Chapter 5 subsection 2** is a clinical study evaluating outcomes from ITx recipients comparing those receiving intraportal infusions to extrahepatic transplant sites including the gastric submucosa, prevascularized subcutaneous space, and omentum. The study demonstrates that, despite promising preclinical data, extrahepatic sites fail to achieve islet engraftment and C-peptide production in human clinical trials. The study has been published and as co-first author I was involved in data collection, analysis, 50% of manuscript drafting and editing GS, DC, KD, DB, BA, AL, PS,

AMJS were involved in patient care. DO'G and TK performed the islet isolations. All co-authors provided revisions. AMJS provided final edits and revisions as the senior corresponding author (Verhoeff, K*; Marfil-Garza, B.A*; Sandha, G; Cooper, D; Dajani, K; Bigam, D.L; Anderson, B; Kin, T; Lam, A; O'Gorman, D; Senior, PA; Ricordi, C; Shapiro, AMJ. Outcomes Following Extrahepatic and Intraportal Pancreatic Islet Transplantation: A Comparative Cohort Study. *Transplantation*. May 2022. DOI: 10.1097/TP.0000000000004180). Subsequently, **Chapter 5 subsection 3** provides a second clinical study evaluating recipients of ITx in Edmonton over the last 20 years; specifically, the study evaluates the median C-peptide levels and optimal C-peptide cut-offs in patients with insulin independence, without insulin independence but with hypoglycemia freedom, and those with persistent hypoglycemia. These cut-offs will provide important benchmarks to target and improve evaluation of current SC ITx clinical trials. The manuscript is published as a first author publication (Verhoeff, K; Marfil-Garza, B.A; Dajani, K; Anderson, B; Bigam, D.L; Kin, T; Lam, A; O'Gorman, D; Senior, P.A; Shapiro, A.M.J. C-peptide Targets and Patient-Centered Outcomes of Relevance to Cellular Transplantation for Diabetes. *Transplantation*. October 18 2022. DOI: 10.1097/TP.0000000000004328). As first author I collected and analyzed data, created figures, and wrote the original manuscript draft; BMG assisted with data collection, and all authors contributed to manuscript edits. Finally, **Chapter 5 subsection 4** offers a review that discusses updated diabetes subclassifications. Using those diabetes subtypes, we provide a review that hypothesizes who, including a subset of patients with type 2 diabetes, may benefit from SC ITx. This review is published in the Journal of Clinical Endocrinology and Metabolism (Verhoeff, K; Marfil-Garza, B; Prus- Czarnechka, Z; Cuesta-Gomez, N; Jasra, I.T; Dadheech, N; Senior, P.A; Shapiro, A.M.J. Stem Cell-Derived Islet Transplantation in Patients with Type 2 Diabetes: Can Diabetes Subtypes Guide

Implementation? *Journal of Clinical Endocrinology and Metabolism*. May 2023. DOI: 10.1210/clinem/dgad257).

Finally, **Chapter 6** provides insight from the collected studies to discuss preclinical and clinical studies required to move SC ITx forward clinically. From this experience, I provide views on key preclinical questions including islet encapsulation, SC islet off-target elimination, and the scalability of SC ITx. I also discuss key questions that remain for SC ITx clinically, specifically highlighting the need to evaluate immune destruction of islets for autologous iPSC ITx versus embryonic allogeneic SC ITx, enrollment in clinical trials, and approaches to broaden recipient pools.

Dedication

I want to dedicate this thesis to my family. This includes my parents, in laws, siblings, friends, and of course my wife Amy. Your support and understanding is what makes this all possible.

Specifically to Amy, I'm not sure how you manage to work so hard and support me at the same time. Your ability to work around our combined ridiculous schedules and always make sure there's time to see family, smile, and have fun is one of many things I love about you. People ask how did you manage to accomplish "X" or "Y", while people that actually know me just ask how I got lucky enough to be with you. They couldn't be more correct.

A last note to our future children. If you manage to find this book on a shelf somewhere don't bother reading beyond this page. Everything in this thesis will probably be outdated by that time anyways. Instead take just one thing away: Whatever you want to do in life, no matter how big or small, **work hard and trust the process**. Don't just work hard in comparison to others or to impress people, do it to challenge yourself, to improve, and most importantly to fail and succeed. Set your own bar and find your own success.

Acknowledgements

I would also like to acknowledge and thank all the individuals and institutions that have supported me before, during, and who I hope to continue learning from in the future.

Dr. Shapiro, an entire chapter could be written about all of the ways you have supported, mentored, and driven my work before and during this PhD. I will attempt to keep this brief, but your work ethic, kindness, and innovation are an inspiration that I hope to emulate. To me, mentoring involves guiding trainees or mentees through a process of self-improvement leading to the success of those mentees. As such, I believe one of the best measures of mentorship is the success of those mentees. Dr. Shapiro's mentorship has a unique ability to bridge the gap between where the trainee sees their success and an often higher goal for the trainee seen by Dr. Shapiro. Dr. Shapiro has an uncanny ability to see the best possible outcome for trainees and to build a pathway for the trainee to achieve that success. That positivity and vision of success for others around him is clear and I have never heard Dr. Shapiro say something negative about another person. Dr. Shapiro bridges the gap and provides mentorship not only with positivity support when needed but guidance and redirection at timely moments. His ability to open a mentees eyes to the possibilities and build a path to that success is something I hope to emulate in the future. Looking back at the trainees that you have mentored, those trainees have gone on to achieve local, national, and international success with numerous world leaders having their roots being trained in your lab. That list includes transplant surgeons from across Canada, leaders in islet cell transplant, those in industry and an amazing list of career successes across broad areas that I believe originate with Dr. Shapiro's mentorship. I hope to continue working and learning from you for many years, and to emulate the work ethic and passion you instill into your clinical practice and research.

Next, I want to thank my graduate committee Dr. Korbutt, Dr. Dajani, and Dr. Bigam. At different times you have all provided insightful thoughts and supported me throughout this endeavor. Dr. Bigam, the big red chair in your office is a place of legend, where innumerable trainees and surgeons have figured out the future of their careers guided by your thoughtful insight. I'm sure I'll land in that chair several more times in my life and equally sure the advice I'll be given will be exactly what I need to hear. Dr. Dajani, your openness and availability to talk at any time over a Keurig coffee in your office will always be appreciated and I hope to lean on your wisdom for years to come.

I also want to thank my external reviewers, Dr. McMullen (candidacy), and Dr. Odorico (defense). Your willingness to book time off and schedule my exams despite being given little scheduling availability helped make these exams possible. Your feedback and insight both in terms of my career and research has also been tremendous.

I also want to sincerely thank all of the members of the Shapiro lab, Rena, Nerea, Braulio, Ila, Haide, and Nidheesh, without whom this work would not have been possible. Rena, your experience and willingness to help, often with very little personal benefit or recognition contributes to so much of the success from the lab. Like Dr. Shapiro, your skills have been passed down through numerous trainees through the lab and are consistently documented in acknowledgements like these, thank you. Nerea, I'm not sure how many times you heard "I have a question" from me, and were happily and willingly available to help; of course, the number of times you told me that you wouldn't help because I had to struggle through it to understand is probably equally as high but also equally as important. You have an innate ability to mentor, teach, and be a leader and you are a terrific scientist; I look forward to seeing you as a PI, hopefully at the University of Alberta. Braulio, you are thoughtful, kind, and you care

tremendously about what you do. Your insight and focus on detail helped kick start my PhD work and I hope our paths will cross again. Ila, Haide, and Nidheesh, your support and positivity helped every day in the lab and the guidance you provided in all of our work will always be valued.

Outside of the Shapiro Stem Cell Diabetes lab, but still always around to help I want to thank the clinical lab and research team including Doug, Tatsuya, Feiyue, Indri, Rayna, Dayne and Rosemary. Doug and Tatsuya, you're always available and extremely helpful running any last minute perifusion samples and half the time would essentially run the machine for me. Feiyue, Indri, Rayna, Dayne, and Rosemary your availability and willingness to help were always just an email away, thank you. Similarly, to all of my collaborators including Dr. MacDonald, Dr. Pepper, Dr. Kallos, Jasmine Maghera, and Tiffany Dang, your help with protocols, data analysis, and completing this work has been invaluable.

I also want to thank the University of Alberta's Department of Surgery including Dr. Berry and Tracey. I was in your office several times a month with paper work, looking for advice and was always greeted with help and quick answers. Dr. Berry, I also thank you for helping chair my candidacy and for navigating/supporting my transition to a PhD program. Tracey, I think you must be in my top 5 most emailed people these last two years and you were always available and always extremely helpful. You do so much for the Department's research commitment and most of your work goes under-recognized.

Finally I want to sincerely thank my funders and those of the Shapiro lab, who have played a substantial role in all of the completed work. This includes the Clinician Investigator's Program, University of Alberta, Faculty of Medicine and Dentistry, Department of Surgery, Alberta Diabetes Institute, the province of Alberta, Juvenile Diabetes Research Foundation of

Canada, the Canadian Stem Cell Network, the Diabetes Research Institute Foundation of Canada, the Canadian Institutes of Heath Research, and the Canadian Research Council.

Table of Contents

List of Figures

List of Tables

Chapter 1: Update on Islet Transplantation and Stem Cell-Derived Islet Transplantation

- Subsection 1.1: Update on islet cell transplantation
- Subsection 1.2: Induced Pluripotent Stem Cells as a Potential Cure for Diabetes

Chapter Summary

The first chapter of this thesis serves as an introduction to both islet transplantation (chapter 1.1) and stem cell-derived islet transplantation (chapter 1.2). The chapter's first subsection offers a review summarizing the last twenty years of advancement in clinical islet transplantation, most notably highlighting the success of anti-inflammatories but also continued barriers including the limited organ supply, immune destruction, and legislation. This is followed by a second review focused on stem cell-derived islet transplantation as a potential solution to those barriers, while also highlighting remaining questions for the field including discussing both the allogeneic and autologous approaches, off-target risks, the scalability of stem cell differentiation processes, and challenges to implementation. These limitations to the stem cell field provide the impetus for this thesis' structure, with each subsequent chapter focused on the aforementioned barriers.

1.1 Chapter 1 subsection 1 – Update on islet cell transplantation

REVIEW

Update on islet cell transplantation

Kevin Verhoeff[a], *Braulio A. Marfil-Garza*[a,b,c], *and A.M. James Shapiro*[d]

Purpose of review
Chronic diabetes-related complications continue to exert a rapidly growing and unsustainable pressure on healthcare systems worldwide. In type 1 diabetes, glycemic control is particularly challenging, as intensive management substantially increase the risk of severe hypoglycemic episodes. Alternative approaches to address this issue are required. Islet cell transplantation offers the best approach to reduce hypoglycemic risks and glycemic lability, while providing optimal glycemic control. Although ongoing efforts have improved clinical outcomes, the constraints in tissue sources and the need for chronic immunosuppression limit the application of islet cell transplantation as a curative therapy for diabetes. This review provides an update on islet cell transplantation, focusing on recent clinical experience, ongoing research, and future challenges.

Recent findings
Current evidence demonstrates advances in terms of long-term glycemic control, improved insulin independence rates, and novel approaches to eliminate chronic immunosuppression requirements after islet cell transplantation. Advances in stem cell-based therapies provide a promising path towards truly personalized regenerative therapies, solving both tissue supply shortage and the need for lifelong immunosuppression, enabling widespread use of this potentially curative treatment. However, as these therapies enter the clinical realm, regional access variability and ethical questions regarding commercialization are becoming increasingly important and require a collaborative solution.

Summary
In this state-of-the-art review, we discuss current clinical evidence and discuss key aspects on the present and future of islet cell transplantation.

Keywords
diabetes, immune reset, immunosuppression, inducible pluripotent stem cells, islet cell transplant

INTRODUCTION

The prevalence of type 1 diabetes (T1D) is increasing. Costs to treat the disease and its complications are rapidly becoming unsustainable [1,2]. Despite technological advances (i.e. continuous glucose monitoring and subcutaneous insulin infusion pumps/closed-loop wearable insulin delivery systems), most patients fail to meet glycemic targets and experience recurrent hypoglycemia. A recent report evaluating 20 000 Americans showed that only 21% of adults and 17% of children achieve HbA1c goals [2]. Islet cell transplant (ICT) represents a robust alternative for a subgroup of these patients, and continued advancements could translate this from treatment to potential cure over time.

Twenty-years ago, Shapiro *et al.* [3] in 2000 revolutionized ICT and realized the potential of a cell-based cure for T1D by achieving 100% insulin independence 1 year post-ICT with glucocorticoid-free immunosuppression in seven consecutive patients. ICT has proven to be a highly efficacious treatment for T1D patients with severe and recurrent hypoglycemia or severe glycemic lability [4▪]. Long-term insulin independence following this initial experience was inconsistent but ongoing improvements in transplant techniques, immunosuppression regimes,

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This chapter subsection was published in its current form as a review in Current Opinion in Organ Transplantation (Impact Factor: 2.30). All figures and tables in this chapter have been adapted from this published work. Full citation: Verhoeff, K; Marfil-Garza, B.A; Shapiro, A.M.J. Update on islet cell transplantation. Current Opinion in Organ Transplantation. August 2021, 26(4), 397-404. DOI: 10.1097/MOT.0000000000000891

1.1.1 **Abstract**

1.1.1.1 Purpose of review:

Chronic diabetes-related complications continue to exert a rapidly growing and unsustainable pressure on healthcare systems worldwide. In type 1 diabetes, glycemic control is particularly challenging, as intensive management substantially increase the risk of severe hypoglycemic episodes. Alternative approaches to address this issue are required. Islet cell transplantation offers the best approach to reduce hypoglycemic risks and glycemic lability, while providing optimal glycemic control. While ongoing efforts have improved clinical outcomes, the constraints in tissue sources and the need for chronic immunosuppression limit the application of islet cell transplantation as a curative therapy for diabetes. This review provides an update on islet cell transplantation, focusing on recent clinical experience, ongoing research and future challenges.

1.1.1.2 Recent findings:

Current evidence demonstrates advances in terms of long-term glycemic control, improved insulin independence rates, and novel approaches to eliminate chronic immunosuppression requirements after islet cell transplantation. Advances in stem cell-based therapies provide a promising path towards truly personalized regenerative therapies, solving both tissue supply shortage and the need for lifelong immunosuppression, enabling widespread use of this potentially curative treatment. However, as these therapies enter the clinical realm, regional access variability and ethical questions regarding commercialization are becoming increasingly important and require a collaborative solution.

1.1.1.3 Summary:

In this state-of-the-art review, we discuss current clinical evidence and discuss key aspects on the present and future of islet cell transplantation.

1.1.2 **Key Points**

- Islet cell transplantation has become a robust therapy for patients with type 1 diabetes and severe hypoglycemia, with 10-year outcomes including near complete abrogation of severe hypoglycemic events, ~80% graft survival, as well as sustained improvements in glycemic control and reductions in insulin doses.
- Anti-inflammatory therapies such as interleukin 1 antagonists (i.e., anakinra) and TNF-α inhibitors (i.e., etanercept and infliximab) have significantly reduced innate inflammatory responses and apoptosis in the immediate post-transplant period, and have markedly improved clinical outcomes.
- Immunomodulatory approaches including gene editing techniques, regulatory T-cell therapies, and immune reset strategies may revolutionize islet cell transplantation by markedly reducing, or completely abrogating the need for chronic immunosuppression.
- Alternative islet cell sources including stem-cell derived islets (i.e., human embryonic and Induced pluripotent stem cells) have consistently demonstrated *in vitro* and *in vivo* success, and early clinical trials show tremendous potential for an immune-protected and unlimited supply of islet cells for transplant.

- Regulatory restrictions and privatization of islet cell isolation products represent important ethical barriers to realizing islet cell transplantation as a true cure for type 1 diabetes, and require urgent collaborative attention.

1.1.3 **Introduction**

The prevalence of Type 1 diabetes (T1D) is increasing. Costs to treat the disease and its complications are rapidly becoming unsustainable [1,2]. Despite technological advances (i.e, continuous glucose monitoring and subcutaneous insulin infusion pumps/closed-loop wearable insulin delivery systems), most patients fail to meet glycemic targets and experience recurrent hypoglycemia. A recent report evaluating 20,000 Americans showed that only 21% of adults and 17% of children achieve HbA1c goals [2]. Islet cell transplant (ITx) represents a robust alternative for a subgroup of these patients, and continued advancements could translate this from treatment to potential cure over time.

Twenty-years ago Shapiro et al. (2000) revolutionized ITx and realized the potential of a cell-based cure for T1D by achieving 100% insulin independence one year post-ITx with glucocorticoid-free immunosuppression in seven consecutive patients [3]. ITx has proven to be a highly efficacious treatment for T1D patients with severe and recurrent hypoglycemia or severe glycemic lability [4]. Long-term insulin independence following this initial experience was inconsistent, but ongoing improvements in transplant techniques, immunosuppression regimes, and stem cell-based islet sources are moving ITx closer towards a more accepted therapy [5,6]. This review provides an update on ITx and discusses recent and ongoing trials since the last *Current Opinion in Organ Transplantation* review of the topic in 2018 [5]. We highlight novel approaches to address chronic immunosuppression and suboptimal engraftment, as well as alternative tissue

sources. Finally, we discuss regional and global challenges limiting ITx access, and touch on the private sector's role in overcoming these challenges.

1.1.4 **Islet cell transplantation: 21st century results**

Current evidence strongly supports the long-term safety of ITx. Twenty-year patient survival after ITx compares to other cohorts of patients with T1D, despite chronic immunosuppression [7]. Reports show 10-year graft survival rates of 78%, coupled with sustained improvements in glycemic control and reductions in insulin doses [8]. The primary indication for ITx remains severe and recurrent hypoglycemia. In this regard, results demonstrate near complete resolution of severe hypoglycemic episodes (SHEs) after ITx [8-10]. Hypoglycemic unawareness, a debilitating consequence of recurrent hypoglycemia, is substantially reduced for up to three years after ITx, in parallel with SHEs, which decrease by 70-100% [8,10]. Resolution of SHEs is not at the expense of glycemic control. A recent multicenter phase 3 clinical trial evaluating islet-after-kidney transplantation demonstrated that 62.5% achieved both abrogation of severe hypoglycemic events and HbA1c ≤6.5%/≥ 1% reduction at one-year post-transplant [10]. A 2020 single-center preliminary report including 272 ITx patients from the University of Alberta, shows a 77.2% insulin independence rate after ITx, slightly lower than after pancreas transplant, but with substantially less morbidity [4]. Simultaneously, advances in islet isolation protocols, immunosuppression regimes and, overall, clinical experience, have demonstrated improved insulin independence rates, with current 5-year insulin independence rates of 50-80% [11]. Ongoing clinical trials hope to further improve outcomes to enable ITx for all T1D patients (Table 1.1.1).

Table 1.1.1 Key Ongoing Islet Transplantation Clinical Trials

Study Name	ClinicalTrials.gov Identifier	Location	Details
Clinical Trials Evaluating Novel Anti-Inflammatory, Immunosuppression, and Immunomodulatory Therapies			
Safety, Tolerability and Efficacy of Immunomodulation With AT-1501 in Islet Cell Transplantation	NCT04711226	University of Alberta, Edmonton, Alberta, Canada	Evaluating the safety of AT-1501 as an immunomodulator after ITx
Anti-inflammatory Therapy to Improve Outcomes After TPIAT	NCT02713997	University of Minnesota, Minneapolis, Minnesota, United States	Evaluating the safety and efficacy of alpha-1 antitrypsin injection after total pancreatectomy autologous islet cell transplant
Islet Transplantation Using PKX-001	NCT03073577	University of Alberta, Edmonton, Alberta, Canada	Evaluating use of PKX-001 during islet preservation to reduce tacrolimus induced graft dysfunction
Stem Cell Mobilization (Plerixafor) and Immunologic Reset in Type 1 Diabetes (T1D)	NCT03182426	University of Alberta, Edmonton, Alberta, Canada	Evaluating immune reset after early type 1 diabetes diagnosis with re-treatment after one year
PolyTreg Immunotherapy in Islet Transplantation	NCT03444064	University of Alberta, Edmonton, Alberta, Canada	Comparing safety and efficacy of ITx using standard immunosuppressants to those injected with regulatory T-cells six-weeks post-transplant
Study to Evaluate Safety and Efficacy of IBsolvMIR in Islet Transplantation	NCT03867851	Karolinska Universitetssjukhuset Huddinge, Stockholm, Sweden	Evaluating safety and efficacy of IBsolvMIR to reduce the instant blood mediated inflammatory reaction
Multicenter Trial of the Effect of AAT on Islet Transplant Engraftment and Durability After Renal Transplant	NCT02464878	University of Iowa, Massachusetts, United States	Assessing addition of alpha-1 antitrypsin to ITx
Clinical Trials Evaluating Stem Cell Therapies and Subcutaneous Device Islet Cell Transplantation			
Sequential Transplantation of UCBSCs and Islet Cells in Children and Adolescents With Monogenic Immunodeficiency T1D	NCT03835312	Children's Hospital of Fudan University, Shanghai, China	Assessing efficacy of injecting umbilical cord blood followed by ITx in newly T1D diagnosed adolescents
A Safety, Tolerability, and Efficacy Study of VC-01™ Combination Product in Subjects With Type I Diabetes Mellitus	NCT02239354	Multicenter, support from California Institute for Regenerative Medicine (CIRM) and Viacyte	Evaluating the safety and efficacy of human embryonic stem cells to mature and eliminate insulin requirements within an

			immune protected subcutaneous device
A Safety, Tolerability, and Efficacy Study of VC-02™ Combination Product in Subjects With Type 1 Diabetes Mellitus and Hypoglycemia Unawareness	NCT03163511	Multicenter, support from California Institute for Regenerative Medicine (CIRM) and Viacyte	Evaluating the safety and efficacy of human embryonic stem cells to mature and eliminate insulin requirements within a non-immune protected subcutaneous device
A Safety, Tolerability, and Efficacy Study of VX-880 in Participants With Type 1 Diabetes	NCT04786262	United Stated, Vertex Pharmaceuticals Incorporated	Evaluating the safety, tolerability and efficacy of ITx from allogeneic human stem cell derived islets (VX-880)
A Safety, Tolerability and Efficacy Study of Sernova's Cell Pouch™ for Clinical Islet Transplantation	NCT03513939	University of Chicago, Chicago, Illinois, United States	Assessing safety and efficacy of the Cell Pouch, a subcutaneous device for ITx
Other Important Clinical Trials			
Health Economic Analysis of Islet Cell Transplantation for the Stabilization of the Severe Forms of Type 1 Diabetes (STABILOT)	NCT02854696	University Hospital, Grenoble, Besancon, France	Cost-utility analysis comparing ITx versus sensor augmented insulin pump therapy

1.1.5 **Anti-Inflammatories, Immunosuppression, and Immunomodulatory Therapies**

Immunosuppression remains the major barrier precluding widespread ITx use [12]. Immunosuppression-related mortality after ITx has been reported at 0.19% [6,13]. Opportunistic infections represent an important concern. Raval et al. (2020) reported the following rates of opportunistic infections after ITx: cytomegalovirus (15%), varicella zoster (5%), and Nocardia sp. (2%), however, severe infections were rare [14,15]. Another issue with chronic immunosuppression is the incidence of neoplasms. Squamous and basal cell carcinoma, the most common neoplasm after ITx, occur in 2% of ITx patients and post-transplant lymphoproliferative disorder occurs in approximately 1% [12,13]. Novel anti-inflammatory and immunomodulatory

agents, including cell-based therapies, are being explored and promise a more nuanced immunosuppression with fewer adverse effects.

Peri-transplant anti-inflammatory therapies to ameliorate innate inflammatory responses, such as the instant blood-mediated inflammatory reaction, and apoptosis have markedly improved ITx outcomes. These include interleukin 1 antagonists (i.e., anakinra), and TNF-α inhibitors (i.e., etanercept, infliximab) [16,17]. Other agents with promising preclinical results, such as reparixin, a CXCR1/2 chemokine receptor inhibitor, failed to demonstrate clinical benefit [18]. Importantly, additional anti-inflammatories to improve islet engraftment, such as alpha 1-antitrypsin, have demonstrated preclinical efficacy with in-human clinical studies ongoing (NCT02713997, NCT02464878).

Conversely, immunomodulatory medications may potentially reduce or eliminate ITx immunosuppression requirements. Gene transfer therapies increasing islet cell expression of specific cytokines (i.e., IL-10) have demonstrated delayed T1D recurrence after transplant in preclinical models [19-21]. Similarly, blocking costimulatory signals and impairing effector T cell responses to prevent allo- and autoimmune responses by overexpressing molecules such as PD-L1 show early promise [21,22]. Both IL-10 and PD-L1 mechanisms are partly mediated through regulatory T-cells (Tregs), so-called "living immunosuppressants," have re-invigorated an interest in these unique immune cells [20,22].

A Treg-related strategy that may be revolutionary is "immune reset", first described in two classical reports from the University of São Paulo, Brazil. These showed that non-myeloablative autologous hematopoietic stem cell transplantation early after T1D diagnosis allowed medication and insulin independence in 87% and 96% of cases, respectively [23,24]. Their approach relies on early elimination of autoreactive cells and repopulation with more tolerant

cells. Islets that remain healthy survive within an immune tolerant milieu, partly mediated by Tregs. A first-in-human clinical trial (NCT03182426) is ongoing, using a novel "immune reset" approach hoping to provide better-tolerated and long-term autoimmunity remission. Newly diagnosed T1D patients are undergoing immune ablation with Alemtuzamab, an anti-CD52 antibody, followed by plerixafor to mobilize hematopoietic stem cells into circulation as an "immune reset" strategy that hopes to enable surviving islets to regenerate in a more immune tolerant milieu. This strategy will be couple with anakinra and etanercept, along with trophic support with liraglutide. As opposed to a single "immune reset" attempted in earlier trials, sustained improvements will be promoted through repeated treatment after one year. This study may foster an immune tolerant system that reverses early T1D and is maintained with a yearly intervention Alternatively, for patients with longstanding T1D, combining this immune reset technique with ITx may enable immunosuppression-free ITx. Hope for this trail is supported by trials demonstrating up to 18-month insulin-free periods after exogenous Treg infusion in adolescents with newly diagnosed T1D, demonstrating effectiveness of Treg-directed islet-specific autoimmunity remission [25,26]. Importantly, Treg induction in other settings, such as renal transplantation, has demonstrated feasibility and safety with substantial immunosuppression reductions [27,28]. Marfil-Garza et al. (2021) provide a thorough review of Treg therapies in ITx and T1D [29].

1.1.6 **Allogeneic Stem Cell-Derived Islets**

In response to tissue supply limitations, investigation into islets derived from human embryonic stem cells (ESC) is evolving rapidly. First described by Kroon et al. (2008), the stepwise process for maturing islet cells from ESCs continues optimization [12,30-32] (Figure 1.1.1).

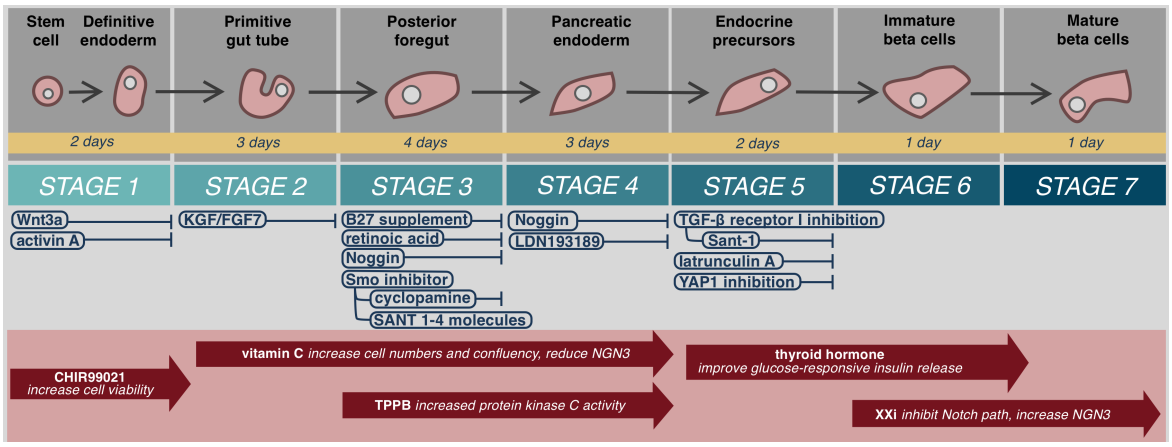

Figure 1.1.1 Embryological differentiation and maturation of islet cells.

Simultaneously, concerns regarding off-target growth led to exploration of implantation sites for ITx that allow easy graft retrieval, such as the subcutaneous space. Preclinical studies demonstrating efficient maturation and optimal function of ESC-based islets in the subcutaneous space have demonstrated proof-of-concept [33]. Alternatively, cellular encapsulation could provide an additional safeguard for off-target growth, while potentially enabling immunosuppression-free transplantation. In this regard, preclinical studies have shown that stem-cell-derived islets and cellular encapsulation approaches are compatible [34], even when combined with a subcutaneous implantation approach [33,35-37]. Recently, ViaCyte Inc. has tested two subcutaneous devices enabling subcutaneous ESC-derived ITx, the PEC-Direct (VC02) and PEC-Encap (VC01). The VC01 subcutaneous macro-encapsulation device enables oxygen and nutrient delivery to contained ESC-derived islets and provides immunoprotection. On the other hand, the VC02 aims to demonstrate maturation and survival of ESC-derived islets within the subcutaneous space but using a perforated (non-immunoprotecting) macro-encapsulation device. Clinical trials with these two approaches are ongoing (NCT02239354 and NCT03163511) with results anticipated in 2021. Preliminary unpublished data is promising and demonstrate that up to one third of T1D

patients have detectable C-peptide in peripheral blood samples after VC01 implantation, which strongly correlates with mature insulin-expressing islet cells within devices.

The foreign body response to these encapsulation devices remains a barrier for long-term insulin independence. Fortunately, research exploring novel low-fowling biomaterials is ongoing. Anderson et al. (2020) have demonstrated immunosuppression-free long-term insulin independence and a minimal foreign body response using subcutaneous and intraperitoneal encapsulated ITx using low-fowling biomaterials [35,36]. Other groups continue to optimize novel encapsulation devices that promote minimal foreign body responses and fibrosis [37,38]. Combining the effectiveness of *in vivo* maturation of ESC-derived islets and immunoprotection from the Viacyte trails with novel biomaterials for encapsulation could provide hope for long-term immunosuppression-free subcutaneous stem cell-based β-cell therapies.

1.1.7 **Induced Pluripotent Stem Cell-Derived Islets**

Human Induced pluripotent stem cell (iPSC)-based ITx also represent a promising avenue to address both chronic immunosuppression and limited organ donor supply. Discovery of the reprogramming factors by Yamanaka et al. and Thomson et al. to induce stem cells from various tissues opened the door to personalized cell-based therapies [39]. T1D represents a prototypical disease for a cell-based cure, as ITx already provides clinical "proof-of-concept", while ESC-derived islets attest to a well-described pathway to differentiate stem cells into islets [30].

Work with iPSCs has enabled efficient maturation processes and improved islet cell purity, building on the thoroughly-studied seven stage maturation process initially described for ESC-derived islets (Figure 1.1.1) [12,31,32]. However, until recently, the final *in vitro* maturation processes (Stages 5-7) have been incompletely understood, as has been demonstrated by the

increased efficiency of *in vivo* or in three-dimensional (3D) culture maturation [31,40]. Recent findings highlight cellular microenvironment process related to the cytoskeleton, cytoarchitecture, and mechanotransduction signals required to advance through these stages, which are relevant to cell culture systems [32,41]. Complete understanding of the maturation process is essential to drive efficient scale-up of iPSC ITx therapies, a *sine qua non* for a true cure for diabetes [31,32].

Simultaneously, iPSC ITx may also enable elimination of immunosuppression. Genetic modification of iPSCs may enable expression of immunotolerant molecules such as IL-10 or PD-L1 [21,22]. A robust theoretical approach currently being pursued is creating non-immunogeneic iPSCs by eliminating typical HLA molecules. Han et al. (2019) and others have recently generated iPSCs without HLA class I molecules and expressing the immunomodulatory factors PD-L1, HLA-G, and CD47, which resulted in with blunted T-cell reactivity, minimal NK cell-mediated death and macrophage phagocytosis [42-44]. Viacyte's PEC-QT multitiered approach takes advantage of these concepts and combines a genetically-modified clonal ESC line expressing PD-L1 and lacking HLA class I molecules (i.e., β microglobulin), with their PEC-Direct maroencapsulation device [45]. PEC-QT is expected to enter clinical trials soon.

The most attractive advantage of iPSC islets is that these could be generated from each T1D patient, echoing autologous ITx after total pancreatectomy. However, recurrent autoimmunity remains a likelihood that will also need to be overcome if this therapy is to be successful in the longer term. Combining autologous ITx with "immune reset" approaches in this context may provide an effective solution to control recurrent autoimmunity. Unfortunately, autologous iPSC ITx demands generation of unique cell lines requiring personalized screening to identify genetic mutations and prevent off-target effects, including abnormal growth. The

resources required for such personalized approach may initially appear prohibitive, but recent advances in process automation, the introduction of large-scale bioreactors, standardized protocols, cell banks and increased efficiency for islet generation may ultimately enable cost-efficient autologous iPSC ITx in the longer term [12].

1.1.8 **Regional and Global Challenges**

An important barrier to widespread ITx pertains to access and regulations. The only countries funding ITx under non-research, clinical care streams are Canada, Australia, the United Kingdom, France, Switzerland, Norway, Sweden, and partial reimbursement in Italy. Only 11 ITxs have occurred between 2016 and 2019 in the United States (USA), compared to 88 in the United Kingdom, and 87 in Edmonton, Canada [46,47]. This lag persists despite consensus statements and national cost-analysis guidelines demonstrating that ITx is cost-effective when provided to patients with labile T1D, or with T1D undergoing kidney transplant [48,49]. Other clinical trials are ongoing comparing the cost-effectiveness of ITx vs. sensor augmented insulin pump therapy, which are of great interest (NCT02854696). Witkowski et al. (2020) describe the highly restrictive regulatory practices limiting ITx in the USA [46], where total pancreatectomy with autologous ITx does not require a biologics license application (BLA) given the "minimal manipulation" of islets, while allogeneic ITx, in which differences in isolation and transplant processes pertain only to the source of the organ (autologous vs allogeneic) and the time in culture (<24 hours vs 24-48 hours), are perceived to represent sufficient evidence against "minimal manipulation", thus requiring a BLA. While it is obvious that autologous and allogeneic islets are not biologically different, substantial evidence demonstrates that islets are not altered during the culture period; the latter being mainly implemented to properly administer induction immunosuppression before ITx [46]. Even after the USA-led, National Institutes of

Health-funded, phase 3 trial demonstrating the effectiveness of ITx to substantially reduce SHEs and improve glycemic control, allogeneic ITx has only been approved as a drug, with a BLA limiting supply by a single private company [10,50]. However, experts suggest that ITx be regulated not by the FDA, but under the Health Resources and Services Administration with oversight by the Organ Procurement and Transplantation Network and United Network for Organ Sharing to enable equitable access. These historic and outdated regulations impose unrealistic barriers to ITx in the USA with ITx costs of approximately $50,000 USD placed on individual institutions. Continuing the advancement of ITx requires international collaboration and standardization of regulations to eliminate these restricting regional barriers.

1.1.9 **Commercialization of Islets**

With current USA regulations, a BLA has recently been discussed and will likely be approved allowing one specific private company (CellTrans) rights to market human islet for transplantation under the Orphan Drug Designation Act [50]. As ITx advances towards being a potential cure for diabetes, this represents an exciting market for commercial investment. Ethically, it remains unclear what role these commercial entities should play in the discovery processes carried within University labs. As discussed by Witkowski et al. (2021), commercialization of organs or their subparts (including islets) raises potential conflicts with transparent and just allocation of these goods [51]. This question will continue to evolve as results continue to demonstrate positive results. The Viacyte and Vertex clinical trials are examples of these relationships and attest to the growth in industry interest that will likely continue to expand. These collaborative schemes will likely be required to expand β-cell replacement therapies and meet demand, yet it will certainly be of utmost importance for academic labs to maintain patient's interests at the forefront of their investigation.

Regardless of the private sector's involvement, providing islets to >8 million T1D patients will remain a challenge [1,12]. Stem cell-based islets will certainly be required to fulfill the demand and just allocation should be considered. The minimum number of islets required for current ITx is approximately >5,000 IEQ/kg and ideally >11,000 IEQ/kg to ensure insulin independence. However, stem cell-based-ITx is expected to improve islet purity from ~ 50% to ~100% allowing ITx with higher IEQs/kg (i.e. a larger functional reserve) with low risk of portal vein thrombosis or complications. Under these circumstances, ~ 9.1×10^8 - 1.4×10^9 cells per patient may be required. Thus, generating islets, differentiating them, and potentially genetically altering them while ensuring quality will require substantial resources that will inevitably require industry support.

1.1.10 **Conclusion**

ITx continues to improve and novel approaches to control engraftment and immune mediated destruction promise a future of immunosuppression-free transplants. Meanwhile, ESC- and iPSC islet cells are generating profound optimism for a potential and accessible cure for T1D. To enable widespread access, integration and commercial relationships will surely be required, however, the ethical considerations for these interactions should continue to be scrutinized to ensure that patient interests remain at the forefront of discovery.

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1.2 Chapter 1 subsection 2 – Induced Pluripotent Stem Cells as a Potential Cure for Diabetes

cells

MDPI

Review

Inducible Pluripotent Stem Cells as a Potential Cure for Diabetes

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Abstract: Over the last century, diabetes has been treated with subcutaneous insulin, a discovery that enabled patients to forego death from hyperglycemia. Despite novel insulin formulations, patients with diabetes continue to suffer morbidity and mortality with unsustainable costs to the health care system. Continuous glucose monitoring, wearable insulin pumps, and closed-loop artificial pancreas systems represent an advance, but still fail to recreate physiologic euglycemia and are not universally available. Islet cell transplantation has evolved into a successful modality for treating a subset of patients with 'brittle' diabetes but is limited by organ donor supply and immunosuppression requirements. A novel approach involves generating autologous or immune-protected islet cells for transplant from inducible pluripotent stem cells to eliminate detrimental immune responses and organ supply limitations. In this review, we briefly discuss novel mechanisms for subcutaneous insulin delivery and define their shortfalls. We describe embryological development and physiology of islets to better understand their role in glycemic control and, finally, discuss cell-based therapies for diabetes and barriers to widespread use. In response to these barriers, we present the promise of stem cell therapy, and review the current gaps requiring solutions to enable widespread use of stem cells as a potential cure for diabetes.

Keywords: islet cell transplant; diabetes; inducible pluripotent stem cells; immunosuppression; immune reset; insulin

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1. Insulin as a Treatment, Not a Cure

In 1889, Oskar Minkowski and Joseph von Mering completed a canine pancreatectomy and induced fatal diabetes mellitus (DM). This experiment demonstrated the central role of the pancreas in glycemic control [1]. In 1893, Williams and Harsant working in Bristol, UK, attempted to transplant pancreatic fragments taken from a freshly slaughtered sheep and placed them subcutaneously in a boy dying of diabetic ketoacidosis, with unsuccessful results [2]. Even throughout the journey to discover insulin, Banting's initial trials focused on subcutaneous injection of an unpurified pancreatic slurry, and the first patient treated developed a sterile buttock abscess [3]. Although Banting, Best, Collip and Macleod subsequently prepared more purified insulin extracts using acid-alcohol to dissolve the insulin and prevent degradation by exocrine enzymes, Banting's acceptance speech for the 1923 Nobel Prize in Physiology and Medicine concluded with these words:

"Insulin is not a cure for diabetes; it is a treatment. It enables the diabetic to burn sufficient carbohydrates, so that proteins and fats may be added to the diet in sufficient quantities to provide energy for the economic burdens of life [3]."

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1.2.1 **Abstract**

Over the last century, diabetes has been treated with subcutaneous insulin, a discovery that enabled patients to forego death from hyperglycemia. Despite novel insulin formulations, patients with diabetes continue to suffer morbidity and mortality with unsustainable costs to the health care system. Continuous glucose monitoring, wearable insulin pumps, and closed-loop artificial pancreas systems represent an advance, but still fail to recreate physiologic euglycemia and are not universally available. Islet cell transplantation has evolved into a successful modality for treating a subset of patients with 'brittle' diabetes but is limited by organ donor supply and immunosuppression requirements. A novel approach involves generating autologous or immune-protected islet cells for transplant from Induced pluripotent stem cells to eliminate detrimental immune responses and organ supply limitations. In this review, we briefly discuss novel mechanisms for subcutaneous insulin delivery and define their shortfalls. We describe embryological development and physiology of islets to better understand their role in glycemic control and finally, discuss cell-based therapies for diabetes and barriers to widespread use. In response to these barriers, we present the promise of stem cell therapy, and review the current gaps requiring solutions to enable widespread use of stem cells as a potential cure for diabetes.

1.2.2 **Insulin as a Treatment, Not a Cure**

In 1889 Oskar Minkowski and Joseph von Mering completed a canine pancreatectomy and induced fatal diabetes mellitus (DM). This experiment demonstrated the central role of the pancreas in glycemic control [1]. In 1893, Williams and Harsant working in Bristol, UK attempted to transplant pancreatic fragments taken from a freshly slaughtered sheep and placed them

subcutaneously in a boy dying of diabetic ketoacidosis, with unsuccessful results [2]. Even throughout the journey to discover insulin, Banting's initial trials focused on subcutaneous injection of an unpurified pancreatic slurry, and the first patient treated developed a sterile buttock abscess [3]. Although Banting, Best, Collip and Macleod subsequently prepared more purified insulin extracts using acid-alcohol to dissolve the insulin and prevent degradation by exocrine enzymes, Banting's acceptance speech for the 1923 Nobel Prize in Physiology and Medicine concluded with these words:

"Insulin is not a cure for diabetes; it is a treatment. It enables the diabetic to burn sufficient carbohydrates, so that proteins and fats may be added to the diet in sufficient quantities to provide energy for the economic burdens of life [3]."

- Banting

Nearly 100 years later this remains true. Despite novel, improved recombinant insulin formulations, the potential of 'smart' insulins that are inactivated in a hypoglycemic environment, the advent of continuous glucose monitoring (CGM) and wearable biomechanical closed-loop pancreas systems, subcutaneous insulin remains a highly problematic treatment. The United States type 1 DM (T1D) exchange registry with >20,000 participants from 2016-2018 demonstrated that only 21% of adults and 17% of children achieve the recommended HbA1c goal of <7% and 7.5%, respectively [4,5]. Current HbA1c levels of 9.0% in 13-17-year-olds are only marginally lower with novel treatment options than the 9.5% seen in the same population during the 1980s [4,5]. Hypoglycemia also remains a significant but often overlooked complication of DM. Hypoglycemia occurs in 31-41% of diabetic patients [6], often at night due to the four-fold

variability of overnight insulin requirements [7-9]. Of 11,061 exchange registry respondents, 6% reported hypoglycemic seizure or loss of consciousness within the previous three months - a risk that increases with age and the presence of hypoglycemic unawareness [4,10]. These events may be life threatening, with an incidence of 320 episodes per 100-patient years in patients that have lived with T1D for more than 15 years [11]. Unfortunately, this risk escalates with intensive insulin therapy and improved control of hyperglycemia [11]. Achieving euglycemia is nearly impossible without flexible, dynamic insulin and glucagon responses and even the most advanced insulin therapies still fail to recreate the precise and physiologic glycemic control orchestrated by almost three million pancreatic islets of Langerhans.

This review briefly discusses novel insulin-based therapies but focuses primarily on the future promise of a potential cure for DM using cell-based therapies and stem cell-derived islet transplantation (SC ITx). We review novel mechanisms for insulin delivery and describe their shortfalls. We describe *in vivo* and *in vitro* islet cell embryological development and physiology to better understand its implications in the generation of functional stem cell-derived islet cells. Finally, we discuss the evolution of islet cell transplantation (ITx) as a cell-based cure for DM and its barriers to widespread use, as well as its importance in the future of stem cell-based therapies. Finally, we present a response to these barriers and review the current gaps requiring further research to enable widespread use of cell-based therapies, including pluripotent stem cells, as a cure for DM.

1.2.3 **Novel Subcutaneous Insulin Delivery**

The use of CGM, continuous subcutaneous insulin infusion (CSII i.e., insulin pump), and closed-loop wearable insulin delivery (i.e., artificial pancreas) devices has increased substantially in recent years, but are still only accessible to a relatively small subset of patients with DM. From 2011 to 2017, CGM use increased from 7% to 30% and CSII from 57% to 63% [4]. CGM, CSII, and artificial pancreas technologies all demonstrate lower HbA1c levels compared to standard insulin treatment [4]. CGM alone improves DM understanding and glycemic control. It guides novel treatment modalities and glycemic optimization by demonstrating real time glycemic targets and time spent in euglycemia, hypoglycemia, or hyperglycemia [5,12,13]. CGM also provides overnight and dynamic readings, and offers hypoglycemic and hyperglycemic alarms. Both independently, and combined with novel insulin delivery tools, CGM users have improved glycemic stability [4]. Advances in wearable insulin pump technologies have also shown clear benefits. A large meta-analysis of 33 randomized controlled trials demonstrated improved glycemic control with CSII compared to standard insulin delivery methods [14]. Bekiari et al. conducted a further meta-analysis comparing artificial pancreas to other forms of insulin therapies, including CSII, and showed the greatest glucose stability using dual hormone artificial pancreas devices [14,15]. Improved overnight glycemic control with artificial pancreas therapy was especially notable, as this has historically been difficult to manage with subcutaneous insulin [14,15]. For those who can access and afford these technologies (CGM, CSII, and closed-loop wearable insulin delivery devices), DM care is clearly improved.

However, despite enhanced glycemic control offered by CGM, CSII, and artificial pancreas technologies, they remain far from a cure (Table 1.2.1). HbA1c reductions with CSII,

although statistically significant, are only 0.3-0.7% [4,14,15]. Even with fully automated, dual hormone artificial pancreas treatment, average daily and overnight glucose improved by only 0.48 mmol/L and 0.81mmol/L respectively compared to standard insulin therapy [15]. Additionally, normoglycemia was only achieved 16.4% of the time for patients using an artificial pancreas technology [15]. When provided structured DM training, patients can achieve similar glycemic control, decreased incidence of hypoglycemia, and improved psychosocial outcomes using self-directed subcutaneous insulin therapy compared to those with CSII [16]. Technical barriers also persist – issues with absorption, lipohypertrophy, rashes and skin reactions from the adhesive devices and extended use in one site can lead to progressively worse glycemic control despite automated insulin delivery [17-19]. Mechanical failure of infusion systems occurs frequently, with catheter kinking or occlusion, leaking, bruising, or infection at the site of insulin instillation occurring in up to 64% of devices over 7 days [5,19-21]. The biggest risk involves unrecognized discontinuity of insulin delivery, which occurs regardless of the needle/injection type, and may lead to diabetic ketoacidosis [20-24]. CGM and CSII also have patient-related factors limiting their utility. Even the most automated artificial pancreas systems require user input for bolus dosing and mechanical errors can occur due to patient misunderstanding or misuse [21]. Additionally, 47% of patients report device discomfort as a barrier to use, and 35% dislike devices on their body [5,25]. Others have reported skin irritation [26], and sleep disruption from bedtime alarms as problems [5,27]. While these therapies offer specific glycemic benefits, the absolute benefit, reliability, and usability concerns limit optimism (Table 1.2.1).

Table 1.2.1 Benefits and drawbacks of novel subcutaneous insulin monitoring and delivery devices.

Technology	Benefits	Drawbacks
Continuous Glucose Monitoring	Immediate glycemic feedback. Improved dynamic glycemic understanding (real time glycemic targets, time spent in euglycemia, hypoglycemia, or hyperglycemia) [5,12,13] Hyper/hypo glycemic alarms.	Device discomfort [25]. Disrupted sleep (alarms) [27].
Continuous Subcutaneous Insulin Infusion (i.e., Insulin Pump)	Improved glycemic control compared to standard subcutaneous insulin [14,15].	Modest HbA1c improvements (0.3-0.7%) Mechanical Failure (64% of devices over 7 days). Device discomfort [25].
Closed Loop, Wearable Insulin Delivery Device (i.e., artificial pancreas)	Improved glycemic control compared to CSII or CGM [15]. Improved nighttime hyper- and hypoglycemic control [15].	Poorly accessible. Device discomfort [25]. Only 16.4% of the time spent in normoglycemia [15].

*CGM: continuous glucose monitor, CSII: continuous subcutaneous insulin infusion

1.2.4 **Islets of Langerhans**

Current injectable insulin technologies fail to recreate physiologic glycemic control with a tight 1-2 mmol/L glycemic variance. *In situ* physiologic intraportal hormone delivery from the pancreatic islets of Langerhans maintains basal normoglycemia with insulin and counterbalances hypoglycemia with glucagon. Insulin output can increase up ten-fold after a meal, and return rapidly to basal levels with no hysteresis. In our opinion, exogenous subcutaneous insulin delivery, even when provided by the most ideal closed loop systems, cannot recreate this degree of dynamic control. Thus, developing a cell-based cure through islet cell generation and transplantation remains an ideal to strive for. Achieving this goal, especially with stem cell therapies, demands complete understanding of embryological differentiation and physiology of the islets of Langerhans.

1.2.4.1 Embryological Development and Structure

Islets form collections of cells that exist uniformly throughout the pancreas but represent only 1-4%, 2g, or 2ml of the pancreatic volume [28]. Person-to-person heterogeneity is common but islets are generally composed of approximately 60% β-cells, 30% α-cells, <10% δ-cells, <5% γ and ε cells producing insulin, glucagon, somatostatin, pancreatic polypeptide, and ghrelin, respectively [28]. Islet mass varies throughout life, and expands during childhood growth and during normal pregnancy. The exact mechanisms that regulate this expansion process remain incompletely understood.

Mature β-cells develop from embryonic stem cells (ESC) in a continual process that may be considered in seven steps beginning from definitive endoderm, to primitive gut tube, posterior foregut, pancreatic endoderm, endocrine precursors, immature β-cells, and finally mature β-cells (Figure 1.1.1) [29-31]. Definitive endoderm forms during gastrulation from epiblast cells undergoing epithelial to mesenchymal transition [32]. This process is initiated by Wnt3a protein signaling [29,31,33,34], followed by Nodal signaling-mediated activation of the TGFβ pathway that ultimately leads to activation of intracellular Smad2 and differentiation into the primitive streak and definitive endoderm [35-37]. Stable, bioactive Nodal does not exist. Fortunately, a similar protein from the TGFβ family, activin-A, acts as an *in vitro* biochemical analogue to activate Smad2 [38-40]. *In vitro*, ESC exposure to Wnt3a and activin-A leads to 95% definitive endoderm cells that express the phenotypic markers SOX17 and FOXA2 [41]. Patterning of anterior-posterior axis occurs with exposure to KGF/FGF7 and creates the primitive gut tube [29,30,34]. Subsequent culture with B27 supplement, retinoic acid, Noggin, and a smoothened inhibitor, such as cyclopamine or Sant 1-4 molecules to prevent Hedgehog (Hh) signaling, induces differentiation into the posterior

foregut that has potential to become pancreatic, hepatic, or duodenal tissues [29,34,42]. Hepatic tissues are favored through bone morphogenetic protein (BMP) signaling pathways, while endocrine differentiation is blocked by FGF10 activation [43,44]. Exposure to Noggin or LDN193189, potent inhibitors of both BMP and FGF10, produces pancreatic endoderm cells ($PDX1^+$) [29,34,43].

Further differentiation of pancreatic endoderm cells into islets has been incompletely understood and until recently, only occurred in three-dimensional (3D) culture *in vitro* [31,41,45]. Differentiation into pancreatic endocrine progenitors ($PDX1^+/NKX6.1^+$) utilizes TGFβ receptor I (TβRI/ALK5) inhibition and continued prevention of Hh signaling with Sant1-4 molecules [30,31,41]. Recent data has helped clarify why 3D culture and *in vivo* differentiation is required at this stage for β-cell differentiation. Failure to produce $NKX6.1^+$ cells prior to expression of endocrine genes such as neurogenin 3 (NEUROG3) produces non-functional poly-hormonal cells [30,46]. Hogrebe et al. (2020) recently demonstrated that the cellular microenvironment, actin cytoskeleton, and cellular attachments, dictate NEUROG3 expression [30]. Firm adhesion of stage 4 ($PDX1^+$) cells to Type-I collagen coated culture plates leads to $NKX6.1^+$ cells, followed by stage 5 actin depolymerization with latrunculin A to allow NEUROG3 expression. Similarly, inhibition of YAP1 function increases NEUROG3 expression and favors endocrinogenesis [47]. Further maturation leads to insulin producing, NKX6.1 expressing, β-cells with islet-like glucose response *in vivo* [30].

Maturation and differentiation specificity and efficiency may be further improved with various compounds (Figure 1.1.1). It should be noted that use and timing of these compounds varies widely by protocol. CHIR99021, a selective glycogen synthase kinase-3β inhibitor, has

been used in stage 1 formation of definitive endoderm to increase cell viability [30,31,48]. Rezania et al. added vitamin C from the primitive gut tube to pancreatic endoderm (stages 2-4), to increase cell numbers and confluency and reduce NGN3 expression, which has demonstrated disruption of pancreatic endoderm [31,49]. Increased protein kinase C activity, demonstrated *in vitro* with -(2S,5S)-(E,E)-8-(5-(4-(trifluoromethyl)phenyl)-2,4-pentadienoylamino)benzolactam (TPPB) has demonstrated improved induction of pancreatic progenitors from primitive gut tube (stages 3-4) [29,30,50-52]. Thyroid hormone acts after stage 5 through the transcription factor MAFA to improve glucose-responsive insulin release in mature cells [30,31,41,53]. Alongside thyroid hormone, gamma secretase XX inhibitor (XXi), which inhibits the Notch pathway and increases NGN3 expression, has been used in step 6 to inhibit PTF1a guided exocrine differentiation to improve β-cell maturation [30,31,38,41,43,49]. Application of these compounds is not standardized, and no author to date has combined all these additives to determine if an ideal, more efficient or specific β-cell differentiation can be achieved. Greater understanding of their role, result replication, and process standardization are needed to determine ideal additive compounds.

A major limitation in our understanding of islet developmental science is that many of the concepts and protocols have been derived from work in murine models – mainly because the relevant human targets and growth factors have yet to be defined. While there may be conservation in the early developmental pathways between species, it seems unlikely that this process will be fully optimized until these pathways are mapped out entirely in human cells. Another important limitation of *in vitro* islet generation is that it only approximates but does not replicate the continuum of cell-to-cell contact, dynamic intracellular signaling and participation of the physiologic extracellular matrix present in the full complexity of a developing human

embryo. Only when we can recapitulate the process with more accuracy will we be able to optimize, perfect and avoid risk of off-target cell growth in this differentiation process.

1.2.4.2 Function

Glucose control is accomplished with both autonomic nervous and hormonal systems (Figure 1.2.1). While interest focuses on β-cells, the α, δ, γ, and ε cells also play increasingly well understood and important roles in glycemic control.

In the fasting state, normoglycemia is achieved through activation of the autonomic nervous system; sympathetic activation leads to glucagon release from α-cells, while parasympathetic activity induces insulin release from β-cells [54]. These actions are directed through glucose-sensing cells located in peripheral locations such as the hepatoportal vein area, and by specialized glucose-excited or glucose-inhibited neurons located in the hypothalamus or brainstem region [54]. This mechanism directs α and β-cells to release basal levels of glucagon and insulin to promote appropriate hepatic gluconeogenesis for anabolism and cellular functions [54]. In anticipation of food, either by sight, mastication, or gastric distention, and prior to any blood glucose changes, parasympathetic release of acetylcholine activates β-cell muscarinic receptors (m3AchR), producing phospholipid-derived messengers to initiate protein kinase C (PKC) directed calcium influx and efficient insulin release through the cephalic response [54,55].

Elevated blood glucose concentrations lead to biphasic insulin release lasting approximately 60 minutes [5,56]. The first phase occurs with GLUT2 facilitated diffusion of glucose into β-cells, which is oxidatively metabolized to produce ATP. In response, ATP dependent K^+ channels (KATP) channels close, leading to cellular membrane depolarization and

opening of voltage-dependent L-type calcium channels. Intracellular calcium promotes SNARE protein mediated exocytosis of insulin-containing secretory granules with release into portal circulation (Figure 1.2.1) [5,56]. Depolarization and exocytosis oscillate every 3-6 minutes to avoid insulin receptor downregulation [5,56].

A second phase of insulin release, accounting for approximately 50% of postprandial insulin secretion, occurs via stimulation from parasympathetic inputs, glucagon-like peptide 1 (GLP-1), glucose-dependent insulinotropic peptide (GIP), free fatty acids (FFA), and somatostatin (Figure 1.2.1) [5,56-59]. GLP-1 and GIP are incretins secreted from pancreatic α-cells, as well as K-cells and L-cells located in the pancreas, ileum, and colonic bowel in response to increase blood glucose concentration [56,59]. This demonstrates the α-cell interaction with β-cells to achieve euglycemia. GLP-1 and GIP act through β-cell G-protein-coupled receptors (GPCR), increasing 3′,5′-cyclic adenosine monophosphate (cAMP) and leading to protein kinase A (PKA) dependent and non-PKA dependent insulin exocytosis [57,59]. Similarly, FFA act through the GP40 GPCR to further stimulate insulin release [57,58]. δ-cell released somatostatin, and γ-cell released pancreatic polypeptide, also play a minor role for glucose homeostasis but mechanisms for such are incompletely understood [60,61]. Ablation of δ-cells impairs islet cell function [60], and infusion of pancreatic polypeptide alongside insulin reduces insulin requirements [62] – further analysis of these mechanisms may assist with improving glycemic control but also highlight the complex interplay of cells required for glycemic control that is often overlooked with single or dual hormone treatment systems.

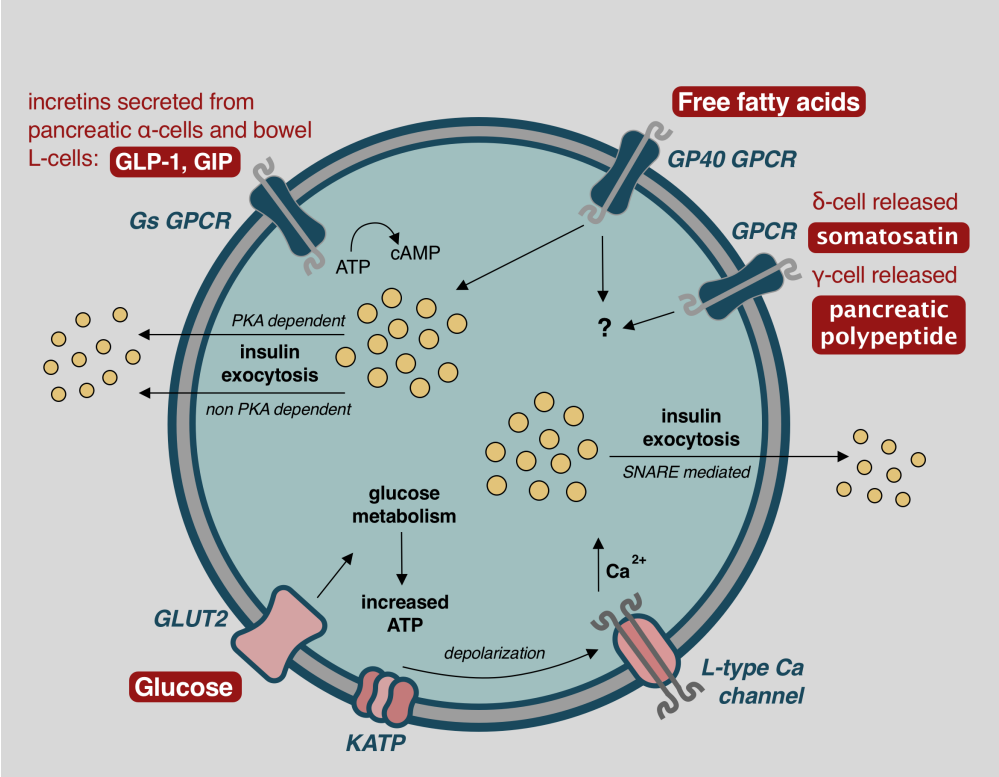

Figure 1.2.1 Mechanisms of β-cell insulin release and glycemic control.
Image adapted from Komatsu et al. (2013) with permission for reuse [56].

1.2.5 Islet Cell Transplantation

In 2000, Shapiro et al. revolutionized clinical outcomes with ITx demonstrating proof-of-concept that cell-based therapy could offer huge potential for the treatment of DM. Their results demonstrated 100% insulin independence at one year in seven T1D patients consecutively treated with glucocorticoid-free immunosuppression using anti-CD25 monoclonal antibody (mAb) induction immunosuppression and maintenance immunosuppression with tacrolimus and sirolimus [63,64]. Unfortunately, long-term insulin independence was not achieved, with most patients returned to low doses of insulin over time. Protocol improvements now demonstrate

five-year ITx insulin independence rates >50%, matching rates observed with whole pancreas transplant, but with significant less morbidity after ITx [11,65,66]. Within-subject, paired comparison of insulin injection versus CSII, and CSII versus ITx demonstrated stepwise improvement of glycemic control, less glycemic variability, and fewer hypoglycemic events, with the best results achieved after ITx [67]. Notably, HbA1c improved from 8.2% using CSII to 6.4% with ITx [67]. Glycemic stability and a lower incidence of hypoglycemia also persisted following ITx regardless of insulin independence [67]. Multicenter phase III clinical trial data also demonstrated that 87.5% and 71% of patients, at one and two years' post-transplant, respectively, achieved a HbA1c <7.0% and median HbA1c of 5.6% [68]. Similar HbA1c results were observed by the Vancouver group with HbA1c of 6.6% following ITx versus 7.5% with intensive insulin treatment; they also reported significantly less retinopathy, nephropathy, and a trend towards less neuropathy with ITx [69,70]. Others have also demonstrated improved retinal blood flow and improved markers of polyneuropathy after ITx [71,72].

ITx has revolutionized the care of patients with DM, with benefits beyond hyperglycemic control. These results have been achieved through optimizing multi donor transplantation, islet isolation [66], good manufacturing practices (GMP) [66,73], and agents to resist the immune and non-immune challenges presented in Figure 1.2.2. Detailed GMP-islet isolation procedures have been made available from the clinical islet transplantation consortium, allowing clinical isolation facilities to utilize >50% of donated organs [66,74]. Once isolated, current islet cell culture techniques have allowed a substantial decrease in the number of apoptotic cells and minimized harmful cytokine release following transplant [75-78]. Specific agents to mitigate inflammation and apoptosis have also increased ITx clinical success, including the interleukin 1 antagonist

anakinra and TNF-α inhibitor etanercept [65,66,79-82]. Similarly, adding manganese superoxide dismutase decreases reactive oxygen species and has shown to enhance *in vitro* islet cell viability with augments *in vivo* murine marginal islet mass engraftment [83,84]. An improved understanding of the blood-mediated inflammatory reaction (IBMIR) following ITx has led to post-ITx heparin infusion to limit tissue factor-related IBMIR, while insulin infusion allows islet rest and reduced inflammation to improve engraftment [85,86]. Finally, depleting T-cell populations with induction therapy using alemtuzumab or thymoglobulin has been more effective that IL-2 receptor (anti-CD25) blockade with less potent daclizumab or basiliximab. All these additions have contributed to enhanced long-term insulin independence rates [66,79]. Other agents that may further improve ITx engraftment and success include liraglutide or pan-caspase inhibitors to further improve insulin independence rates [11,65,87-90]. Ongoing research promises to elucidate additional modifications to improve graft success. Immunogenic protection with regulatory T cells (Tregs) may enable optimal engraftment and a decrease (or complete elimination) of lifelong pharmacologic immunosuppression [91,92]. Achieving this would closely resemble a true cure for DM.

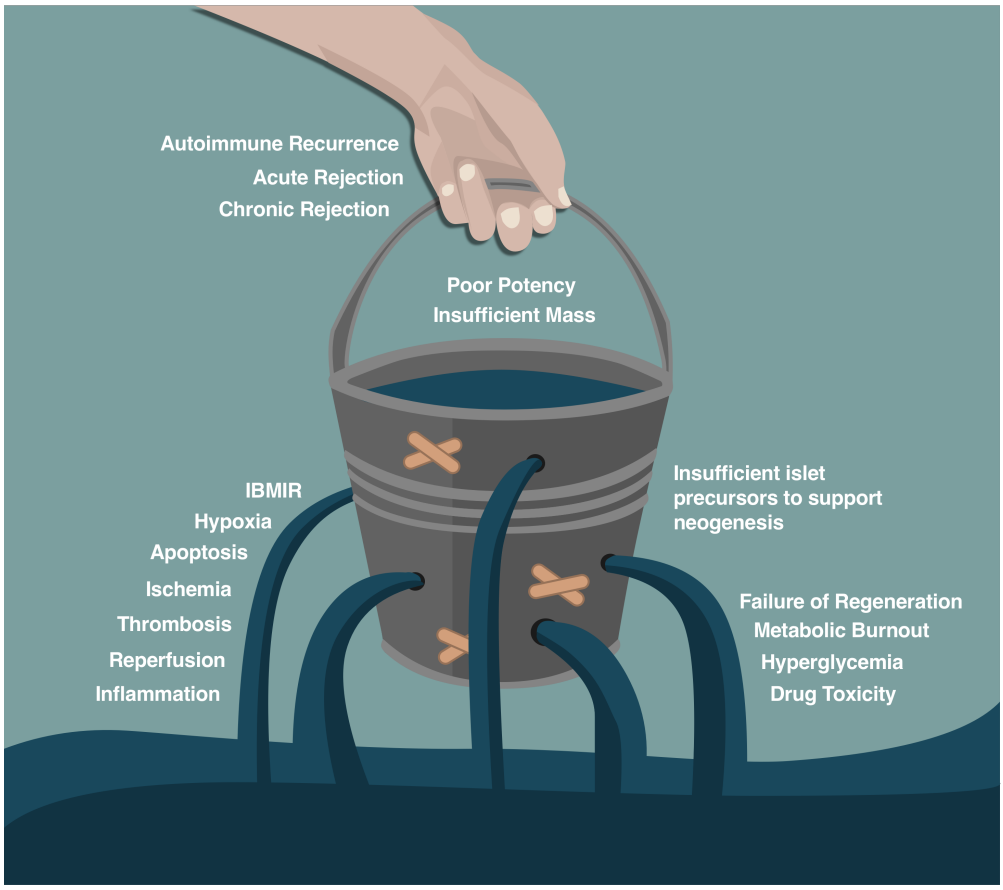

Figure 1.2.2 Limiting factors for islet cell engraftment after islet cell transplant. Adapted from Shapiro et al. (2011) with permission for reuse [11].

1.2.5.1 Barriers to Islet Cell Transplant

Despite excitement, numerous barriers to widespread use of ITx persist. The only current islet cell source is human deceased donor pancreata, and the supply of potential organ donors is severely limited in the context of the prevalence of DM. Each recipient requires > 5,000 islet equivalents (IEQ) per kg and ideally >11,000 IEQ/kg for insulin independence, and typically 2-4 pancreata per recipient, further straining a small donor pool [66]. Access is also limited by funding. In 2012, the only countries that funded ITx under non-research, clinical care streams were

Canada, Australia, the United Kingdom, France, Switzerland, Norway, Sweden, and parts of Europe [11,93]. Even in countries with access, lifelong immunosuppression requirements and associated complications mean that strict recipient criteria must be met for islet-alone transplant (i.e. without kidney). Patients must have recurrent severe hypoglycemic episodes with hypoglycemic unawareness, glycemic lability not managed with intensive insulin, pumps and/or continuous glucose monitoring therapies [11]. They should also have had T1D for >5 years, be over the age of 18, have normal renal function, and have a BMI (<30 kg/m2) and/or weight <90 kg and/or daily insulin requirement < 1.0 U/kg.

Even when patients access ITx, alloimmunity, and autoimmunity in type 1 diabetes (T1D), mean patients must remain on lifelong potent immunosuppression. Infectious risks and toxic effects from immunosuppression have improved but persist and must be balanced against ITx benefits. Timing of the ITx must also be considered, as earlier ITx prior to diabetic complications is ideal but increases length of immunosuppression exposure, in-turn increasing the risk of infection, cancer and drug toxicity. Risk of opportunistic infections include cytomegalovirus (15%), cytomegalovirus retinitis (20%), varicella zoster (5%), and nocardia (2%), amongst other infections [94,95]. Severe infection remains rare and more commonly patients experience minor concerns including acne, mouth ulcers, and diarrhea [94]. Calcineurin-inhibitors are especially notable in that they are both nephrotoxic and diabetogenic [65,96,97]. Malignancy, namely squamous and basal cell carcinoma of the skin, occurs in 2% of ITx patients, and post-transplant lymphoproliferative disorder (PTLD) occurs in approximately 1%. Mortality related to immunosuppression in the context of ITx is 0.19% [66,98]. These risks occur despite approximately 50% of ITx failing to achieve long-term insulin independence. Insulin independence is limited by

auto- and alloimmunity, but also imperfect engraftment that decreases functional islet cell mass. Engraftment is limited by apoptosis, thrombosis, ischemia, inflammation, and instant blood-mediated inflammatory reaction (IBMIR) [66,99,100]. While many ITx recipients benefit irrespective of complete insulin independence, dynamic risk-benefit analysis should be contemplated and individualized in every case. Considering evolution of artificial pancreas technology, carefully designed randomized control trials with intention-to-treat analysis are required to compare ITx to novel subcutaneous delivery systems.

1.2.6 **The Promise and Future Challenges for Stem Cells**

Islet/β-cell stem cell-derived therapies offer the potential to overcome many of the barriers emphasized above to widespread application of ITx. SC ITx has the potential to resolve limited access, donor shortage, and need for immunosuppression. Human embryonic stem cells (ESC) and Induced pluripotent stem cells (iPSC) can be differentiated into mature β-cells that co-express PDX1, NKX6.1, MAFA, Insulin, C-peptide, that have prohormone processing enzymes, and most importantly, that are glucose responsive *in vivo* [29-31,34,41]. Stem cell differentiation and expansion can now occur in 2D and 3D growth media following the seven-step embryological process shown in Figure 1.1.1, with resultant islet-like cell clusters capable of consistently reversing diabetes in murine models [30,31,41].

Specific challenges relating to ESC/iPSC ITx approaches remain if this therapy is to one day be applied as a widespread cure for all forms of DM. Determining the ideal source for islet generating stem cells (allogeneic versus autologous iPSC), the optimal transplant site, and identifying an approach to eliminate immunoreactivity remain unanswered (Figure 1.2.3). Lastly,

if these therapies are to be used as a true cure for DM, economically viable scale up and supply with standardized GMP protocols to generate these cells is vital (Figure 1.2.4).

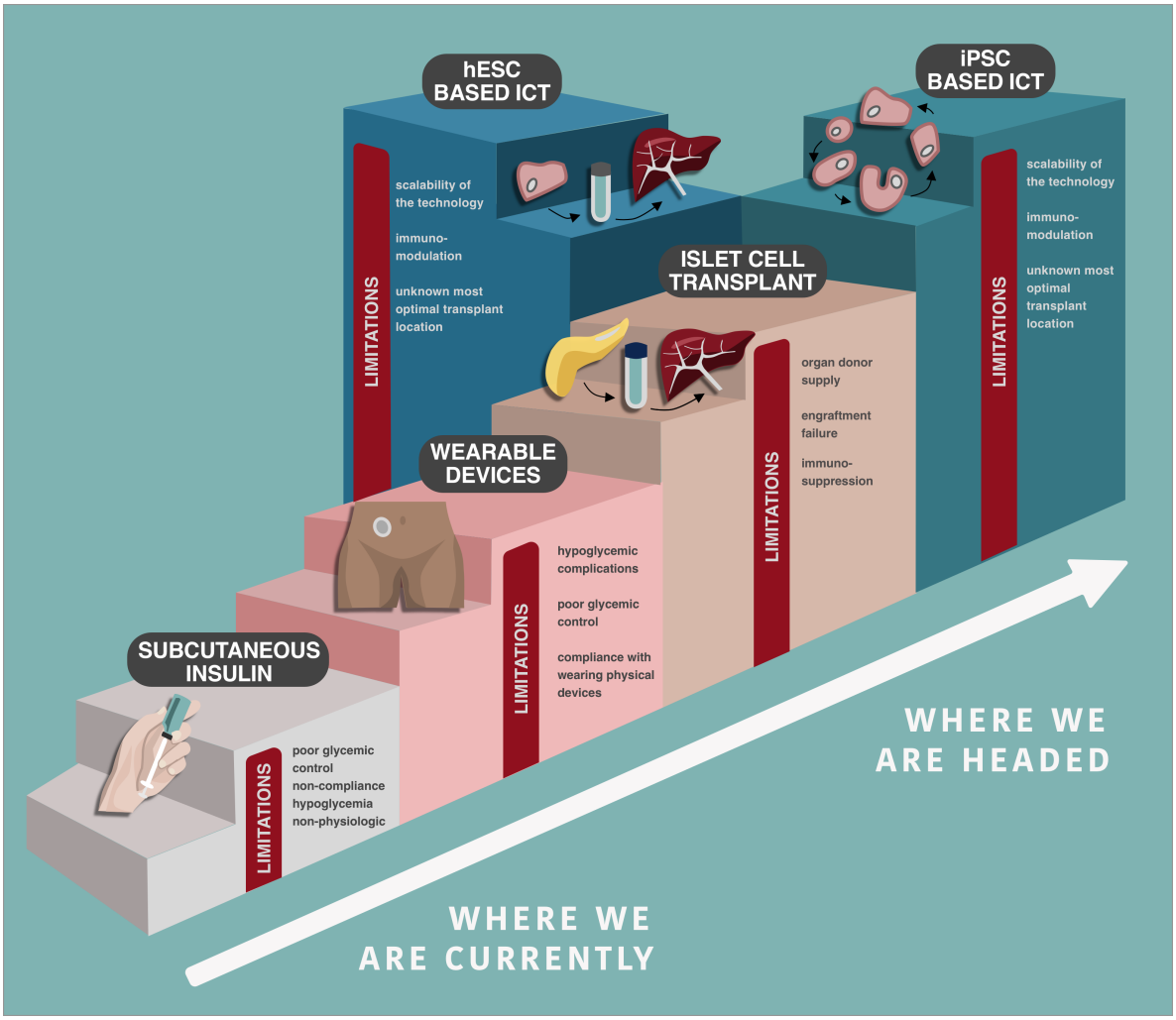

Figure 1.2.3 Comparison and advancement of subcutaneous insulin delivery, islet cell transplant, and novel Induced pluripotent stem cell-based islet cell transplant for cure of diabetes.

1.2.6.1 Stem Cell Source

The two primary sources of iPSC are allogeneic and autologous, both offer benefits and drawbacks. Allogeneic sources allow for mass generation of islet-like cells from a single,

optimized iPSC source. Large pools of HLA-specific iPSC-generated cell lines could be generated to provide 'haploidentical' islet-like cells. This may offer a homogenous cell source with optimal glycemic control, less off-target growth, and easily accessible HLA-matched islets for SC ITx. However, despite major HLA matching, patients will certainly require some degree of immunosuppression as inevitable minor HLA mismatches will still generate immunoreactivity if not otherwise modified [101]. The most significant barrier to allogeneic transplant is therefore immunoreactivity and post-SC ITx immunosuppression requirements. ViaCyte (previously NovoCell) has been at the forefront of technologies attempting to resolve this barrier. They hope to demonstrate successful allogeneic SC ITx engraftment, hormonal release, and immunoprotection through clinical trials evaluating the PEC-Encap (VC01) and PEC-Direct (VC02) devices. Albeit these clinical trials use ESCs as the cellular substrate for differentiation and transplant, outcomes obtained from these groundbreaking efforts could prove valuable for iPSC-based therapies. The VC01 is a planar subcutaneous macro-encapsulation device for pancreatic progenitors with oxygen and nutrient transport capacity but also allo and auto immunoprotection to enable SC ITx without immunosuppression [102]. Phase 1/2 clinical trials have demonstrated pancreatic progenitor maturation without off-target growth. In ViaCyte's most recent clinical trials summarized in oral form, up to one third of patients demonstrated detectable human C-peptide in peripheral blood in previously C-peptide negative individuals with T1D. This correlated strongly with the persistence of polyhormonal insulin-expressing islet cells contained within the subcutaneous devices over time (unpublished data). The perforated VC02 device does not provide immunoprotection, but ongoing clinical trials are assessing efficacy of pancreatic progenitors to provide in-human insulin independence (Clinicaltrials.gov

Identifier: NCT03163511). Although long-term results were limited by the foreign body response [29,103-106]; discovery of novel biomaterials for encapsulation that abrogate this reaction would provide promise for immunosuppression-free SC ITx. Anderson et al. (2020) have demonstrated long-term insulin release in immunocompetent mice, without immunosuppression requirements or foreign body response, using microspheres and selectively permeable silicone devices coated with a synthetic polymer [107,108]. Previously successful microsphere and synthetic polymers that have enabled islet cell survival and immunoprotection in murine models have failed in humans due to a vigorous foreign body response [109]. Testing novel polymers in humans will certainly be required [107].

Autologous iPSC islet cell generation may allow for personalized SC ITx. Islet cell auto-transplantation following pancreatectomy in the context of chronic pancreatitis is a crude first representation of the potential of this approach. Zhao et al. have raised concerns that islet cell maturation may alter cellular immunogenicity and thereby confer acute rejection [110]. Further investigation has revealed that immunoreactivity is only conferred in retrovirus derived iPSCs due to leakage of transgenes and activation of neighboring genes, whereas plasmid derived iPSCs demonstrate negligible immune reaction [111-113]. iPSC ITx without immunosuppression requirements would therefore be technically possible but remains to be tested. The costs and time of generating person-specific iPSC and then maturing them into islet-like cell clusters confers an astronomical barrier, but the hope is that with economies of scale, process automation and increased efficiency, mass iPSC ITx manufacture will indeed be possible at reasonable cost. This will be critically dependent on advances in robotic engineering, artificial intelligence and machine learning, and collaboration with industry to take this from single patient to mass

manufacture over time. HLA screening of individual autologous iPSC islet cell clusters would be another barrier. Rather than a single screened HLA-specific pool of transplantable iPSC islet cells, each patients autologous iPSC and matured islets would requiring screening for genetic mutations and off target growth prior to transplantation [113]. Additionally, variability exists between different iPSC lines, mostly due to genetic background differences, and their ability to differentiate into functional cells of a given lineage [114,115]. Overall, a better understanding of the *in vivo* immune response to HLA-matched iPSC islet cell clusters, or other alternatives to immune acceptance (as discussed below), and calculation of the cost/time feasibility and optimization for personalized autologous SC ITx is needed to better determine the best source of iPSC islet cells.

Xenogeneic islet cell sources should not be overlooked. We have not reviewed them thoroughly here, but O'Connell et al (2013) provide a complete review of this solution to ITx [116]. It is important to be aware that xenogeneic (porcine) sources provide a potentially large source of mature, insulin producing islet cells for transplantation. Two concerns for this islet cell source are xenogeneic immune reaction and the risk of zoonotic infection of porcine endogenous retrovirus. Genetic engineering and encapsulation devices have been utilized to prevent these reactions and clinical trials may be within reach [117-121]. Two trials by a single group have evaluated encapsulated porcine ITx, both showing potential therapeutic benefit; unfortunately, this is yet to be replicated by others [122,123]. Despite advances, xenogeneic sources currently remain futuristic and require replication and larger scale studies to evaluate their clinical benefit.

1.2.6.2 Transplant Sites

The ideal implantation site for SC ITx should first and foremost provide hormone release in a physiologic location; other desired characteristics in decreasing importance include: vascular and environmental support for islet cell engraftment, easy access for transplantation, immunoprotection, and retrieval capacity. Potential sites include renal subcapsular, subcutaneous (within devices or modified spaces), omental, and intraportal [124,125]. The renal subcapsular space has demonstrated promising results in murine models, but has failed to achieve euglycemia due to limited subcapsular space and exocrine contamination in larger animals and humans [125-127]. The subcutaneous and intramuscular sites have been also investigated due to their easy transplant procedures, easy resection in case of off-target growth, and easy monitoring with non-invasive imaging. Major issues include non-physiologic release of hormones, poor vascular and environmental islet cell support, and immunoprotection. Recent unpublished results from ViaCyte are encouraging and the PEC-Encap (VC01) and PEC-Direct (VC02) devices may enable viable subcutaneous SC ITx, as long as the foreign body response can be mitigated through the use of novel biomaterials. Alternatively, Pepper et al. (2017) utilized this foreign body response to create a subcutaneous transplant site with neovascularization and collagen support for islet cell engraftment [124]. This technique enables optimized subcutaneous engraftment; however, immunosuppression remains a barrier to its applicability for widespread use. Overall, encapsulation devices offer a unique tool to study iPSC islet cell maturation and insulin release for DM reversal. Their greatest benefit is enabling in-human evaluation of off-target growth with easily retrievable devices and demonstrating applicability of iPSC islet cell cluster maturation and survival *in vivo*.

Insulin independence necessitates adequate islet engraftment without fibrosis, which is currently only offered with omentum and intraportal ITx. Omental ITx has demonstrated positive early results in animal and human studies [125,128,129]. Stice et al. (2018) demonstrated successful omental autologous ITx in four patients confirming prior promising animal studies. The omentum releases hormones into portal circulation, supports islet engraftment, and is relatively accessible if resection is needed [128,129]. The omentum also limits IBMIR, since no direct blood contact occurs[129]. A limitation of this site is that surgical placement is required, which may limit widespread use due to cost and access to operative time, but all clinical trials to date involving omental implantation have used minimally invasive laparoscopic approaches [128,129]. Clinical trials have begun to further evaluate the omentum, but direct comparisons with the intraportal site are needed to guide future endeavors. Intraportal ITx remains the clinical gold standard because it has demonstrated adequate hormone release into the portal circulation, islet cell engraftment, and accessibility via radiologically-guided injection. Initial concerns regarding an 11% risk of portal venous thrombosis and bleeding following intraportal ITx has been diminished and nearly eliminated through well-described techniques that ablate the hepatic catheter tract and post-transplant heparinization to limit thrombosis [130,131]. The remaining barrier to intraportal iPSC ITx, particularly due to the intrinsic inability to remove the infused islets from the liver, is uncertain off-target growth and teratoma formation. Off-target growth and teratoma formation has been demonstrated in 15-45% of cases when pancreatic progenitor cells were transplanted [29,34]. Off-target growth is likely reduced with more mature stage 6 cell-derived products but longer term follow up is ongoing, as is investigation of treatment for off-target intraportal growth with ablation techniques [30,31]. Overall, the omentum and liver remain potential

sites for transplantation but larger in-human trials of omental transplant have yet to be completed; intraportal transplant remains the most viable long-term option with physiologic hormone release, adequate islet cell engraftment, easy transplantation techniques, and is only limited by the IBMIR, post-injection complications, and graft irretrievability. Novel genetic techniques to biochemically eliminate transplanted cells with kill switches may enable intraportal transplant without concerns for off target growth as we discuss below [132,133].

1.2.6.3 Immunoreactivity

Immunosuppression requirements remain one of the largest limitations to ITx. Autologous iPSC transplant offers a solution but may be limited due to its high costs. Alternatively, HLA-matched allogeneic iPSC ITx would still require immunosuppression – likely at least as potent as current immunosuppression protocols used in islet transplantation today [100]. Approaches to managing or eliminating immunoreactivity for allogeneic iPSC islet cells are under examination. Liu et al. demonstrated that sourcing iPSCs from less immunogenic sources, such as umbilical mesenchymal cells instead of skin fibroblasts, could limit immunogenicity [134]. These iPSCs had statistically significant less immune reactivity, with less HLA expression and less T-cell expression of perforin and granzyme B, but results were modest and unlikely to enable immunosuppression-free transplant [134]. Micro and macro encapsulation allow immunoprotection for first-in-human safety and off target growth assessment, but are unlikely to provide a long-term solution with metabolic control and insulin independence due to the foreign body response. More definitive options for eliminating immunosuppression include immunomodulation, and iPSC gene editing.

Gene editing may offer the most robust option for eliminating immunosuppression requirements. It benefits from leaving the recipient's immune system untouched and capable of immune regulation and effective infection control. The CRISPR/Cas9 system has been widely used to create and study genetic disease states such as Rett syndrome [135], HIV [136], and Parkinson's [137], but has also been used to modify iPSCs and reverse genetic disease states *in vitro* [138-140]. These techniques may allow transplanted islet cell expression of tolerogenic cytokines, and immunomodulatory proteins. Increased interleukin-10 (IL-10) expression has demonstrated less immune activation, and improved graft survival without immunosuppression, in animal models for liver, lung, and corneal autologous transplant [141-143]. However, results demonstrate that although graft rejection is limited, it still occurs. On the other hand, complete elimination of HLA class-I molecules from stem cells offers a cellular transplant source readily available to all recipients independent of their genetic background or HLA type. HLA-silenced iPSC lines have been generated by targeted disruption of both alleles of the Beta-2 Microglobulin gene, and produce non-reactive iPSC cells in lymphocyte reaction assays with retained ability to differentiate into multiple cell lineages [144-146]. Further analysis with HLA-silenced iPSC islet cells for transplantation is required to determine long-term efficacy.

Immunomodulation or immune protection with genetic alteration may also offer protection from autoimmune re-activation. In patients transplanted with autologous or allogeneic HLA-silenced islet cells, patients with T1D will likely still suffer from autoimmune graft destruction. Exogenous IL-10 supplementation [147], and more recently, gene transfer and increased islet cell IL-10 expression has demonstrated delayed recurrence of DM after syngeneic islet transplantation [148-150]. Similarly, increased PD-L1 expression may block effector T-cell

mediated islet destruction and prevent autoimmune re-activation after SC ITx [151-154]. Both IL-10 and PD-L1 mechanisms typically occur *in vivo* through the action of Tregs [149,150]. Therefore, increasing this cell population could provide SC ITxs a similar immune protection. Studies have demonstrated alloantigen-specific immunosuppressive capacity of Tregs after transplant [155], and clear GMP protocols now exist to generate protective Tregs specific for recipient alloantigen's under GMP conditions [156]. Unfortunately, these protocols would require patients to receive numerous Treg doses to maintain autoimmune protection.

An alternative to exogenous Treg infusions is a technique termed "immune reset" where the inappropriately activated immune system is eliminated and replaced with one with decreased effector T cells and proportionally more Treg cells to eliminate islet cell autoimmunity. This method was first discovered through evaluation of bone marrow-derived hematopoietic and mesenchymal stem cells (BMSC) as a source of Induced islet cells [157]. Although iPSCs have largely supplanted BMSC as a stem cell source, evaluating the pathway of islet cell regeneration though BMSC transplant inadvertently led to immune reset discovery. Following experimentally induced DM in streptozotocin-treated mice [158,159], streptozotocin-treated rats [160], E2f1/E2f2 mutant mice [161], and non-insulin-dependent KKAy mice [162], early BMSC treatment induced DM reversal. Despite insulin production and DM reversal, Hasegawa et al. (2007) demonstrated that BMSC did not differentiate into islets but instead initiated islet regeneration from pre-existing pancreatic progenitor cells [163]. Voltarelli et al. (2007) tested these techniques clinically; they mobilized patient's CD34+ (hematopoietic BMSC), collected them via leukapheresis, and then intensively immunosuppressed patients for five days with cyclophosphamide and rabbit antithymocyte globulin for immune ablation. CD34+ cells were then re-introduced to patients

and 87% medication independence and 96% insulin-independence was achieved in 23 patients with newly diagnosed T1D [157,164]. Evaluation of this technique demonstrated that it not only leads to maturation of pancreatic progenitor cells into islets, but also resets the immune system to prevent cytotoxic T-cell activation through extended duration CD4+ T cell depletion [165].

Current immune reset techniques do not offer long-term insulin independence, primarily due to recurrence of autoimmunity. However, we currently have an ongoing clinical trial in Edmonton that is exploring the potential of the drug plerixafor to mobilize CD34+ stem cells into the peripheral blood. This trial, approved for adults and adolescent children with new onset T1D uses a single dose of T cell-depleting therapy, dual anti-inflammatory medications and a long-acting GLP-1 analogue to promote immune reset. Using this technique, BMSC are mobilized from a patient's own bone marrow and may enable yearly doses to maintain autoimmune protection.

Genetic modification may also resolve other barriers to iPSC ITx. Enabling non-immunogenic islet cells eliminates cost of personalized medicine but may also eliminate concerns regarding off-target growth. Off-target growth could be controlled using gene-edited cell lines with drug-inducible kill switches. Liang et al. (2018) demonstrated effective drug activation of an essential cell-division gene (CDK1), while Di Stasi et al. (2011) took this further and genetically expressed a drug-inducible caspase-9 (iCasp9) that allowed complete apoptosis of transplanted T-cells, even when they were not proliferating [132,133]. Incorporating a similar, inducible mechanism for apoptosis in iPSC islet cells has not been demonstrated, but would allow for mitigation of concerns for off-target growth and enable safe intraportal transplantation.

Many of these solutions to immunoreactivity have been proven but have yet to be trialed specifically for SC ITx and work remains to be done. Combining allogeneic protection with HLA-silenced iPSCs, autoimmune protection with IL-10 or PD-L1 expression for Treg activity upregulation, and immune reset together provides promise immunosuppression free SC ITx. Meanwhile, successfully demonstrating drug-induced apoptosis and safe intraportal transplantation may eliminate fears of off-target SC ITx growth. This would allow for a single source of allogeneic, but HLA-silenced and autoimmune protected islet cells, with controlled safety switches to enable immunosuppression-free intraportal transplant.

1.2.6.4 Scale out, Scale up, and Increased Culture Surface per Volume

As we move closer to a cell-based cure for DM, a significant challenge will be providing them to >8 million T1D patients [5,166]. A parallel can be drawn to chimeric antigen receptor (CAR)-T-cell oncologic immunotherapy; once CD19-targeted CAR-T-cell therapy demonstrated remarkable benefit for acute lymphoblastic leukemia, a significant bottleneck for widespread use developed [167,168]. Personalized CAR-T-cellular therapy demonstrates remarkable similarity to allogeneic iPSC-based therapies, with cell collection from patients, genetic modification using CAR cDNA and then subsequent cellular expansion and selection with quality control prior to patient use [167,168]. We expect a similar supply and demand bottleneck that will limit initial widespread use of iPSC ITx once therapeutic benefit is demonstrated and the complex manufacture processes have been stabilized. This bottleneck will be amplified if iPSC sources are autologous, since each patient will require unique iPSC generation and expansion; however, even if allogeneic sources are used, few labs currently exist that can make GMP iPSCs-based

islet cell clusters. A step-by-step approach to enable scale up and treatment of the hundreds of million patients with DM is needed. Learning from barriers faced by CAR T-cell therapy, we suspect that iPSC supply shortage may be overcome by generating a consistent GMP protocol for islet stem cell production, regionalization of iPSC islet cell generation, and technological solutions for mass production to create an economy of scale and inexpensive DM cure (Figure 1.2.4).

Figure 1.2.4 Steps to achieve widespread use of Induced pluripotent stem cell-based islet cell transplant.

The first step of scale up will be consistently demonstrating a safe, and efficient GMP protocol for iPSC ITx. CAR T-cell therapies initially struggled to achieve widespread use due to product heterogeneity caused by variability in "manufacturing processes, source materials, viral vectors, ancillary reagents, quality control, post-treatment immune monitoring, and government regulation [168]." iPSC ITx technologies should use this experience as a learning opportunity to standardize GMP protocols including processes, reagents, and quality control (Figure 1.2.4). Current iPSC islet cell production is variable, especially with regards to additives to improve specificity and efficiency [30,31,41]. Having standardized processes will enable creation of consistent, homogenous products and facilitate government approval with common international production standards and regulations. Standardization will also enable definition of critical quality standards forand quality by design, which sets out required attributes of the final products

and guarantee them via assurance of the design process rather than necessitating testing of each product, thus saving money [169]. Well defined, standardized GMP protocols will enable economically viable and consistent products for use.

Once standardized and approved, regionalization of processes should be then implemented. CAR T-cell therapy outcomes are limited by "vein to vein" time, whereas islets are capable of being preserved in culture [170]. CAR T-cell limitations initially forced patients to travel long distances for treatment, which reduced production capacity and made CAR T-cell transport difficult with specialized couriers required to maintain handling quality and proper therapy identification [170]. With standardized GMP protocols, iPSC generation and purification expertise may shift to regional centers to reduce laboratory production costs and ensure product consistency – a concept already proven in islet isolation for ITx [66,171-174]. This will require significant collaboration between the iPSC laboratories, transplant coordinators, researchers, technicians, physicians, and patients at the recipient's center, but will significantly reduce costs of producing GMP stem cell-derived islet cell clusters [174].

Lastly, aligning production and remuneration with demand and healthcare budgets may be the final barrier to making SC ITx a first line therapy for DM. This will require creating economies of scale. Centralized production lowers costs by spreading the initial monetary investment of an approved GMP facility but limits production capacity. Maximizing the production capacity of centralized facilities will become of utmost importance. Doses for most cell-based therapies are approximately 10^7 to 10^9 cells [169,174]. As above, ITx requires minimum >5,000 IEQ/kg and ideally >11,000 IEQ/kg for insulin independence [66]. Improved islet purity from the current 30-50% up to 100% with iPSC based islets will enable safe SC ITx of minimum

13,000 IEQ/kg and potentially up to 20,000 IEQ/kg to allow for functional reserve and improved long-term insulin independence. With a standard 70kg patient, and estimated 1,000 cells/islet [175], at least 9.1 x 10^8 to 1.4 x 10^9 cells would be required. Engineering collaborations will be required to enable single laboratories to create these large volume cell therapies within the size constraints of a lab; this will be emphasized if autologous cells are used, where one person's cells are reverted into iPSCs and then expanded exponentially prior to islet differentiation to provide a personalized cure, as opposed to a set number of HLA-matched, or HLA-silenced, allogeneic iPSC lines that could be expanded and banked. Keys to achieving an economy of scale with high-throughput and large-scale iPSC expansion (autologous or allogeneic) will be to identify an appropriate growth medium, extracellular matrix (ECM), and environment for mass production [176].

1.2.6.4.1 Growth Medium

Growth media provides important nutrients and cell signaling factors for iPSC expansion and differentiation. An ideal growth medium for commercialization would allow cheap, ethical, and easily reproducible products to be formed. This largely eliminates serum and animal-sourced media. Historically, fetal bovine serum was required for stem cell expansion but more recently, Chen et al. (2011) described the TeSR-E8 medium, which allows growth of various iPSC lines with improved reprogramming and experimental consistency [177]. Sui et al. (2018) have used a similar media (StemFlex), which claims to have fewer components and enables superior single-cell passaging, gene editing, and reprogramming [41]. Direct comparison of these two media is required to help guide iPSC expansion standardization.

1.2.6.4.2 Extracellular Matrix

Until recently, 2D culture was the only method for stem cell expansion. Stem cells grow as adherent colonies and upon detachment, degrade into embryoid bodies (EB) [178]. This is due to the requirement of ECM-integrin interactions to maintain pluripotency and continued expansion. Matrigel and Geltrex are two commonly used basement matrices used to allow ECM interactions in cell culture for iPSC expansion [30,31,41]. Unfortunately, these are semi-chemically defined, xenogeneic substrates, that are difficult to sterilize with standard techniques and have significant variability limiting them from clinical use [176,178]. Growth with recombinant laminin-511, a xeno-free recombinant protein, represented a significant advancement of our understanding of ECM importance [176,179]. It led to the discovery that the interaction between laminin -111, -332, and -511 and its primary receptor integrin α6β1, supports stem cell expansion and blocks differentiation into EB [178,180]. Unfortunately, many of these recombinant protein surfaces were limited by cost, therefore, novel synthetic surfaces have been developed to mimic these ECM interactions. The hydrogel poly[2-(methacryloyloxy)ethyl dimethyl-(3-sulfopropyl)ammonium hydroxide] (PMEDSAH), and polymer coating aminopropylmethacrylamide (APMAAm) have demonstrated iPSC expansion with maintained pluripotency, but only APMAAm is capable of being sterilized using common techniques but required growth with fetal bovine serum to enable stem cell expansion [178,181]. Overall, no ideal 2D ECM has been discovered; moreover, 2D cellular expansion is limited by cell growth surface area and would likely not be capable of expanding iPSCs up to the required scale of 10^7 to 10^9 cells. However, discovery that stem cells could be grown and expanded as spheroid clumps in 3D suspension culture has enabled significant scale-up. Previously, stem cells required micro carriers to enable suspension culture

and expansion, which significantly limited cell concentrations and expansion capabilities. However, suspension culture with Rho-associated coiled-coil kinase inhibitors (ROCKi) such as Y-27632 has allowed iPSC re-aggregation and prevention of apoptosis [182-184]. Enabling suspension-based iPSC culture and expansion will substantially increase scale-up capabilities, with limitations primarily driven by environmental factors, as discussed below.

1.2.6.4.3 Environment

The ideal environment for commercial scale cellular expansion will be automated with ideal oxygen, temperature, pH, and chemical factor conditions. To expand iPSC on a small scale, planar plasma-treated polystyrene tissue culture flasks are a viable, economical option [169]. However, for larger-scale expansion, planar growth is expensive, requires highly trained operators, and necessitates large GMP facilities [169,174]. With the advent of 3D stem cell expansion, bioreactors present a favorable option to allow automation, standardization, and reproducibility [169]. More importantly, they allow increased culture surface per volume by removing all the gas layers typically required for oxygenation when using cell stacks. They do this by conducting continuous nutrient replenishment, biochemical (pH, temperature, etc.) control, and waste disposal with recirculated culture medium. This also eliminates open processes, which require large clean rooms as necessitated in flask-based cultures [169]. Complete automation, as with the Lonza Cocoon platform, may provide adequate expansion, with personnel savings, standardization, and cost efficacy provided through an economy of scale [169,174]. It appears that with large volume demand, micro carrier technology is most economical with costs approximately \$700/dose of 10^9 cells, a value that could be further improved with

greater growth concentrations and lower growth media costs [174]. One barrier that remains is recovery of expanded iPSC cells. Following expansion, cells are washed and centrifuged for recovery. Typical centrifuges technologies shear cells and are not suitable for iPSC retrieval, requiring new technology such as closed continuous fluidized bed centrifuges to be optimized for retrieval [174]. With the advent of 3D iPSC expansion bioreactors will almost definitely be used to produce the large volume cells for transplant. Once therapeutic success has been achieved with iPSC ITx, bioreactor-based proof-of-concept first-in-human trials will occur soon after.

1.2.7 **Conclusion**

Subcutaneous insulin treatment remains the mainstay of T1D treatment. It enables sufficient carbohydrate metabolism for patients to survive, but remains far from ideal. T1D patients suffer from hypoglycemia, hyperglycemia, and associated complications that limit their quantity and quality of life. These complications persist despite novel technologies for glycemic monitoring and control. ITx has long provided hope for a cell-based cure. It continues to demonstrate advances with improved glycemic stability, less hypoglycemia, and improved DM-related complications. However, islet engraftment and long-term insulin independence remains approximately 50% and patients must be exposed to potential risks associated with lifelong immunosuppressant therapy. Deceased donor islet sources and funded access also remain limited.

Stem cells derived from ESCs or iPSCs, can be differentiated into mature insulin producing islet cell clusters capable of fully reversing diabetes in mice and rats. However, as ITx transforms from a deceased donor to ESC- or iPSC-based source, several questions will need

resolution. Autologous versus allogeneic iPSC sources face unique challenges. Immunosuppression remains a barrier for allogeneic iPSCs, whereas allogeneic sources facilitate scale up with creation of HLA-matched iPSC islet cell cluster banks that can be standardized. Results from ViaCyte's clinical trials demonstrating successful allogeneic islet maturation and resultant detectable C-peptide levels that correlate with persistence of polyhormonal islet cells within subcutaneous devices provides enthusiasm that immunoprotected allogeneic SC ITx is within reach. On the other hand, autologous iPSCs enable immunosuppression-free SC ITx, but may be difficult to scale up with such personalized medicine. Further evidence is also required to determine the safety and efficacy of other transplantation sites for SC ITx in comparison with the intraportal site, particularly in terms of its potential for off-target growth. Generation of iPSC islet cell clusters with inducible kill switches is also important to consider in this discussion. With these answers, clinicians will require collaboration with multiple parties in the government and industry to standardize GMP protocols, enable consistent international regulations, and create economies of scale. This will likely be enabled with bioreactors utilizing 3D culture expansion of iPSCs, regardless of allogeneic or autologous sources. Regionalization with economies of scale will then enable economic generation of curative therapy for DM. It is certainly an exciting time as we border a new frontier in diabetes care, transitioning from treatment to cure.

1.2.8 **References**

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Chapter 2: Optimizing Scalability of Stem Cell-Derived Islet Transplantation

- Subsection 2.1: Scaling Stem Cells to cure Millions of Patients with Diabetes: Approaches, Technology, and Future Directions
- Subsection 2.2: Suspension culture improves iPSC expansion and pluripotency phenotype

Chapter Summary

The second chapter of this thesis is composed of one review manuscript and one preclinical study, both aimed at resolving issues with regards to the scalability of stem cell-derived islet generation. Chapter 2.1 provides a review that discusses approaches that are being investigated and may be useful to improved scale up and scale out of stem cell products, focusing primarily on automation, artificial intelligence, three-dimensional culture, and bioengineering approaches. This is followed by Chapter 2.2, whereby we demonstrate that three-dimensional culture of induced pluripotent stem cell lines can generate millions of cells, is scalable into large vessel formats, and an ideal environment for cells to maintain their pluripotency and achieve a naïve pluripotency phenotype that is superior for differentiation.

2.1 Chapter 2 subsection 1 – Scaling stem cells to cure millions of patients with diabetes: approaches, technology, and future directions

This chapter subsection is *in press* as a chapter within the book "Handbook of Stem Cells: From Basic to Clinical Sciences." All figures and tables in this chapter have been adapted from this published work. Full citation: Verhoeff, K; Shapiro, A.M.J. Scaling Stem Cells to Cure Millions of Patients with Diabetes – Approaches, Technology, and Future Directions. Handbook of Stem Cells: From Basic to Clinical Sciences.

2.1.1 **Abstract**

Stem cells offer a renewable and safe source of islets to enable widespread immunosuppression-free islet cell transplantation as a potential cure for diabetes mellitus (DM). Preliminary reports from current stem cell-derived islet cell transplantation clinical trials show promise. More clinical trials are expected to report similarly favorable outcomes in the near future that hope to drive stem cell-based technology from consideration into reality. Although optimization is ongoing and proof of concept from these trials is crucial before implementation to become standard care, early consideration of process scalability to enable accessibility for millions of patients with diabetes is crucial. Planning for the success of these technologies means considering approaches, techniques, and technology early during the introduction of stem cell-derived treatments to optimize current investigation and maximize future utility.

Herein, we discuss the two top contenders for stem cell-derived islet cell transplantation: Induced pluripotent stem cell-based autologous islets and allogeneic embryonic stem cell-derived islets, and evaluate their potential for scalability. Further, we introduce the current investigation of artificial intelligence approaches to optimize cell selection and differentiation. We also review two-versus-three dimensional culture techniques and technological advances that hope to enable mass production of stem cell-derived islets in the future. Finally, we discuss an essential real-life consideration for these technologies – the cost and accessibility to the 40 million patients with DM worldwide. We intend to highlight the importance of scalability concerns early during the investigation to combat such problems and diminish potential scalability barriers in promoting early widespread stem cell accessibility to millions of patients.

2.1.2 **Introduction**

Type 1 diabetes mellitus (T1D) involves autoimmune destruction of the islets of Langerhans, which are responsible for the endocrine function of the pancreas. Islets constitute α- and β-cells, which respond to their local environment and are responsible for dynamic, responsive, glycemic control. In simple terms, when blood sugar is too high (i.e. hyperglycemia), insulin is released from β-cells to decrease blood sugar, and when blood sugar is too low (hypoglycemia), glucagon is released from α-cells to increase blood sugar. In this way, islets allow humans to achieve euglycemia (i.e. a glucose level from 3.9-10 mmol/L), in a physiologic rapidly responsive fashion, regardless of their glucose intake. Therefore, in patients with T1D, without islets to facilitate glycemic control, both hyperglycemia and hypoglycemia can occur, leading to substantive consequences from both states. With hyperglycemia, patients acutely experience increased urination (i.e. polyuria), thirst (i.e. polydipsia), nausea, vomiting, abdominal pain, fatigue, and fruity smelling breath. Physiologically, diabetic ketoacidosis (DKA) can occur, where the body fails to utilize energy sources appropriately, and transitions into a catabolic state to generate non-glucose sourced energy [1]. DKA rapidly becomes a life-threatening condition due to increasing dehydration, acidosis, and confusion, with a mortality rate of 0.3-1.3% [2,3]. Chronically, hyperglycemia leads to important microvascular and macrovascular complications that drastically reduce quality and quantity of life, including coronary artery disease, peripheral vascular disease, neuropathy, nephropathy, and retinopathy. On the other hand, hypoglycemia leads to immediate autonomic and neuroglycopenic complications including trembling, drowsiness, vision and speech problems, palpitations, anxiety, and most concerningly, loss of consciousness (i.e. diabetic coma) and death [4].

Chronically, hypoglycemia can lead to patients losing awareness of these events (i.e. hypoglycemic unawareness) and confers a substantial morbidity and mortality risk in patients with T1D [4,5].

Prior to 1922, T1D had an exceedingly high death rate, often within weeks of patient's demonstrating symptoms, with no available cure or treatment. In January 1922, after demonstrating success in animal models, Dr. Banting, Best, and Macleod, in Toronto, Canada treated the first patient with T1D using isolated insulin who then survived 13 years with ongoing treatment, a remarkable feat at the time. For their work, Banting and Macleod were awarded the 1923 Nobel Prize in medicine [6]. Despite the remarkable discovery Banting understood that insulin represented only a treatment, rather than a cure, noting this in his acceptance speech:

"Insulin is not a cure for diabetes; it is a treatment. It enables the diabetic to burn sufficient carbohydrates, so that proteins and fats may be added to the diet in sufficient quantities to provide energy for the economic burdens of life [6]"

- Banting

Our utilization of insulin over the last 100-years has provided lived experience of this statement. While the discovery of insulin allowed patients to survive the immediate complications from hyper and hypo glycemia, glycemic control secondary to exogenous insulin remained imperfect. Patients began experiencing the chronic complications of T1D and work to optimize insulin, its delivery, and technologies has occurred in response. Although this has led to substantive improvements over the last 100-years, even current technologies fail to achieve the dynamic, physiologic glycemic control achieved by endogenous endocrine function provided by

islet cells [5]. Using optimized insulin treatments, only 21% of adults in the United States with T1D achieve HbA1c goals (<7%) [5,7]. Furthermore, even with current technologies and insulin formulations, the mean HbA1c levels of children aged 13-17 with T1D remains 9.0%, only marginally lower than the 9.5% seen in the same population during the 1980s [5,7]. Hypoglycemia also remains prevalent, occurring in 31–41% of patients with T1D [8], often at night when morbidity risk is the greatest [4,9-11]. Of 11,061 patients in the American diabetes exchange, 6% reported hypoglycemic seizure or loss of consciousness during the previous three months from the survey [5,12].

The morbidity and mortality of diabetes mellitus (DM) is not restricted to those with T1D. More than 400 million patients worldwide are diagnosed with type 2 DM (T2D), and also experience substantial effects from hyper and hypo glycemia [4,7]. Patients with T2D experience glycemic variability because of deficient insulin secretion, relative tissue insulin resistance, and poor compensatory insulin secretion [13]. While these patients don't experience absolute lack of islets, the long-term metabolic outcomes remain similar. Patients with T2D experience similar hyper and hypo glycemia, symptoms, and complication s[4]. Most patients with T2D are treated with oral therapies [14]. Oral agents act to increase the increase the physiologic insulin production from remnant islets, or by increasing insulin sensitivity of peripheral tissues. When oral agents are inadequate due to worsening insulin resistance or further functional islet loss, insulin is introduced. Nearly 15% of patients with T2D require insulin therapy, a proportion that is continuing to grow [14]. Regardless of the therapy, oral agent or insulin, current treatments are aimed at increasing insulin function. Therefore, islet transplantation (ITx) could provide a similar result in these patients; however, ITx it is currently rarely applied for patients with T2D due to

the risk associated with lifelong immunosuppression, and because of easily accessible oral therapies. Despite those current limitations, as we discuss below, optimization of ITx techniques may eliminate immunosuppression requirements, and open the door for ITx in patients with T2D. This further highlights the need to optimize scalability during development processes, as the number of patients who may benefit from these approaches far exceeds only patients with T1D.

In hopes of optimizing glycemic control by providing patients with dynamic, physiologic glycemic control, transplant researchers have developed techniques to restore islet mass including whole pancreas transplant and ITx. Both techniques have shown promise, each with their own benefits and drawbacks [15-17]. While pancreas transplant offers a robust restoration of endocrine function, the procedure and post-operative immunosuppression confers risk to patients. On the other hand, ITx has limited perioperative complications, but also remains limited due immunosuppression requirements, and sometimes fails to provide endocrine function due to limited available islet mass, compounded by immune destruction. Further, both procedures are limited by donor supply, and therefore cannot meet the needs of >400 million patients with DM. Because of these limitations, and to optimize pancreas donor allocation, specific indications have been constructed to delineate who should receive each of these approaches [18-20]. For ITx, only patients with severe hypoglycemia, hypoglycemic unawareness, or brittle T1D with substantive glycemic irregularity are currently candidates.

Stem cell-derived ITx offers an unparalleled opportunity to eliminate donor supply limitations, immunosuppression, and further improve ITx outcomes. This would enable consideration of a greater patient demographic for transplant, including those with T2D. In this

approach, stem cells (SCs), including induced pluripotent stem cells (iPSCs) that we discuss in this chapter, are guided through differentiation to produce large numbers of islets for ITx. Currently, research to optimize these processes, and clinical trials evaluating their efficacy in humans are underway and showing promising preliminary results [21-27]. Using these approaches, the hope is to provide a potential cure for DM that can be available widely to all affected patients. While current deceased donor allogeneic ITx practices have shown remarkable success, they also remain limited to patients with T1D due to the associated risks of lifelong immunosuppression. However, using SC-derived ITx with the potential for immunosuppression-free transplant the opportunity to treat all patients with diabetes, including those with T2D, is a possibility. In fact, autologous or immune protected SC-derived ITx may offer the best results to those patients, as their islet grafts won't be impacted by the potential for recurrent autoimmune attack. Therefore, the aim of SC-derived ITx isn't just to cure approximately 8 million patients with T1D, but to potentially >400 million patients living with all forms of DM [7].

In order to meet that aim, scalability must be integrated into research and development processes. Consideration of scalability when evaluating the two primary approaches to stem cell derived ITx (i.e. allogeneic and autologous) should be considered. Similarly, early consideration and evaluation of techniques to optimize cell selection, including artificial intelligence, are needed. Complete understanding of stem cell growth conditions and expansion conditions are also required to produce a functional and rapidly expandable product. Finally, collaboration to achieve integration of engineering and automated systems need to be promoted to achieve efficient production. By considering and optimizing techniques for compatibility with these

essential components early in process development, the goal of providing ITx as a cure to DM can hopefully be recognized and provided to patients efficiently and economically (Figure 2.1.1).

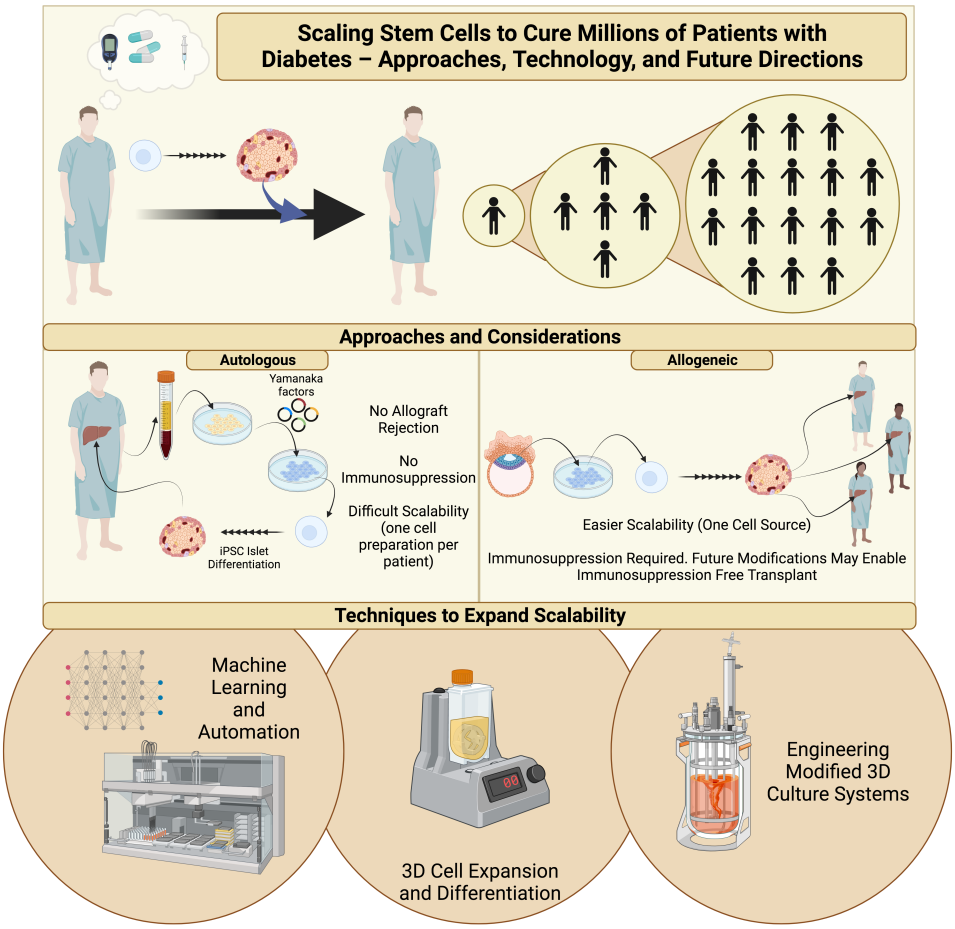

Figure 2.1.1 Chapter Summary and Overview of the Approaches to Scale Stem Cell-Derived Islet Transplantation.

2.1.3 Scaling Allogeneic Versus Autologous Stem Cells

Two approaches to SC-derived ITx are currently under investigation [22,23]. Each has their own benefits and drawbacks and have unique considerations for scalability. Within this section of the chapter subsection we will discuss the scalability considerations for both of these approaches. Allogeneic SC-derived ITx involves a small number of SC donors as the source for

differentiation and islet generation [24]. While potentially easier to scale for widespread application because of fewer SC sources, the requirement for immunosuppression or immune protection may limit its overall risk-benefit profile. Alternatively, autologous iPSC ITx involves generating patient specific iPSCs for islet generation, in turn eliminating any immunosuppression requirements; however because of the individualized iPSC and islets required for this approach, techniques to optimize scalability will be key to enable widespread use.

2.1.3.1 Allogeneic Stem Cell-derived ITx

Allogeneic SC-derived ITx involves a small number of embryonic or induced pluripotent SCs as the source for islet generation for all recipients. These SC sources are expanded and then differentiated into islets and provided as an allogeneic ITx to patients. Using this technique, SCs could potentially be expanded in large bioreactors to generate millions of cells for differentiation. Similarly, differentiation from this expanded SC source could more easily produce a large number of differentiated islets for ITx. While the scalability of this approach could eliminate the islet source limitation for ITx, without further process modification, patients would still require immunosuppression due to the allogeneic source of islets. Despite this limitation, recent clinical trials have demonstrated success from this approach. Vertex Pharmaceuticals Inc. has initiated their first-in-human phase 1/2 clinical trial, with promising early phase results testing the VX-880 embryonic SC-derived islet product. In their study, following intraportal transplantation and applied alongside immunosuppression, improved glycemic control and near complete insulin independence has been shown in a patient living with T1D, demonstrating proof-of-concept for these approaches [28]. Similarly, ViaCyte Inc's embryonic-derives stem cell islet like product has

recently demonstrated C-peptide production following in-human transplant into subcutaneous devices [24]. Future peer reviewed evaluation of more patients from these studies are needed to better evaluate these outcomes, however, these preliminary results suggest very promising potential for these therapies.

Currently, process optimization using the allogeneic approach hopes to either generate an human leukocyte antigen (HLA) islet bank to allow matched allogeneic transplant, modify SCs-derived islets to create a hypo-immune or immune protected product, or to transplant cells in extrahepatic sites that are immunoprotected. While generating islet banks to allow HLA matched ITx would likely be feasible, it is no different than current allogeneic ITx procedures where recipients receive matched deceased donor islets. This process would therefore not reduce immunosuppression requirements from current processes. Alternatively, generating a genetically modified SC product to enable hypoimmunogenic SC-derived islets may reduce or eliminate immunosuppression requirements. Genetically modifying ESCs to express immunotolerant molecules such as interleukin-10 (IL-10) or programmed death ligand-1 (PD-L1) [29,30], has been completed with ensuing reduction in T-cell and macrophage reactivity, and minimal NK cell-mediated death of islets [31-33]. Similarly, genetic modification has shown capacity to eliminate HLA class 1 expression with a similar reduction in immunoreactivity [31-33]. Combining multiple genetic approaches with both immunoprotective insertions and HLA elimination is also being considered, for example in ViaCyte Inc's PEC-QT system [22,23,34], but outcomes remain unreported. Finally, transplantation of these SC-derived allogeneic islets into immune protected devices or environments is also being evaluated [16,35]. Preclinical evaluation of devices capable of engrafting islets, while protecting them from immune destruction have had promising

preliminary results. For example, Anderson et al. (2020) have demonstrated insulin release in immunocompetent mice following immunosuppression free ITx into microspheres within selectively permeable silicone devices coated with a synthetic polymer [36,37]. However, long-term results and in-human evaluation are needed; previous encapsulation devices have initially shown promising results in animal models but unfortunately failed to translate clinically into humans secondary to the foreign body response [38-45]; discovery of novel biomaterials for encapsulation that abrogate this reaction would provide promise for immunosuppression-free ITx. More recently, a clinical trial (NCT03162926) evaluating safety and tolerability of ViaCyte Inc's VC-02 combination product, enabling subcutaneous immunoprotected SC-derived ITx has shown promising safety data with positive C-peptide production, suggesting potential to offer SC-derived transplant within subcutaneous devices if further optimization can be achieved [24,46].

While investigation of an allogeneic SC line that can be rapidly expanded and differentiated to create islets for allogeneic ITx initially appears feasible and scalable, more studies are needed. HLA matched ITx is unlikely to enable widespread SC-derived ITx due to immunosuppression requirements. Further, while genetic modification or immunoprotection may be possible, it remains uncertain whether immunosuppression will remain a barrier and whether genetic manipulation will affect the ability to produce functional, safe β-cells after differentiation. In terms of scalability, it also remains unclear whether modified SCs will be capable of exponential expansion and growth that currently enables generation of millions of cells for differentiation. Finally, although extrahepatic sites and devices offer potential immunoprotection, ITx into sites including the subcutaneous space, omentum, and gastric submucosa have been trialed clinically, but often fail to match results achieved pre-clinically

[45,47-54]. Ongoing studies and experiences with these techniques will certainly be valuable to guide future directions

2.1.3.2 Autologous iPSC-derived ITx

The second alternative approach for SC-derived ITx involves individualized iPSC generation that could be differentiated into islets and transplanted autologously. This technique, first involves patient specific generation of iPSCs. To accomplish this, somatic cells are reprogrammed into pluripotent cells by over-expressing the reprogramming transcription factors (Oct3/4, Sox2, Klf4 and c-Myc) discovered by Yamanaka et al. and Thomson et al. [55-57]. Most labs currently use peripheral blood mononuclear cells (PBMC) and a commercially available Sendai virus transduction kit to efficiently produce good manufacturing practice (GMP) compliant iPSCs [58]. Currently, this is one of the only mechanisms to generate iPSCs in a good manufacturing practice compliant manner. Once iPSCs are generated, differentiation can occur to generate completely autologous islets [56,57,59,60]. This approach benefits from completely personalized cellular therapy, thereby eliminating any immunosuppression requirements. Because differentiation protocols and transplant techniques have been well described for allogeneic SC-derived ITx with clinical success, these methods will almost certainly translate to iPSC ITx and allow immunosuppression free ITx. In fact, preclinical studies have already demonstrated capacity to generate iPSCs, differentiate them, and reverse diabetes in animal models [56,57,59,60]. However, because each patient would require a unique iPSC line to be generated, differentiated, and quality checked prior to transplant, scalability represents a

substantial barrier to widespread use. Scaling approaches and technologies are therefore of utmost importance to the applicability of this approach.

In order to enable widespread, scalable application of iPSC-derived autologous ITx, optimization, standardization, and automation of each step will likely be required. As we discuss below, generation of iPSCs involves optimal clone selection, which may be able to be accomplished with artificial intelligence (AI) technology. Once iPSCs are generated, large scale expansion in commercial bioreactors, and automated cell processing during differentiation may enable this technique to be applied broadly for all patients with DM.

2.1.4 **The Role of Artificial Intelligence to Optimize Cell Selection and Differentiation**

As discussed above, the first step to autologous SC-derived ITx involves generation of an individualized patient-specific iPSC line. To achieve this, somatic tissues (often PBMCs) are collected and expanded *in vitro*. Once an adequate number of cells is grown, cells are then cultured with Sendai virus that enables expression of reprogramming factors and development of iPSCs. Sendai virus transduction efficiently delivers transgenes to recipient cells without genomic integration [61-64]. Despite being the most efficient method, only 1-3% of PBMCs successfully become iPSCs [65-67]. Additionally, cells must be passaged approximately ten times for the Sendai virus to be undetectable in reprogrammed cells [65-67]. Therefore, once cells are transfected, colonies must be selected, passaged up to ten times, and then characterized to determine the optimal iPSC clone. To accomplish that, each colony is selected manually based on its morphology and transferred to a culture dish for expansion as a unique clone. Once clones have been passaged ~10 times and reach adequate confluence (i.e. expansion over 2-4 weeks)

each individual clone must undergo complete characterization including flow cytometry, genetic analysis, viral screening, and RNA sequencing. Using these characteristics, an optimal clone, with the best expression of pluripotency markers and without viral infection or genetic shift, is selected as the final patient iPSC line. Cells from the optimal clone then require further expansion until an adequate number is available for differentiation, again taking approximately 1-2 weeks. Finally, the expanded iPSC line undergoes a 27-day differentiation protocol to generate islets for transplant. Prior to transplant, those cells then undergo re-characterization to evaluate differentiation success, off-target growth, and to ensure product safety. Together, the process takes approximately two-months, with cell maintenance, evaluation, and differentiation actions required nearly daily.

Currently iPSC generation and differentiation is completed manually. However, there are specific morphologic and growth kinetic cellular characteristics that may enable automated selection of optimal PBMCs for iPSC generation. In similar fashion, the optimal iPSC clone may be able to be selected with automated technology. Our own lab has shown capacity to identify, track, and select optimal iPSC clones using artificial intelligence (AI) technology (Figure 2.1.2). Artificial intelligence (AI) is capable of learning characteristic morphologic and growth features of iPSCs to allow optimal clonal selection [68,69]. Cellino Biotech is providing label free imaging combined with AI algorithms to select optimal iPSC clones for expansion in a closed cassette format [21]. Additionally, work to automatize these techniques has been ongoing since the mid 2000s, with several preliminary prototypes being reported from 2007-2015. This work was further improved and compacted by Paull et al. (2015) who demonstrated an automated system capable of growing somatic cells, selecting optimal cells for iPSC generation, and isolating the

best iPSC clones for expansion prior to differentiation [68]. Similarly, Konagaya et al. (2015) demonstrated the capacity to maintain and expand iPSCs in completely automated fashion for 60-days using similar techniques [70]. The StemCellFactory represents another recently described model system to optimize these techniques using commercially available systems and economically viable start-up costs [69]. Using the StemCellFactory, iPSCs can be thawed and cultured with the optimal clone selected for expansion [69]; this is all achieved robotically without manual requirements and within a closed system to maintain sterility and GMP conditions. Using technology such as these, automated, optimized iPSC generation has not only shown to be feasible, but has also led to improved cell quality. Cells grown in automated systems have less variability and improved capacity for differentiation [68-70]. Continued optimization and integration of these technologies into labs worldwide to enable scale up and SC product consistency will be crucial as SC-derived islets move from a preclinical setting towards clinical implementation.

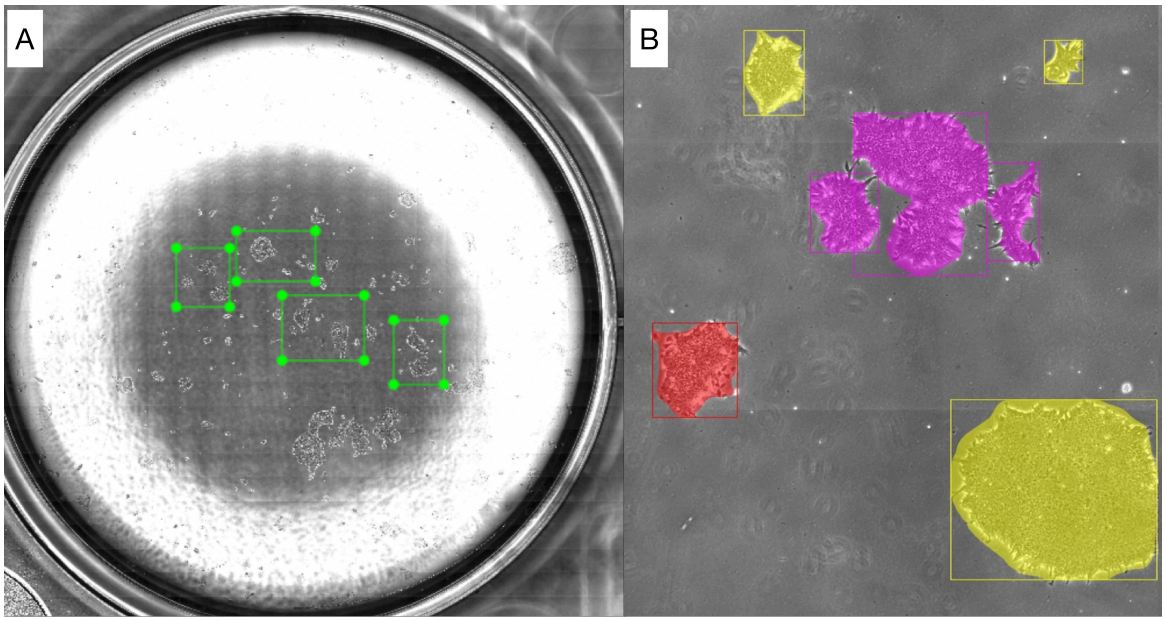

Figure 2.1.2 In vitro demonstration of induced pluripotent stem cell identification, monitoring, and selection using artificial intelligence technology.
A) Automated selection of iPSC colonies to track growth and expansion. B) Artificial intelligence selecting the optimal iPSC clone for selection according to cell morphology, growth, and expansion rate. (Figure from the Shapiro Lab, Edmonton, Alberta, Canada)

In addition to selecting optimal iPSC lines for autologous ITx approaches, automated AI directed technology also offers benefits to ESC-derived allogeneic ITx. While allogeneic approaches for SC-derived ITx don't face the challenge of first generating pluripotent lines, they do require cell maintenance and expansion. If a single modified ESC is used for all patients, that line must be grown, maintained in culture, and expanded prior to differentiation. While automated systems have shown capacity to select optimal iPSC lines, they can also be used to expand cells. Therefore, applying techniques and technology similar to these could be applied for maintenance and expansion of ESCs to also improve scalability of allogeneic SC-derived ITx approaches. These approaches are likely to maintain more consistent cell lines and reduce costs associated with daily manual cell maintenance, improving the potential for allogeneic SC-derived ITx.

2.1.5 Three-Dimensional Culture and Differentiation

Once iPSC or immunoprotected ESC lines are generated and maintained, expansion to achieve adequate cell numbers prior to differentiation represents the next hurdle. As discussed above, robotic and technological approaches have already demonstrated capacity for cell expansion; however, these technologies currently remain limited to two-dimensional (2D) cell culture conditions [71-74]. More recently, the potential for exponentially greater expansion using

three-dimensional (3D) cell culture systems has demonstrated promise for process scalability [75,76]. While 2D cell expansion with automation enables expansion without manual action, cell growth in these conditions is limited to the plated surface area. When cultured in 3D settings, supported initially with ROCK inhibitor (i.e. Y27632) to allow aggregation and survival, cells can expand within uniform cell clusters and achieve 50-100 fold expansion per week [75]. Similarly, Kallos et al. and PBS Biotech have demonstrated the capacity to achieve 30-50 fold expansion per week using vertical wheel 3D bioreactors, which can be introduced into labs without any significant setup requirements [77,78]. Cells expanded in this way are of high quality and capable of differentiating into islets and reversing murine diabetes [75,76]. Further, more recent islet cell differentiation protocols have suggested that 3D culture conditions enable optimized islet generation, especially during the later stages of cell maturation [56,60,79,80].

Due to the success of 3D SC culture and differentiation, optimization of previous automated cell culture technologies is now underway. Tristan et al. (2021) recently demonstrated an automated SC culture platform that is adaptable to 2D or 3D culture conditions [81]. While their technology allows 3D culture, the current design is limited to free-floating growth within T175 flasks. Further optimization of these technologies, with potential integration of the unique three dimensional culture systems discussed below, will be of great interest to the field as we hope to provide cells for millions of patients with DM.

2.1.6 **Engineering Modern Approaches to Scaling Three-Dimensional Stem Cell Culture**

Due to the recent success and publication of 3D cell culture from several labs, technologies to enable commercial scale cell expansion in these conditions are now being

developed and investigated. Commercial for profit organizations including Lonza, and Treefrog therapeutics have begun to take note, with development commercial sized 3D cell expansion technologies. For example, Lonza has developed a cocoon platform capable of generating personalized cell lines for hundreds to thousands of patients within confined spaces for patient-specific cell therapies [82]. Although these cocoons were originally developed for Chimeric antigen receptor T (CAR-T) cell expansion, investigation with regards to their applicability for SC expansion and islet differentiation is of great interest. Alternatively, Treefrog therapeutics has recently demonstrated preliminary findings evaluating their C-stem technology™, showing capacity to generate 15 billion cells per week in large 10 L suspension bioreactors (Figure 2.1.3). This accounts for 276-fold hiPSC expansion per week, which is the largest fold expansion reported to date [83].

Figure 2.1.3 Commercial sized bioreactor from TreeFrog therapeutics providing an example of expansion capacity for stem cells within large 3D culture conditions.
Image reproduced with permission from TreeFrog therapeutics [83].

The primary concern with these expansion technologies and commercial sized expansion techniques remain their efficacy to produce similar quality SCs and islets as demonstrated in individual labs. Using these large scale volumes and cell expansion techniques, it is possible that important reaction and differentiation chemodynamics are altered leading to variable product output. Further evaluation of these techniques, specifically to expand SCs and differentiate islets will certainly be valuable to guide future directions. Optimizing these scalable technologies simultaneously during initial clinical trials and investigation will enable direct implementation of SC-derived ITx broadly once proof of concept is demonstrated. In turn, this will lead to widespread application and improved accessibility of this revolutionary treatment.

2.1.7 **Patient Accessibility and Costs**

Fortunately, economic assessment of AI approaches, automated technologies, 3D culture conditions, and technological advancements has already demonstrated substantial expected savings. Economic analysis has demonstrated that although up-front costs are greater, automated systems have an overall savings of 42% over the expected 8-year lifespan of the machine compared to manual techniques when generating and maintain iPSC lines (Figure 2.1.4) [84]. Similarly, expanding cells under 3D culture conditions has shown to improve expansion capacity by at-least 10-fold compared to 2D conditions, further reducing costs. By compounding the potential 90% savings achieved through optimized 3D cell expansion, and 42% savings with

automated systems, we suspect that delivery of a cost-efficient approach for SC-derived ITx is achievable. Parallels may be drawn to the cost reductions recognized with CAR-T cell therapies, where initial costs for each patient cell therapy treatment was >1 million dollars but has now been reduced to < $100,000 per patient and with capacity for cost-efficient production in many University settings. We suspect a similar trend for SC-derived ITx, with hopes of providing widespread use to millions of patients. Considering the current cost of treating diabetes and its complications are the leading health expense for most nations, the potential savings are substantial.

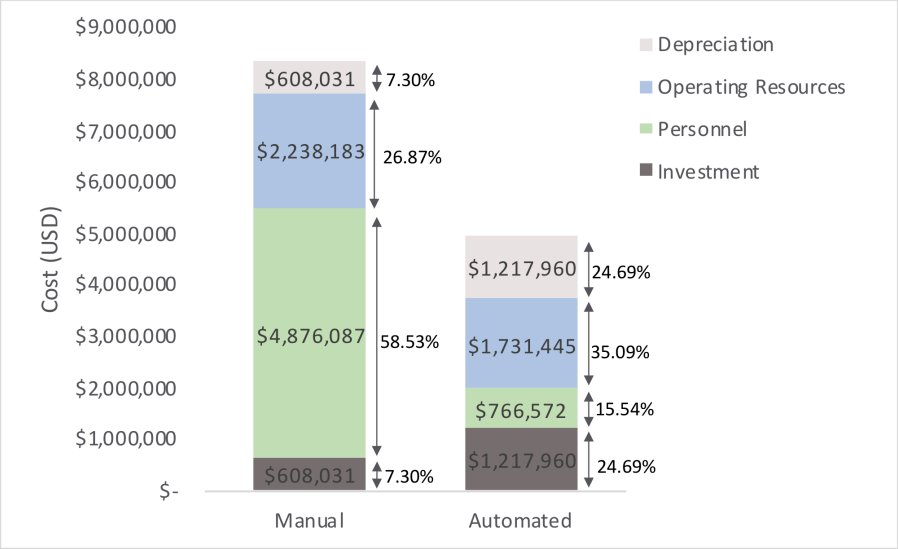

Figure 2.1.4 Cost analysis of automated versus manual generation, maintenance, and expansion of induced pluripotent stem cells using the StemCellFactory over 8 years. Image generated from data published by Nießing et al. (2021) [84] with costs modified from Euros to United States Dollars (USD) based on the exchange rate (1.2119 USD to 1 Euro) on January 28, 2021 (date of study publication).

By combining AI, automation, and optimized 3D culture systems, the cost of generating SC-derived islets for transplant is likely to decrease dramatically in the near future. Preclinical

studies has already garnered interest from corporate partners including ViaCyte Inc. and Vertex Pharmaceuticals to implement clinical trials. If clinical trials continue to show promising results, that investment is likely to grow, with the capacity to cover up-front costs for automation and large-scale commercial bioreactors. Despite the potential for accessible cost-appropriate SC-derived ITx, researchers and funders alike should be understand the investment potential of these therapies and consider accessibility to patients when partnering with the private sector. As discussed throughout this text, millions of patients stand to benefit from these therapies; providing an economically accessible therapy to all these patients is within grasp and remains in the hands of researchers currently investigating and implementing these approaches.

2.1.8 **Conclusion**

As cell therapies continue to be optimized for a potential cure for diabetes mellitus, we must continue to consider the scope of disease that we face. More than 8 million patients are currently living with T1D, and >400 million patients are affected with DM. Developing a therapy or potential cure that is inaccessible or non-scalable for the majority of patients should not be considered an option. Throughout research, development, and clinical trials we must continue to investigate and optimize techniques that enable scalable approaches. Early optimization and collaboration to enable technological and automated scaling should remain a priority alongside process development. If we continue to consider these techniques, generating a cell product that is accessible and cost-efficient is possible with the goal of curing millions of patients being the goal to aim for.

2.1.9 **References**

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2.2 Chapter 2 subsection 2 – Suspension Culture Improves iPSC Expansion and Pluripotency Phenotype

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2.2.1 **Abstract**

2.2.1.1 Background:

Induced pluripotent stem cells (iPSCs) offer potential to revolutionize regenerative medicine as a renewable source for islets, dopaminergic neurons, retinal cells, and cardiomyocytes. However, translation of these regenerative cell therapies requires cost-efficient mass manufacturing of high-quality human iPSCs. This study presents an improved three-dimensional Vertical-Wheel® bioreactor (3D suspension) cell expansion protocol with comparison to a two-dimensional (2D planar) protocol.

2.2.1.2 Methods:

Sendai virus transfection of human peripheral blood mononuclear cells was used to establish mycoplasma and virus free iPSC lines without common genetic duplications or deletions. iPSCs were then expanded under 2D planar and 3D suspension culture conditions. We comparatively evaluated cell expansion capacity, genetic integrity, pluripotency phenotype, *in vitro* and *in vivo* pluripotency potential of iPSCs.

2.2.1.3 Results:

Expansion of iPSCs using Vertical-Wheel® bioreactors achieved 93.8-fold (IQR 30.2) growth compared to 19.1 (IQR 4.0) in 2D ($p < 0.0022$), the largest expansion potential reported to date over 5 days. 0.5 L Vertical-Wheel® bioreactors achieved similar expansion and further reduced iPSC production cost. 3D suspension expanded cells had increased proliferation,

measured as $Ki67^+$ expression using flow cytometry (3D: 69.4% [IQR 5.5%] vs. 2D: 57.4% [IQR 10.9%], $p = 0.0022$) and had a higher frequency of pluripotency marker ($Oct4^+Nanog^+Sox2^+$) expression (3D: 94.3 [IQR 1.4] vs. 2D: 52.5% [IQR 5.6], $p = 0.0079$). qPCR genetic analysis demonstrated a lack of duplications or deletions at the 8 most commonly mutated regions within iPSC lines after long-term passaging (> 25). 2D-cultured cells displayed a primed pluripotency phenotype, which transitioned to naïve after 3D-culture. Both 2D and 3D cells were capable of trilineage differentiation and following teratoma, 2D-expanded cells generated predominantly solid teratomas, while 3D-expanded cells produced more mature and predominantly cystic teratomas with lower $Ki67^+$ expression within teratomas (3D: 16.7% [IQR 3.2%] vs.. 2D: 45.3% [IQR 3.0%], $p = 0.002$) in keeping with a naïve phenotype.

2.2.1.4 Conclusion:

This study demonstrates nearly 100-fold iPSC expansion over 5-days using our 3D suspension culture protocol in Vertical-Wheel® bioreactors, the largest cell growth reported to date. 3D expanded cells showed enhanced *in vitro* and *in vivo* pluripotency phenotype that may support more efficient scale-up strategies and safer clinical implementation.

2.2.2 **Background**

Human induced pluripotent stem cells (iPSCs) possess the potential to revolutionize the field of regenerative medicine, offering the capacity to generate autologous tissues such as islets, cardiomyocytes, retinal cells, or dopaminergic neurons [1-11]. However, to implement iPSCs and their ensuing islet, cardiomyocyte or other differentiated cell products clinically, up to 10^8-10^{10} cells per patient would be required [12,13]. Producing the required cells in a cost-effective and scalable manner remains a challenge. Further, to ensure cell product safety, expanded cells should ideally display a naïve pluripotency phenotype and maintain consistent differentiation capacity over time [12-14]. While substantial work has evaluated the ideal approach for iPSC generation [15,16] and tissue differentiation [1-11], few studies have comparatively assessed iPSC expansion protocols in terms of scalability, pluripotency phenotype, and differentiation potential [17-21].

After the discovery of human iPSCs in 2007 [22], initial iPSC expansion and differentiation experiments utilized two-dimensional (2D) planar expansion using feeder layers or supporting extracellular matrices, which resulted in approximately 10-fold expansion [1,2,6,17,23-25]. More recently, iPSC growth within three-dimensional (3D) suspension conditions using stirred suspension bioreactors has been suggested to provide a superior environment for expansion. This is due to improved mixing effects, which effectively distributes dissolved gasses and nutrients throughout the culture environment [18-21]. While initial studies achieved 20 to 40-fold expansion using suspension culture, some authors cautioned that the introduction of complex hydrodynamic forces in the bioreactor could adversely affect cell viability and pluripotency [20,26-28]. To mitigate these potentially deleterious effects, Vertical-Wheel® bioreactors have recently been

investigated because of their unique geometry which reduces shear stress effects and improves vessel content homogenization [27-30]. Despite the proposed benefits of suspension culture using Vertical-Wheel® bioreactors, protocol optimization with direct thorough comparison to 2D planar expansion techniques are needed. Additionally, while cells grown in both conditions are capable of differentiating into tissues of interest [1-11], comparison of their pluripotency phenotype remains elusive. Optimization of 3D suspension expansion protocols to achieve maximal iPSC expansion of homogeneous high-quality cells is essential to achieve adequate cell yield for safer, efficient, and cost-effective in-human clinical implementation of iPSC-derived cell therapies.

Herein, we present a modified iPSC expansion protocol using 3D suspension culture within Vertical-Wheel® bioreactors that achieves the largest cell expansion to date, in a single passage, while maintaining a high-quality cell product. We evaluate the expansion potential of this 3D suspension protocol in terms of growth kinetics, viability, genetic stability, pluripotency phenotype, *in vitro* and *in vivo* pluripotency against iPSCs expanded in 2D planar conditions. Results from this study elucidate opportunities and impediments for scalability of iPSC expansion to improve future clinical implementation of iPSC-derived cell therapies.

2.2.3 **Methods**

2.2.3.1 Experimental model and subject details

All procedures and protocols were approved by the Stem Cell Oversight Committee (SCOC), Canada and the University of Alberta Institutional Health Research Ethics Board (PRO00084032). All animal protocols were conducted in accordance with the Canadian Council on Animal Care Guidelines and Policies with approval from the Animal Care and Use

Committee (Health Sciences) for the University of Alberta. Animals were euthanized under anesthesia (5% isoflurane) by a combination of thoracotomy and exsanguination. Patients recruited as blood sample donors provided written consent for the use of tissue, cell reprogramming, and result disclosure. All experiments were planned *a priori* and completed in technical and biological triplicates based on standard experimental procedures without exclusion of experimental groups. The scientist performing analysis was blinded to the group allocation of samples. Other confounders were not controlled for.

2.2.3.2 Cell culture

Cell culture was completed using good manufacturing practice (GMP) compliant materials, where available, to replicate clinical conditions [31]. Cell processing was performed in a Class-II biocontainment compliant lab with the manipulation of cells taking place in a sterile environment with high efficiency particulate air filtration. Cells were maintained at 37°C with 5% CO_2 within humidified incubators.

2.2.3.2.1 Generation of induced pluripotent stem cell lines

In this study, 4 human iPSC lines were generated from peripheral blood mononuclear cells (PBMCs) from healthy donors (patient demographics in **Appendix** Table S2.2.3). Donor blood (20.0 mL) was collected into BD vacutainer spray coated K2EDTA tubes (Thermo Fisher Scientific cat.13-680-61). Collected blood was diluted equally with Ca^{2+}/Mg^{2+} free phosphate buffer solution (PBS, pH 7.2) with 2 mM ethylenediaminetetraacetic acid (EDTA; EMD Millipore cat. 324506). The PBS-Blood solution (20.0 mL) was carefully layered over top of

15.0 mL histopaque density gradient solution (Sigma, cat. 10771) in 2 tubes and centrifuged at 800 g for 30 minutes without breaks. The solution was washed with 20 mL PBS-EDTA and centrifuged at 300 g for 10 minutes to create a density gradient from which the PBMCs were isolated using a serological pipette. Isolated PBMCs were cultured in StemPro-34 Serum Free Complete Media (Gibco, cat. A14509) supplemented with human recombinant cytokines (10 ng/mL IL3, IL6, SCF and FLT3; R&D, cat. 203-GMP, 206-IL, 7466-SC, and 308E-GMP respectively) for 4 days. PBMCs were reprogrammed using the CytoTune iPS 2.0 Sendai Reprogramming Kit (Thermo Fisher Scientific cat. A16517), whereby 500,000 PBMCs were infected with the appropriate combination of Sendai virus particles (KOS, C-Myc, Klf4) for 24-hours (h) as per manufacturer recommendations. This was followed by cell culture with StemPro-34 Complete Media, supplemented with human recombinant cytokines (10 ng/mL IL3, IL6, SCF and FLT3) for 2 days. Single floating virus infected cells were pooled and transitioned to BioLite cell culture treated plates (Thermo Fisher Scientific cat. 130181) coated with human recombinant vitronectin (rhVTN) as per manufacturer recommendations (Thermo Fisher Scientific cat. A27940) and grown with StemPro-34 Complete Media from days 3-6 with daily media replacement. From day 7 onwards, attached cells were cultured using StemFlex media (Thermo Fisher Scientific cat. A3349401). Between days 15-20, individual colonies (hereafter referred to as clones) were handpicked under 10x phase objective (using ECHO inverted Rebel microscope and ECHO image acquisition application). Each clonal cell line was scrutinized for viral clearance and pluripotent stem cell quality control criteria (immunohistochemistry and flow cytometry for Oct4, Sox2, SSEA4, Nanog, Tra-1-81, and Tra-1-60, expression of alkaline phosphatase (ALP), and lack of duplications or deletions at the 8 most commonly mutated

regions within iPSC lines) with the best clone used to establish an iPSC cell line for this study. During this process of colony development and expansion, the entire cell culture dish was imaged to assess colony position and number of colonies reprogramed using the Cell Observation System Biostudio-T microscope (Nikon, MLA10000); image acquisition and processing was performed using NIS-element AR version 5.30.02 (Nikon, MQS31000) combined with PCR-AR-02 iPSC Colony Area Package (Nikon, MQS60002) software.

2.2.3.2.2 Induced pluripotent stem cell culture maintenance

iPSC lines were maintained in 60 mm rhVTN coated tissue culture plates (Thermo Fisher Scientific cat. 130181) with StemFlex media. rhVTN plates in this study were used only once to maintain GMP compliance, but we have also been successful using this technique with reuse of plates for up to five passages. Cultures were monitored daily using a Nikon TE300 Inverted Fluorescence Phase Contrast Microscope. Upon 80% confluency, cells were subcultured. For subculture from rhVTN plates, StemFlex media was removed and plates were washed with 2.0 mL of PBS. PBS was removed and plates were incubated for 2 minutes at 37°C and 5% CO_2 with CTS EDTA Versene Solution (Thermo Fisher Scientific, cat. A4239101) supplemented with 2 µl/mL Rho-kinase inhibitor (RockI; Y-27632 STEMCell Technologies cat. 72304). After incubation, the EDTA solution was removed and detachment of the cells was performed with mechanical disruption using StemFlex media supplemented with RockI (2 uL/mL). Cells were then collected into a 1.5 mL tube and spun down at 450 g for 2 minutes. The supernatant was removed, and cells were resuspended in culture media for subculture or used for experimental purposes.

Throughout this text, a cell passage is described as cell detachment from a culture dish in 2D planar conditions as described above, or dissociating clusters into single cells in 3D suspension conditions as described below. Quality control of the cell lines was routinely performed every 5 passages and prior to experimentation. Following each passage, cells were counted and viability was assessed using the Thermo Fisher Scientific Invitrogen Countess II AMQAX1000 Cell Counter. To accomplish this, 20 µL of single cell solution was combined with 20 µL of 0.4% trypan blue (Thermo Fisher Scientific cat. 15250061) and placed in a Countess cell counting chamber. Live cell numbers were used to calculate cell requirements for all processes.

2.2.3.2.3 Induced pluripotent stem cell expansion in 2D planar and 3D suspension conditions

Following cell passaging, iPSCs allocated for expansion were randomly assigned to 2D planar or 3D suspension conditions.

For 2D planar expansion $2x10^6$ live cells were seeded into 150 mm plates coated with Geltrex (Thermo Fisher Scientific cat. A1413302) in 20 mL of StemFlex media with RockI (2µL/mL). Geltrex coating was prepared at 6 µg/mL concentration using cold DMEM-F12 media (Thermo Fisher Scientific cat. 10313021) and incubated for 1h at 37°C prior to cell seeding as per manufacturer recommendations. 24 h post-seeding, media was removed and 20.0 mL of fresh pre-warmed StemFlex without RockI was added. Media was replaced daily for 5 days during cell expansion. Media was collected daily to assess pH, glucose, lactate, lactate dehydrogenase, ammonia, and glutamine using the Cedex bio analyzer (Roche cat.

06395554001). Cells were lifted for evaluation by incubating them with CTS EDTA Versene Solution for 8 minutes at 37°C.

The 3D suspension expansion protocol was modified from previously published reports by Borys et al. (2020), Rohani et al (2020), and Dang et al. (2021) [27,32]. For expansion, $2x10^6$ live iPSCs were seeded into 0.1 L Vertical-Wheel® bioreactors (PBS Biotech Inc.) in 55.0 mL of pre-incubated StemFlex media with RockI (2 µL/mL) (day 0) with constant rotational speed of 60 revolution per minute (rpm). Pre-incubation of StemFlex media is critical to allow temperature and pH stabilization. After 24 h, clustered iPSCs were then supplemented with 45.0 mL of StemFlex media without RockI (day 1). On day 3, clusters were allowed to gravity settle and the upper 50.0 mL of StemFlex was replaced with 50.0 mL of fresh pre-incubated media. On day 5, clusters were harvested for experimental purposes or were dissociated for further expansion. Media was collected on days 2-5 for 3D suspension when cells would settle by gravity to allow media sampling.

For passaging of 3D suspension iPSCs, clusters were allowed to gravity settle and StemFlex was removed. Clusters were washed with 30 mL of PBS with RockI (2 µL/mL) and were allowed to gravity settle. Supernatant was removed and clusters were incubated within the bioreactor with 10.0 mL of StemPro Accutase enzyme supplemented with RockI (2 µL/mL) for 10 minutes at 20 rpm and 37°C. Following incubation, clusters were immediately disrupted with mechanical forces aspirating the clusters up and down with a 10 mL serological pipette. Single cells were transferred into a 15 mL conical tube and centrifuged at 450 g for 2 minutes. Supernatant was discarded and cells were resuspended in 10 mL of StemFlex with RockI. Finally, cells were counted for live and dead cells with trypan blue solution described above and

subsequently cultured or prepared for experimental purposes. During media replenishment and exchanges, spent media was collected for assessment.

To further compare the efficiency of our expansion protocol to other 3D protocols, we replicated the protocol described by Dang et al. (2021) that previously reported the highest fold expansion using Vertical-Wheel® bioreactors. To reduce inter-protocol variability, we cultured cells using StemFlex rather than the modified B8 media initially reported by Dang et al. (2021). In this protocol, $2x10^6$ live iPSCs were seeded into 0.1 L Vertical-Wheel® bioreactors (PBS Biotech Inc.) in 100.0 mL of StemFlex media with RockI (2 μL/mL) (day 0) with a constant rotational speed of 60 rpm (day 0). On days 3, 5 and 6, clusters were allowed to gravity settle and the upper 50 mL of StemFlex was replaced with 50 mL of fresh pre-warmed StemFlex. Cell counts and aggregate sizing samples were taken daily from the bioreactors to assess growth kinetics and aggregate morphology. On day 7, clusters were harvested for experimental purposes or were dissociated for further expansion.

2.2.3.2.4 Embryoid Body Formation

Embryoid body (EB) formation was performed using AggreWell 400 plates (STEMCell Technologies, cat. 34425) as per manufacturer instructions. Briefly, each well of the AggreWell 400 plates was rinsed twice with 2.0 mL of Anti-Adherence Rinsing Solution (STEMCell Technologies cat. 07010) followed by centrifugation at 1300 g for 5 minutes in a swinging bucket rotor fitted with plate holders. Anti-Adherence Rinsing Solution was replaced with 5.0 mL of DMEM/F12 before use. Following preparation of the AggreWell 400 plates, 2D planar cells or 3D suspension cells were lifted and/or dissociated and a single cell suspension in

StemFlex media with RockI (2 µL/mL) was prepared. Next, $4x10^6$ cells were seeded per well and plates were incubated at 37°C for 24 h. After 24 h, the EBs were gently collected from the AggreWell 400 plates by pipetting the media up and down with a wide bore 10 mL pipette and were transferred into a 50 mL conical tube where they were allowed to gravity settle. Supernatant containing StemFlex media with RockI was removed and EBs were resuspended into fresh StemFlex media and transferred into a 100 mm low adhesion plate. EBs were kept in culture for an additional 4 days with media change on day 3.

2.2.3.2.5 Trilineage differentiation

To evaluate the pluripotency potential of 2D planar and 3D suspension expanded cells we completed trilineage differentiation using the Human Pluripotent Stem Cell Functional Identification Kit (R&D cat. SC027B). 2D planar cells were seeded into a Geltrex-coated plate (or coverslip for immunohistochemistry samples) then grown and differentiated according to manufacturer instructions. We attempted to differentiate 3D suspension cell clusters by placing 2000 clusters into a 6-well suspension culture plate alongside 2.0 mL of differentiation media; unfortunately, the trilineage differentiation kits were not capable of maintaining cells in 3D culture leading to 100% cell death. Instead, 3D cell clusters were dissociated and seeded into a 60 mm rhVTN coated plate (or coverslip for immunohistochemistry samples), allowed to grow until appropriate confluency, and differentiated as per kit instructions (R&D cat. SC027B).

2.2.3.3 Molecular biology

2.2.3.3.1 Ribonucleic acid (RNA) extraction and reverse transcription

Prior to RNA extraction, all materials were cleaned with RNase AWAY to decontaminate surfaces (Thermo Fisher Scientific cat. 10328011). A pellet of a maximum of $5x10^6$ cells was lysed with 350 µL RLT buffer (Qiagen cat. 79216) and frozen at -80°C until RNA extraction. Suspension of lysed cells in RLT buffer was thawed and cells were disrupted and homogenized using the QIAshredder system (Qiagen) and total RNA was then extracted with the RNeasy Mini Kit (Qiagen cat. 74104) according to the manufacturer instructions. Concentration and purity of the isolated RNA samples was evaluated using spectrophotometry with the Multiskan SkyHigh Microplate Spectrophotometer and µdrop plate (Thermo Fisher Scientific cat. A51119600DPC) by assessing the 260/280 nm and 260/230 nm absorption of samples. Samples were then stored at -80ºC until needed; RNA was quantified after each defrost.

RNA was reverse-transcribed using the RevertAid First Strand cDNA Synthesis Kit as per manufacturer guidelines (Thermo Fisher Scientific, cat. K1621). Complement DNA (cDNA) was stored at -20ºC until required for PCR.

2.2.3.3.2 Polymerase chain reaction (PCR)

cDNAs were thawed and combined with PCR mix as described in **Appendix** Table S2.2.4. GoTaq G2 Hot Start Colorless Master Mix (Promega, cat. M7422) was used alongside forward and reverse primers as described in **Appendix** Table S2.2.5. Samples were placed in a thermocycler and underwent the sequence specified in **Appendix** Table S2.2.6**.** For mycoplasma

detection, Mycoplasma PCR Detection Kit (ABM cat. G238) was used as per manufacturer instructions. Samples were loaded into a 2% agarose gel (Invitrogen cat. 16520-050) with GelRed 6X loading Dye (RCD cat. 41003) and ran for 35 minutes at 100 volts. Gels were visualized using the Image Quant 300 Gel documentation station under ultraviolet light.

2.2.3.3.3 Genomic DNA extraction

Whole genomic DNA (gDNA) was extracted by lysing a maximum of $5x10^6$ cells for 18-24 h at 55°C in 487.5 µL TENS buffer (10 mM Tris-HCl (Sigma cat. T3253) pH 8.0, 25 mM EDTA (Sigma cat. 324506) pH 7.5, 100 mM NaCl (Sigma cat. S1679), 0.5% SDS (Sigma cat. 71736)) with 12.5 µL of proteinase K (20 mg/mL; Sigma cat. 70663-4). Proteins were precipitated using 250 µL of 6 M NaCl followed by centrifugation for 5 minutes at 12,000 g. The supernatant was recovered, and gDNA was precipitated with 900 µL isopropanol followed by centrifugation at 12,000 g for 10 minutes. The pellet was collected and washed with cold 70% EtOH and allowed to dry. gDNA was resuspended in 50 µL of TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA pH 8.0) and purity was assessed using the Multiskan SkyHigh Microplate Spectrophotometer and µdrop plate (Thermo Fisher Scientific). Sample purity was measured by determining the 260/280 nm and 260/230 nm absorption ratios with samples achieving 1.7-2.0 and 2.0-2.2 respectively being used.

2.2.3.3.4 Quantitative PCR (q-PCR)

For genetic analysis, reactions were set up in 96-well plates using the hPSC Genetic Analysis Kit (STEMCell technologies cat. 07550) as per manufacturer instructions. qPCR was

performed using the StepOnePlus Real-Time PCR System (Thermo Fisher Scientific cat. 4376600) and gDNA was amplified as per **Appendix** Table S2.2.7. Samples were analyzed using chromosome 4p as reference using the calculations below:

$$\Delta C_t = C_t\ (target\ region) - \ C_t(chromosome\ 4p)$$

$\Delta\Delta C_t$ was calculated by subtracting the average ΔC_t of the gDNA from the control sample (supplied with the kit) for each sample to be tested.

$$\Delta\Delta C_t = C_t\ (\Delta sample\ of\ interest) - \ \Delta C_t(chromosome\ 4p)$$

Data were represented as $2^{(-\Delta\Delta CT)}$ x 2, which enables the visualization of copy number for the specific chromosomal regions of each sample.

$$Copy\ number = \ 2^{(-\ \Delta\Delta C_t)} \cdot 2$$

The median of the RQ values of each sample was used for statistical analysis.

2.2.3.3.5 Quantitative reverse transcription PCR (qRT-PCR)

Custom designed gene TaqMan Low Density Array Cards were used as per manufacturer instructions (Thermo Fisher Scientific cat. 4342253); gene array set up is described in **Appendix** Table S2.2.8. Briefly, 500 ng of cDNA was combined with 55 µL of nuclease free water and 55 µL TaqMan Universal PCR Master Mix (Thermo Fisher Scientific cat. 4305719). The combined solution was loaded into the gene array cards, centrifuged, and processed using the FAST-384 well array program via the QuantStudio 12K Flex Real-Time PCR system.

Alternatively, pairs of primers were designed (sequences detailed in **Appendix** Table S2.2.9) to quantify the amount of specific cDNA by SYBR Green qRT-PCR (Thermo Fisher Scientific cat. 4385612). qRT-PCR assay was performed using the Applied Biosystems 7900HC

Fast Real-Time PCR Systems detection system (Applied Biosystems). Samples were analyzed using B2M as reference for data normalization.

In all cases, data was analyzed and represented as a heat map and/ or $2^{(-\Delta\Delta CT)}$ using GraphBio [33] or GraphPad Prism version 9.3.1 for Mac, GraphPad Software, www.graphpad.com.

2.2.3.4 Protein biology

2.2.3.4.1 Alkaline phosphatase staining

iPSCs were seeded and grown on Geltrex-coated 24x24 mm glass coverslips in 6-well plates. iPSC colonies were washed with PBS and fixed in 4% paraformaldehyde (PFA) for 20 minutes. Colonies were washed three times with PBS and ALP-substrate staining solution (Abcam cat. Ab242287) was added as per manufacturer recommendations. Cells were incubated for 20 minutes in the dark at RT and then washed with PBS. Colony images were acquired and analyzed with the ECHO Rebel inverted microscope (ECHO).

2.2.3.4.2 Flow cytometry

$1x10^6$ live cells were filtered through a 40 μm cell strainer (Thermo Fisher Scientific cat. 22363547) and fixed with 4% PFA for 20 minutes at RT. Upon fixation, cells were centrifuged at 700 g for 2 minutes and supernatant was removed. Cells were then permeabilized and stained using the BD Cytofix/Cytoperm Fixation/Permeabilization Solution Kit (BD Biosciences cat. 554714) as per manufactures instructions. Primary antibodies were incubated for 1 h and secondary antibodies for 30 minutes according to the dilutions in **Appendi**x Table S2.2.10. Cells

were resuspended in fluorescence – activated cell sorting buffer (2% FCS, 2 mM EDTA in DPBS) and kept on ice until flow cytometry acquisition and analysis.

Isotype controls were used to accurately gate positive staining and data were acquired using the CytoFLEX S flow cytometer and analyzed using the CytExpert software (Beckman Coulter).

2.2.3.4.3 Immunohistochemistry

For 2D planar cell immunohistochemistry, cells were grown on coverslips and fixed with PFA as above. Cover slips were stored in PBS until staining. 3D suspension clusters were washed with PBS and fixed with 4% PFA for 30 minutes on ice. The PFA was removed and clusters were suspended in 1% low melting agarose within a silicone histology mold. The solidified agarose-cluster preparation was removed from the mold, placed in wax on a histology cassette for paraffin embedding and processing. Sections of 8 μm on glass slides were used. Slides were incubated for 40 minutes at 60°C to melt the paraffin and allow cell adherence to the slides followed by rehydration. Slides underwent antigen retrieval in warmed citrate buffer (0.0126 M citric acid, Sigma cat. C-0759; 0.0874 M sodium citrate, Sigma cat. S-4641; pH 6.0) for a total of 20 minutes. Slides were then ready for staining.

Slides and cover slips were blocked for 1 h at RT with 5% normal donkey serum (Sigma cat. S30-M) in FoxP3 permeabilization buffer (Biolegend cat. 421402). Primary antibodies were diluted in FoxP3 permeabilization buffer as per **Appendi**x Table S2.2.10 and were incubated for 2 h at RT in a humid dark chamber. Slides and cover slips were washed 3 times with 0.1% Tween 20 in PBS followed by incubation with secondary antibodies diluted in FoxP3

permeabilization buffer for 40 minutes at RT in the dark. Slides and coverslips were washed 3 times in 0.1% Tween 20 in PBS prior to incubation with DAPI (Sigma, D1306) for 4 minutes at RT in the dark. Slides or cover slips were then washed with PBS and mounted with fluoromount-G (Thermo Fisher Scientific cat. 00-4958-02). Slides were visualized using the Zeiss Observer Z1 inverted fluorescence microscope and images were processed using Zeiss software.

2.2.3.5 Teratoma assay

Male immunocompromised SCID beige mice, aged 16-18 weeks (Charles River Laboratories) were used. iPSCs were transplanted under the kidney capsule for 60-days (8 weeks), recovered, and assessed (n = 6 per group). For cells expanded in 2D planar, cells were lifted as per passaging protocols and transferred to a 15 mL conical tube with StemFlex supplemented with RockI (2µL/mL). Cells were aliquoted at $1x10^6$ cells per tube, which were centrifuged to remove media. Cells were combined with 15µL of matrigel (Sigma cat. CLS354277) and placed on ice. For 3D suspension preparations, cell clusters were collected from the bioreactor into microcentrifuge tubes and were ready for transplant (as clusters and without matrigel). In both cases, 3D expanded iPSC clusters or 2D expanded single cells embedded into matrigel were aspirated into polyethylene-50 tubing with a microsyringe. A left lateral paralumbar incision was made and the left kidney was delivered. The kidney capsule was incised and the cells were infused [34,35]. Mice were anesthetized with 5% isoflurane. Buprenorphine (0.1 mg/kg subcutaneous) was administered for post-operative analgesia. Mice were assessed daily for humane end-points described by any mouse distress or change in physiologic condition. Throughout care, mice were housed within GM500 Mouse IVC Green

Line cages in the Health Sciences Laboratory at the University of Alberta, in compliance with the Canadian Council on Animal Care guidelines.

On post-operative day 60, non-recovery nephrectomy was performed. Kidney cross-sections were performed, fixed in 10% formalin, and paraffinized. 8 μm sections were stained with hematoxylin and eosin (H&E) or prepared for immunohistochemistry as above. H&E-stained slides were assessed by a board-certified pathologist.

2.2.3.6 Statistical Analysis

Normality testing was performed using the D'Agostino-Pearson normality test, which determined the need for non-parametric testing. Between group comparisons were carried out using the non-parametric Mann–Whitney U test or Kruskal–Wallis test with the alpha value set at 0.05. Continuous values are presented as medians with interquartile ranges (IQR), and with discrete values presented as absolute values with percentages. All statistical analysis was completed using GraphPad Prism version 9.3.1 for Mac, GraphPad Software, www.graphpad.com.

2.2.4 **Results**

2.2.4.1 Generation of iPSC lines from human peripheral blood mononuclear cells

Following Sendai virus infection of human donor PBMCs, iPSC-like colonies were screened to select an optimal clone for iPSC line establishment (Figure 2.2.1A). PBMCs grew independently with round shape, while iPSC-like colonies displayed compact cell-to-cell connections, rounded colony margins, and condensed nucleus with minimum cytoplasm (Figure

2.2.1B). Manually picked and individually isolated clones (10-12 clones) were characterized at passage 3-5 according to current standards [22,36]. Clones were assessed for expression of alkaline phosphatase (ALP) (Figure 2.2.1C), Nanog, Tra-1-81, Sox2, Tra-1-60, and SSEA4 (Figure 2.2.1D and **Appendix** Figure S2.2.8A-B). A single ALP-stained clone attaining 99.9% Tra-1-60^{+}SSEA4^{+} and 98.3% Sox2^{+}Nanog^{+} was selected for iPSC line establishment (**Appendix** Figure S2.2.8A). Immunohistochemistry demonstrated the selected clone to be positive for Oct4, Sox2, SSEA4, Nanog, Tra-1-81, and Tra-1-60 (Figure 2.2.1E). The clone's lack of duplications or deletions at the 8 most commonly mutated regions within iPSC lines was demonstrated by lack of alteration in copy number (Figure 2.2.1F) [37,38]. Upon establishment of the iPSC line, genomic profiling of 48 key human pluripotency targeted genes using TaqMan low density array cards displayed downregulation of somatic cell markers, like *Sox17* and *IL6*, while observing pronounced upregulation of pluripotency marker expression such as *Lin28*, *Sox2* and *PODXL* (Figure 2.2.1G-L). Furthermore, *SEV* and *SEV-KOS* levels were identical to uninfected PBMC levels, which do not express *SEV* and *SEV-KOS* genes, ensuring lack of Sendai virus host-genome integration (Figure 2.2.1M-N). PCR of the established iPSC cell lines at passage 10 using primers specific for the amplification of the Sendai virus further confirmed lack of Sendai viral vector integration (**Appendix** Figure S2.2.8C). PCR of the genetic material present in the supernatant confirmed lack of mycoplasma contamination (**Appendix** Figure S2.2.8C). These results demonstrate efficient reprogramming of PBMCs into iPSCs. The process was repeated to generate iPSC lines from four healthy volunteers.

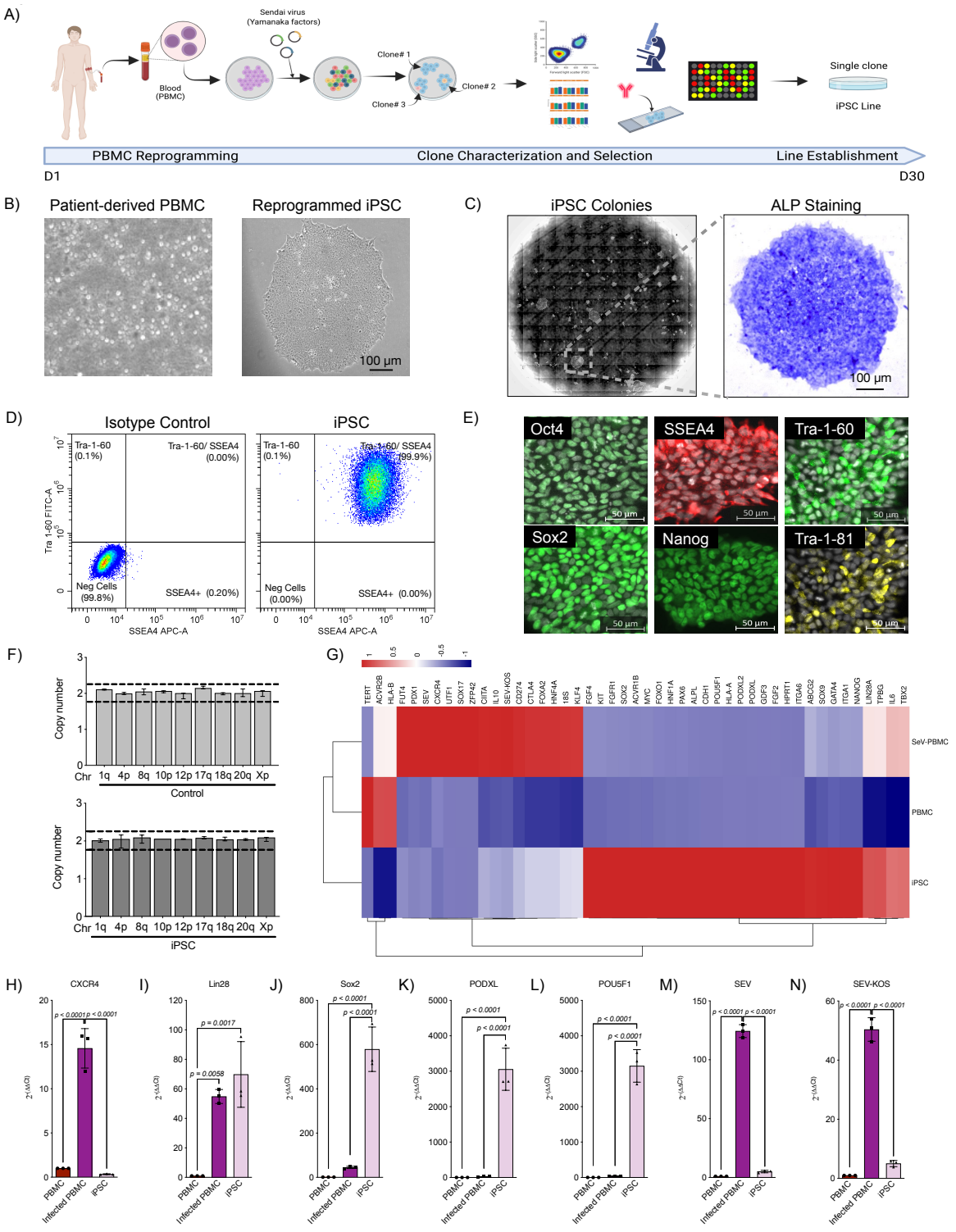

Figure 2.2.1 Establishment of iPSC line from human peripheral blood mononuclear cells.

A) Overview of processes for generating an induced pluripotent stem cell line including patient blood collection (day 1), peripheral blood mononuclear cell isolation, infection with reprogramming factors discovered by Yamanaka et al. and Thomson et al., optimal clone selection, and iPSC line establishment (day 30). B) Microscopy of peripheral blood mononuclear cells and established iPSCs. C) Characterization of pluripotency of the established iPSC line using alkaline phosphatase (ALP) staining. D) Flow cytometric analysis of the selected iPSC line with isotype control and characterization of Tra-1-60 and SSEA4 expression. E) Immunohistochemistry of the established iPSC line with expression of Oct4, Sox2, SSEA4, and Tra-1-60. F) Quantitative PCR evaluation of the established iPSC line frequently for genetic abnormalities within iPSCs comparing to commercially available control DNA (n = 9, 3 per iPSC line) G) Genetic microarray results comparing established iPSCs to fibroblasts and peripheral blood mononuclear cells (n = 3, 1 per iPSC line). H Differential expression of CXCR4, I Lin28, J Sox2, K PODXL, L POU5F1, M SEV and N SEV-KOS in PBMC, infected PBMC and iPSC (n = 3).

2.2.4.2 3D suspension condition supports increased iPSC expansion

Following iPSC line establishment, $2x10^6$ cells from 60 mm dishes were cultured in either 2D planar or 3D suspension conditions for a 5-day expansion cycle followed by cell harvest and head-to-head comparative assessment (Figure 2.2.2A). Cells expanded in 2D planar conditions grouped tightly together to form compact colonies with well delineated borders, which generated a monolayer sheet of cells upon confluency. Comparatively, cells grown in 3D suspension formed tight clusters that grew outwards in all directions with a central cavity (similar to epiblast structure during embryo formation), allowing cell microstructure support from nearby cells (Figure 2.2.2B). 2D planar and 3D suspension expanded cells demonstrated no difference in single cell size (Figure 2.2.2C) or viability (2D: 89.5% [IQR 6.5] vs. 3D: 86.0% [IQR 7.0], $p = 0.75$; Figure 2.2.2D) throughout expansion. Notably, when 3D expanded cells were dissociated and re-plated they acquired identical architecture to 2D expanded cells; they generated compact colonies with delineated borders. The majority of iPSC clusters in 3D

suspension were 175-250 μm (range: 125-324 μm) by the end of the 5-day expansion cycle (Figure 2.2.2E). Following 3 days of expansion, comparatively more cells were generated using 3D suspension (2D: $13.5x10^6$ [IQR $3.7x10^6$] vs. 3D: $54.2x10^6$ [IQR $14.7x10^6$], $p < 0.0001$), with even greater expansion in 3D suspension after 5 days (2D: $40.1x10^6$ [IQR $8.5x10^6$] vs. 3D: $187.5x10^6$ [IQR $60.4x10^6$], $p < 0.0001$; Figure 2.2.2F). Fold expansion was significantly greater under 3D suspension condition both on day 3 (2D: 6.7-fold [IQR 1.9] vs. 3D: 27.1-fold [IQR 7.4], $p < 0.0001$) and day 5 (2D: 19.1-fold [IQR 4.0] vs. 3D: 93.8-fold [IQR 30.2]; $p < 0.0001$; Figure 2.2.2G). Results were similar for all iPSC lines (n = 4, **Appendix** Figure S2.2.9). At day 5, the number of cells generated per consumed mL of media was significantly higher for cells grown in 3D suspension compared to 2D planar conditions (2D: $4.0x10^5$ cells/ml [IQR $5.7x10^4$ cells/ml] vs. 3D: $1.2x10^6$ cells/ml [IQR $4.0x10^5$ cells/ml], $p < 0.0001$; Figure 2.2.2H). Extrapolating from these data, the generation of $1x10^6$ iPSCs would cost significantly less in 3D suspension compared to 2D planar (2D: $417.7 [IQR $270.8] vs. 3D: $196.0 [IQR $58.9], $p < 0.0001$; 2022 Canadian Dollars; Figure 2.2.4). Similarly, the population doubling level was significantly higher in 3D suspension condition (2D: 14.1 [IQR 1.0] vs. 3D: 21.4 [IQR 1.19], $p = 0.0022$; Figure 2.2.2I). Increased population doubling level was confirmed by a significantly increased percentage of proliferative ($Ki67^+$) cells in 3D suspension condition on day 5 (2D: 57.4% [IQR 10.9%] vs. 3D: 76.6% [IQR 6.5%], $p = 0.0022$, Figure 2.2.2J-K).

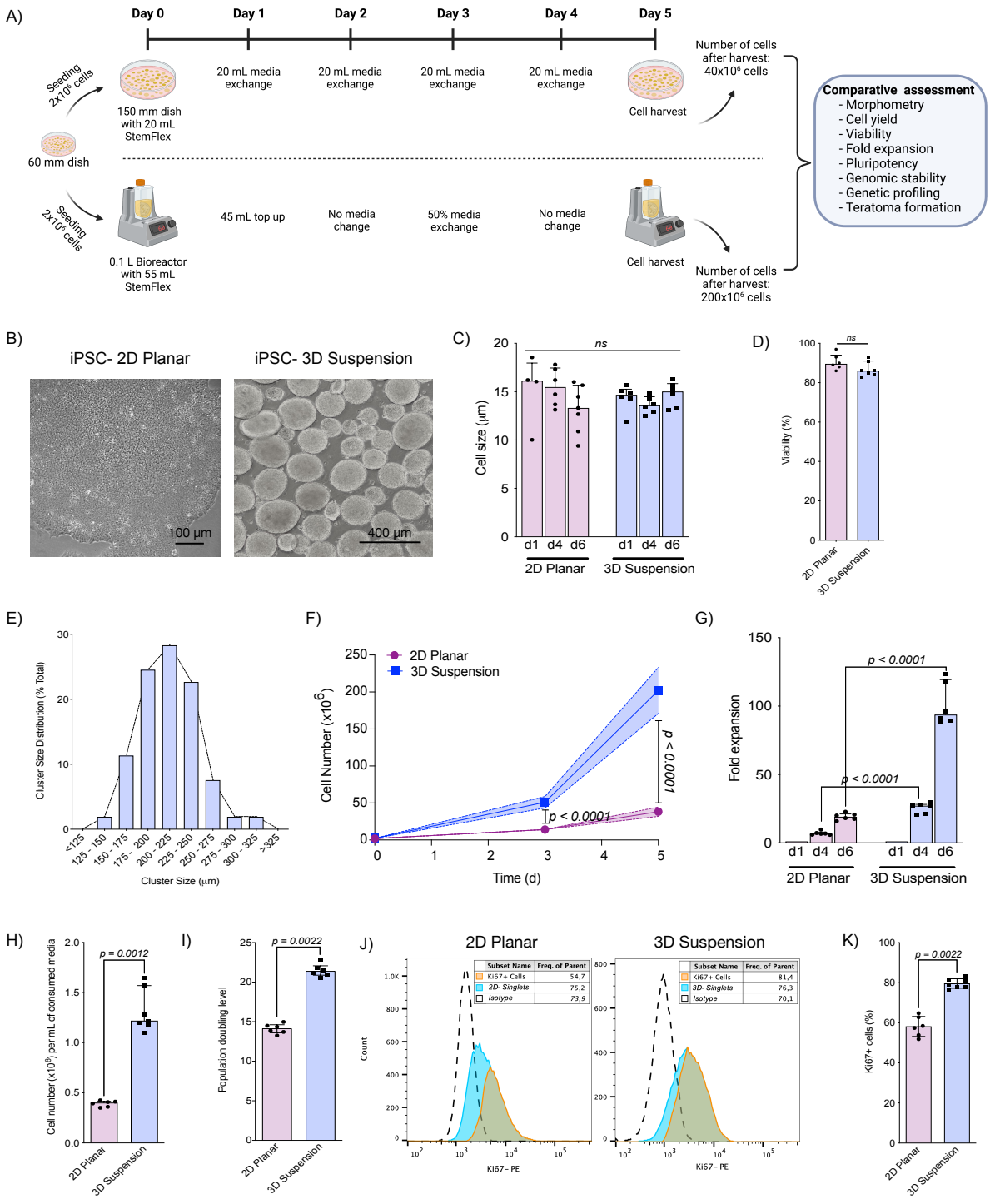

Figure 2.2.2 Evaluation of iPSCs expanded in two-dimensional planar (2D) and three-dimensional suspension (3D) cell culture.

A) Schematic representation of the expansion protocols for 2D and 3D suspension conditions with summary of techniques used to compare cells. B) Morphology of cells expanded in 2D planar cell culture and 3D suspension expansion within Vertical-Wheel® bioreactors C) Cell size following 3D cluster dissociation and 2D cell passaging on days 0, 3, and 5 of expansion (n = 6). D) Cell viability following 5 days of cell expansion comparing 2D and 3D conditions (n = 6). E) Cluster size for cells grown in 3D conditions with frequency of clusters characterized (n = 3). F) Absolute cell number expansion using 2D and 3D cell culture (n = 6). G) Fold expansion following 3 and 5 days of cell expansion in 2D and 3D cell culture (n = 6). H) Cell expansion per milliliter of consumed media following 5 days of cell expansion in 2D and 3D cell culture (n = 6 per group). I) Population doubling level for cells expanded in 2D and 3D conditions (n = 6 per group). J) Representation of the gating strategy followed for the quantification of Ki67+ cells in 2D and 3D conditions. K) Ki67 expression of cells expanded in 2D and 3D conditions (n = 6 per group).

Media collected during 2D planar and 3D suspension iPSC expansion showed no statistically significant difference in pH or glucose, lactate or glutamine concentrations (**Appendix** Figure S2.2.10A-H). Supernatant from iPSCs expanded in 3D suspension had significantly lower concentrations of lactate dehydrogenase and ammonia than those from iPSCs expanded in 2D planar condition (**Appendix** Figure S2.2.10I-L).

In addition, we compared our expansion protocol (current protocol from hereon) to the previously published 7-day 3D expansion protocol within Vertical-Wheel® bioreactors by Dang et al (2021) [28]. We performed parallel experiments using both protocols; to reduce inter-protocol variability, we cultured cells using StemFlex rather than the modified B8 media initially reported by Dang et al. (2021). The primary differences between the two protocols include inoculation procedure (Current protocol: $2x10^6$ cells into 55.0 mL of StemFlex with RockI (2 µL/mL) on day 0 then top up to 100 mL on day 1; Dang et al (2021): $2x10^6$ cells into 100 mL of StemFlex with RockI (2 µL/mL) on day 0) and feeding regime (Current protocol: 50% media replacement on d3 and harvest on d5; Dang et al (2021): 50% media replacement on day 3, day 5 and day 6,

and harvest on day 7) (Figure 2.2.3A). Regardless of the protocol used, cells were cultured with a constant rotational speed of 60 rpm. In both cases, cells formed tight clusters that grew outwards in all directions with a central cavity allowing cell microstructure support from nearby cells (Figure 2.2.3B). Single cell size and viability were not different between cells from each protocol (Figure 2.2.3C-D). However, replication of the Dang (2021) protocol produced iPSC clusters with median size of 607.7 μm [IQR 271.1 μm] after 7 days compared to 229.3 μm (IQR 10.8 μm] after 5 days of expansion using our protocol ($p < 0.001$, Figure 2.2.3E); furthermore, the cluster size distribution was wider following the Dang protocol (Figure 2.2.3F). Additionally, comparatively more cells were generated using our protocol following 3 days of expansion (Current protocol: $54.2x10^6$ [IQR $14.7x10^6$] vs Dang (2021): $18.96x10^6$ [IQR $6.83x10^6$], $p = 0.0095$) and after 5 days (Current protocol: $187.5x10^6$ [IQR $60.4x10^6$] vs Dang (2021): $97x10^6$ [IQR $18.17x10^6$], $p = 0.0095$; Figure 2.2.3G). Similarly, fold expansion was significantly greater following this study's 3D suspension expansion protocol both on day 3 (Current protocol: 27.1-fold [IQR 7.4] vs Dang (2021): 9.48-fol [IQR 4.5], $p = 0.0095$) and day 5 (Current protocol: 93.8-fold [IQR 30.2] vs Dang (2021): 48.5 [IQR 7.7], $p = 0.0095$; Figure 2.2.3H). Reduced fold expansion after 5 days of the Dang (2021) expansion protocol is a result of reduced population doubling (Current protocol: 21.4 [IQR 1.2] vs Dang (2021): 17.1 [IQR 2.9], $p < 0.0001$; Figure 2.2.3I) which resulted in decreased cell number per mL of consumed media (Current protocol: $1.2x10^6$ cells/ml [IQR $4.0x10^5$] cells/ml vs Dang (2021): $0.65x10^6$ cells/ml [IQR $1.1x10^5$] cells/ml, $p = 0.0061$; Figure 2.2.3J) and increased cost per 100 million cells (Current protocol: $196.0 [IQR $58.9] vs Dang (2021): $308.0 [IQR $46.37], $p = 0.0061$; Figure 2.2.3K). However, when the Dang protocol is continued until day 7, a significantly greater number of cells are

generated ($361.59x10^6$ [IQR $116.81x10^6$], and a higher fold expansion is achieved (Current protocol: 93.8-fold [IQR 30.2] vs Dang (2021): 180.8 [IQR 78.11], $p = 0.0022$), compared to our 5-day protocol (Figure 2.2.3G-H). This leads to a reduced cost per 100 million cells using the complete 7-day Dang protocol compared to our protocol (Figure 2.2.3K). Interestingly, despite reduced population doubling level on day 5, there were no differences in the population doubling levels between protocols upon replication of the Dang et al. (2021) protocol all the way to day 7 (Current protocol: 21.4 [IQR 1.19] vs Dang (2021): 21.57 [IQR 2.03]; Figure 2.2.3I).

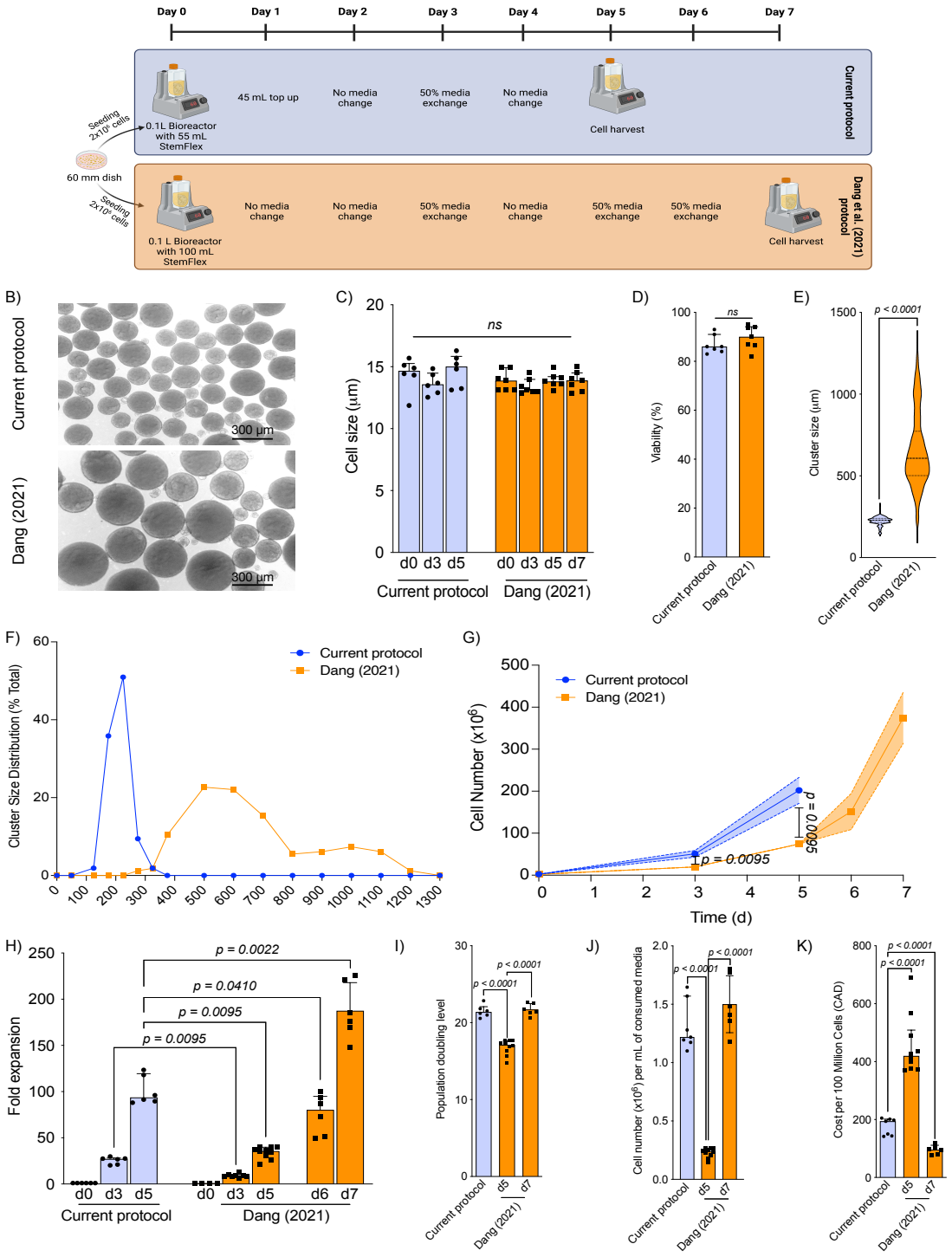

Figure 2.2.3 iPSC expansion comparison within Vertical-Wheel® bioreactors using the current protocol and replication of Dang (2021) protocols.

A) Schematic representation of the current protocol and the replicated Dang (2021) expansion protocol. B) Cluster morphology at termination of iPSC expansion protocol using our current protocol or replicated Dang (2021) protocols. C) Cell size following 3D cluster dissociation after expansion using the current protocol and replicated Dang (2021) protocol on days 0, 3, and 5 of expansion (n = 6). D) Cell viability following 5 days of cell expansion comparing the current protocol and replicated Dang (2021) protocol (n = 6). E) Cluster size for cells grown using the current protocol and replicated Dang (2021) protocol with frequency of clusters characterized (n = 6). F) Cluster size distribution at termination of iPSC expansion protocol using the current protocol and replicated Dang (2021) protocol. G) Absolute cell number expansion using the current protocol and replicated Dang (2021) protocol (n = 6). H) Fold expansion following 3 and 5 days of cell expansion using the current protocol and 3, 5, 6 and 7 days of cell expansion using the replicated Dang (2021) protocol (n = 6). I) Population doubling level for cells expanded using the current protocol and replicated Dang (2021) protocol (n = 6 per group). J) Cell expansion per milliliter of consumed media following 5 days of cell expansion using the current protocol and 5 and 7 days of cell expansion using the replicated Dang (2021) protocol (n = 6 per group). K) Cost of producing 100x106 cells in 2023 Canadian Dollars following 5 days or 5 and 7 days of cell expansion using the current protocol and replicated Dang (2021) protocol (n = 6 per group).

2.2.4.3 3D suspension condition using Vertical-Wheel® bioreactors enables scalability

Following one passage of 3D suspension culture, iPSC clusters were dissociated and $10x10^6$ cells from 0.1 L Vertical-Wheel® bioreactors were seeded into 0.5 L Vertical-Wheel® bioreactor with a constant rotational speed of 60 rpm and cultured for a 5-day expansion cycle (Figure 2.2.4A). 3D suspension expanded cells in 0.1 L or 0.5 L Vertical-Wheel® bioreactors demonstrated no difference in single cell size (Figure 2.2.4B) or viability (0.1 L: 86.0% [IQR 7.0] vs. 0.5 L: 86.5% [IQR 12.0], $p = 0.5979$); Figure 2.2.4C) throughout expansion. Volume capacity of the Vertical-Wheel® bioreactor did not alter the cluster size distribution; most iPSC clusters in 3D suspension were 175-250 μm (range: 125-324 μm) by the end of the 5-day expansion cycle regardless of the size of bioreactor used (Figure 2.2.4D). Following 3 days of expansion, comparatively more cells were generated using 0.5 L Vertical-Wheel® bioreactors

(0.1 L: 54.2x10^6 [IQR 14.7x10^6] vs. 0.5 L: 272.7 x10^6 [IQR 30.4 x10^6], $p < 0.0001$), with even greater expansion in 3D suspension after 5 days (0.1 L: 187.5x10^6 [IQR 60.4x10^6] vs. 0.5 L: 997.1 [IQR 164.3], $p < 0.0001$; Figure 2.2.4E). Scale up to 0.5 L Vertical-Wheel® bioreactor did not affect fold expansion at day 3 (0. 1L: 27.1-fold [IQR 7.4] vs. 0.5 L: 28.3-fold [IQR 10.3], p = 0.3676) or day 5 (0.1 L: 93.8-fold [IQR 30.2]; vs. 0.5 L: 94.5 [IQR 34.7], $p = 0.4923$; Figure 2.2.4F). The number of cells generated per consumed mL of media at day 5 was significantly higher for cells grown in 3D suspension, regardless of the size of Vertical-Wheel® bioreactor used, compared to 2D planar condition (2D: 4.0x10^5 cells/ml [IQR 5.7x10^4 cells/ml] vs. 0.1 L: 1.2x10^6 cells/ml [IQR 4.0x10^5 cells/ml], $p < 0.0001$; 0.5 L: 1.3x10^6 cells/ml [IQR 2.7x10^5 cells/ml], $p < 0.0001$; Figure 2.2.4G). Scale up did not affect the number of cells generated per consumed mL of media at day 3 or 5. Extrapolating from these data, the generation of 1 x10^6 iPSCs would cost significantly less in 0.5 L Vertical-Wheel® bioreactors compared to suspension culture in 0.1 L Vertical-Wheel® bioreactors or 2D planar culture (2D: $417.7 [IQR $270.8] vs. 0.5 L: $70.4 [IQR $ 18.4], $p < 0.0001$; 0.1 L: $196.0 [IQR $58.9], $p < 0.0001$; 2022 Canadian Dollars; Figure 2.2.4H). The cost breakdown and comparison between 2D planar and 3D suspension conditions using 0.1 L and 0.5 L Vertical-Wheel® bioreactors is shown in Figure 2.2.4I.

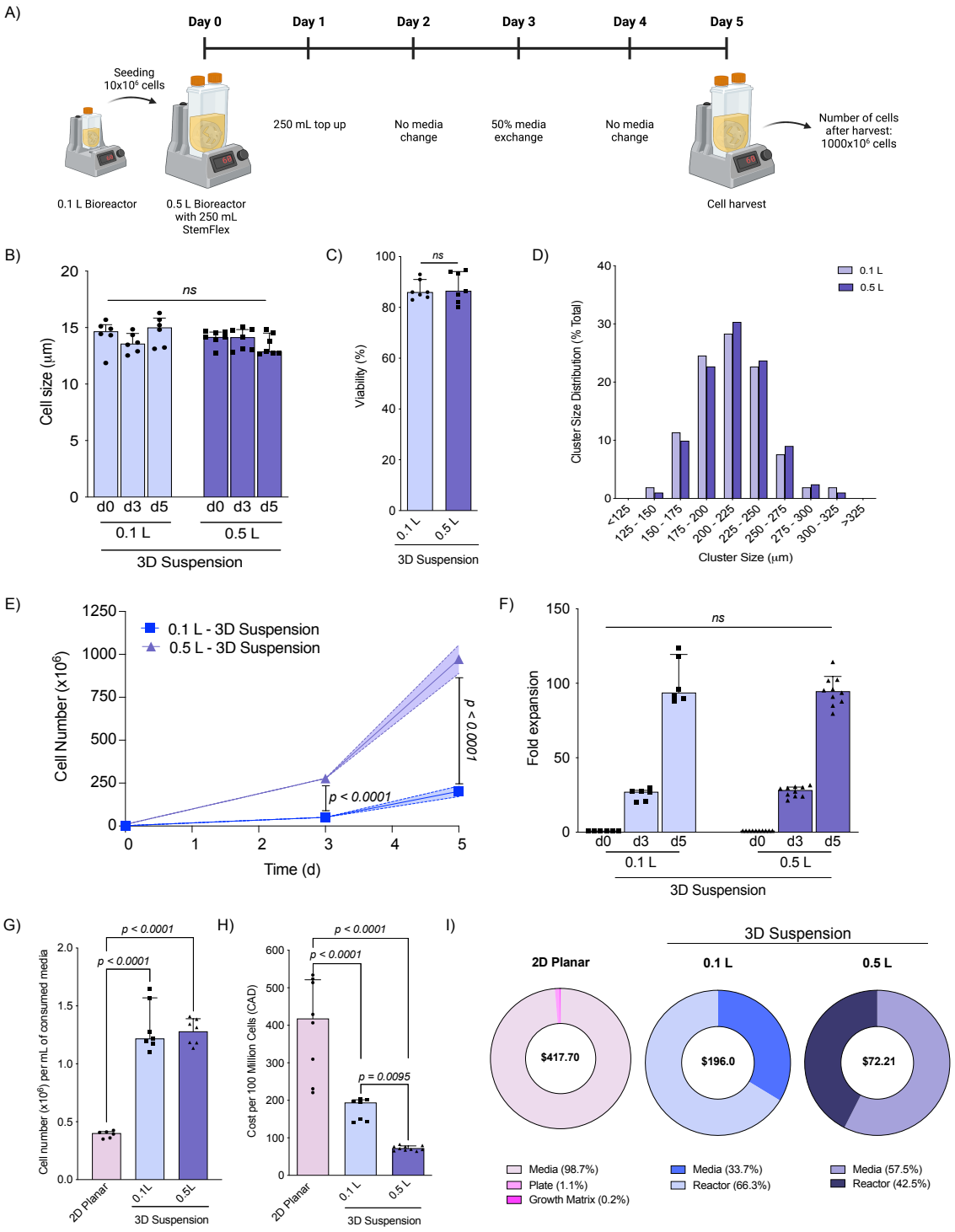

Figure 2.2.4 Comparison of expansion potential between 0.1 L and 0.5 L Vertical-Wheel® bioreactors.

A) Schematic representation of the expansion protocol used with 0.5 L Vertical-Wheel® bioreactor. B) Cell size following 3D cluster dissociation from 0.1 L and 0.5 L Vertical-Wheel® bioreactor on days 0, 3, and 5 of expansion (n = 6). C) Cell viability following 5 days of cell expansion comparing 2D and 3D conditions (n = 6). D) Cluster size distribution for clusters grown in 0.1 L and 0.5 L Vertical-Wheel® bioreactors with frequency of clusters characterized (n = 3). E) Absolute cell number expansion using 0.1 L and 0.5 L Vertical-Wheel® bioreactors (n = 6). F) Fold expansion following 3 and 5 days of cell expansion in 0.1 L and 0.5 L Vertical-Wheel® bioreactors (n = 6). G) Cell expansion per milliliter of consumed media following 5 days of cell expansion in 0.1 L and 0.5 L Vertical-Wheel® bioreactors and 2D planar conditions (n = 6). H) Cost associated to the generation of 100x106 cells following 2D planar or 3D suspension conditions using 0.1 L or 0.5 L Vertical-Wheel® bioreactors. I) Representation of cost associated to media, plate and growth matrix or reactor.

2.2.4.4 3D suspension condition promotes superior pluripotency phenotype

Qualitative assessment of protein level pluripotency marker expression by cells expanded under both conditions was completed using immunohistochemistry and demonstrated that both 2D planar and 3D suspension expanded iPSCs displayed classical markers of pluripotency including Oct4, Nanog, SSEA4, Sox2, Tra-1-60, and Tra-1-81 (Figure 2.2.5A). Quantification of pluripotency markers using flow cytometry demonstrated that significantly more 3D expanded cells co-express Oct4, Nanog, and Sox2 (2D: 52.5% [IQR 5.6%] vs. 3D: 94.3% [IQR 1.4%], p = 0.0079, Figure 2.2.5B-C), with more cells expanded in 2D planar culture failing to co-express Tra-1-60 and Tra-1-81 (2D: 3.3% [IQR 0.9%] vs. 3D: 0% [IQR 0%], p = 0.0476, Table 2.2.1) Complete gating strategies and quantification can be found in **Appendix** Figure S2.2.8 and Table 2.2.1 respectively.

Table 2.2.1 Pluripotency Marker Expression of 2D and 3D expanded cells.

	2D planar	3D Suspension	p-value
Tra-1-60$^-$ Tra-1-81$^-$	3.31% (IQR 0.95%)	0.00% (IQR 0.00%)	0.0476
Tra-1-60$^-$ Tra-1-81$^+$	0.08% (IQR 0.04%)	0.00% (IQR 0.00%)	0.0476
Tra-1-60$^+$ Tra-1-81$^-$	11.05% (IQR 2.83%)	1.20% (IQR 0.87%)	0.0952
Tra-1-60$^+$ Tra-1-81$^+$	85.57% (IQR 3.75%)	98.80% (IQR 0.87%)	0.0952
Oct4$^-$ Nanog$^-$ Sox2$^-$	5.47% (IQR 2.21%)	0.09% (IQR 0.09%)	0.0476
Oct4$^-$ Nanog$^-$ Sox2$^+$	26.92% (IQR 5.98%)	1.13% (IQR 0.42%)	0.0079
Oct4$^-$ Nanog$^+$ Sox2$^-$	0.09% (IQR 0.04%)	0.01% (IQR 0.01%)	0.0873
Oct4$^-$ Nanog$^+$ Sox2$^+$	5.89% (IQR 0.85%)	3.95% (IQR 1.30%)	0.3095
Oct4$^+$ Nanog$^-$ Sox2$^-$	0.27% (IQR 0.16%)	0.01% (IQR 0.01%)	0.0873
Oct4$^+$ Nanog$^-$ Sox2$^+$	6.46% (IQR 2.19%)	0.25% (IQR 0.08%)	0.0079
Oct4$^+$ Nanog$^+$ Sox2$^-$	0.32% (IQR 0.15%)	0.00% (IQR 0.00%)	0.0476
Oct4$^+$ Nanog$^+$ Sox2$^+$	52.45% (IQR 5.61%)	94.25% (IQR 1.40%)	0.0079

Quantitative pluripotency marker expression on day 5 characterized by flow cytometry of cells expanded in 2D planar and 3D suspension conditions with percent of total cells and p-value.

To evaluate cells from both conditions for spontaneous trilineage differentiation cells were stained for ectoderm, endoderm and mesoderm markers (Figure 2.2.5D) and transcripts for these markers were evaluated. Immunochemistry assessment showed that neither 2D planar or 3D suspension expanded cells expressed markers for ectoderm (Pax6 and OTX2), endoderm (Sox17 and FoxA2) or mesoderm (CD31 and TBXT) (Figure 2.2.5D). Similarly, qRT-PCR demonstrated that both 2D planar and 3D suspension cells did not demonstrate expression of trilineage transcripts (Figure 2.2.5E and **Appendix** Figure S2.2.11B-D). Additionally, embryoid bodies generated from 2D planar and 3D suspension expanded cells did not express trilineage markers on immunohistochemistry or within their transcripts (**Appendix** Figure S2.2.11A-D). Assessment of pluripotency marker expression at transcript level can be found in **Appendix** Figure S2.2.11E-K. Although no spontaneous differentiation was noted, both 2D planar and 3D suspension cells were capable of trilineage differentiation (Figure 2.2.5D-E).

To study the effect of 2D planar and 3D suspension expansion on pluripotency status we evaluated cells for primed (CD24 and CD90) and naïve (CD130 and CD75) pluripotency phenotype markers using flow cytometry. The differential expression of these markers has previously been reviewed by Collier et al. (2017) demonstrating these to represent the most specific markers for naïve and primed pluripotency phenotypes [39]. Under 2D planar conditions, 98.5% (IQR 1.0%) and 99.6% (IQR 0.3%) of cells were $CD24^{+}CD130^{-}$ and $CD90^{+}CD75^{-}$, respectively (Figure 2.2.5F). Following the first 3D suspension passage, iPSCs began expressing naïve iPSC markers and became $CD24^{+}CD130^{+}$ and $CD90^{+}CD75^{+}$. Gradual transition of iPSCs from primed ($CD24^{+}CD130^{-}$ and $CD90^{+}CD75^{-}$) to naïve ($CD24^{-}CD130^{+}$ and $CD90^{-}CD75^{+}$) occurred with continued 3D suspension culture, with 98.40% (IQR 1.14] of cells being $CD24^{-}CD130^{+}$ and 99.10% (IQR 0.75%) being $CD90^{-}CD75^{+}$ after 5 passages under 3D suspension conditions (Figure 2.2.5). Complete flow cytometric analysis including pluripotency and primed/naïve marker expression upon 10 subsequent passages in 2D planar or 3D suspension conditions can be found in **Appendix** Figure S2.2.12. Due to these findings, further 2D planar and 3D suspension comparisons were made on cells expanded using each condition for at least 5 passages.

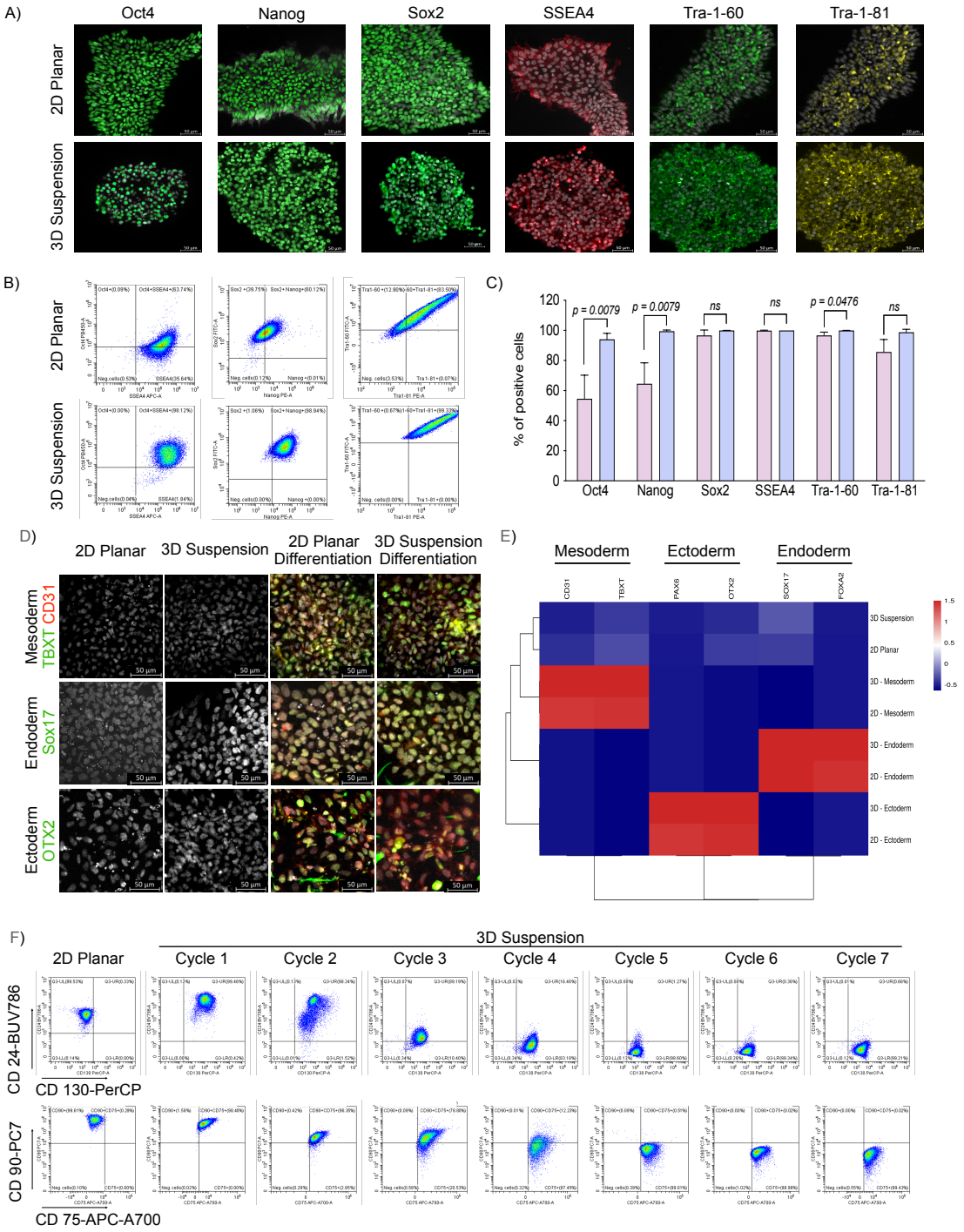

Figure 2.2.5 Comparative quantification of pluripotency marker expression.
A) Immunohistochemistry evaluation of pluripotency marker expression for cells expanded using 2D and 3D cell culture. B) Flow cytometric analysis to quantify pluripotency marker expression of iPSCs expanded in 2D and 3D conditions (n = 6 per group). Single stained results for the right

panel can be found in Appendix Figure S2.2.8B. C) Quantification of pluripotency marker expression of iPSCs expanded in 2D and 3D conditions (n = 6 per group). D) Flow cytometric analysis to show the expression of CD24, CD130, CD90 and CD75 upon the transition of iPSCs from 2D to 3D conditions (n = 3 per group). E) Comparison of the expression of key mesoderm, ectoderm and endoderm lineage associated genes among iPSCs cultured in 2D and 3D conditions as well as differentiated cells cultured under 2D and 3D conditions. F) Flow cytometric analysis of the transition of primed to naïve cells. Single cells were selected and examined for the expression of CD24, CD130, CD90 and CD75.

2.2.4.5 3D suspension conditions induce transcriptional changes without promoting copy number variations

Genetic analysis evaluating the most frequently mutated genomic locations during iPSC expansion showed that both 2D planar and 3D suspension conditions did not demonstrate any deletions or duplications (Figure 2.2.6A). Transcriptomic analysis of pluripotency genes showed that iPSCs expanded under both conditions had upregulated pluripotency gene transcription, including *POU5F1*, *Nanog* and *Sox2*, compared to the patient derived PBMCs and the PBMCs 4-days after Sendai virus infection (Figure 2.2.6B). More importantly, under 2D planar and 3D suspension conditions biological replicates clustered independently from each other, highlighting the effect that culture conditions have on iPSC transcriptomics. 2D planar cells transcribed significantly more *FGF2* (2D: 31114.0 [IQR 11024.0] vs. 3D: 6909.0 [IQR 3901.0], $p = 0.0049$), *DNMT3B* (2D: 9.3 [IQR 3.8] vs. 3D: 3.7 [IQR 0.9], $p = 0.0038$), *ID01* (2D: 1.8 [IQR 0.6] vs. 3D: 0.24 [IQR 0.2], $p = 0.0131$), and *XIST* (2D: 0.4 [IQR 0.08] vs. 3D: 0.08 [IQR 0.08], $p = 0.0083$) than 3D suspension cells, while 3D suspension cells transcribed significantly more *GDF3* (2D: 2852.0 [IQR 610.0] vs. 3D: 20211.0 [IQR 4420.0], $p = 0.0068$), *KLF4* (2D: 38.0 [IQR 2.4] vs.

3D: 57.6 [IQR 9.6], $p = 0.0019$), *Nanog* (2D: 234.7 [IQR 15.2] vs. 3D: 405.0 [IQR 37.9], $p = 0.0002$) and *c-Myc* (2D: 55.8 [IQR 18.1] vs. 3D: 145.5 [IQR 39.6], $p = 0.0046$) (Figure 2.2.6C).

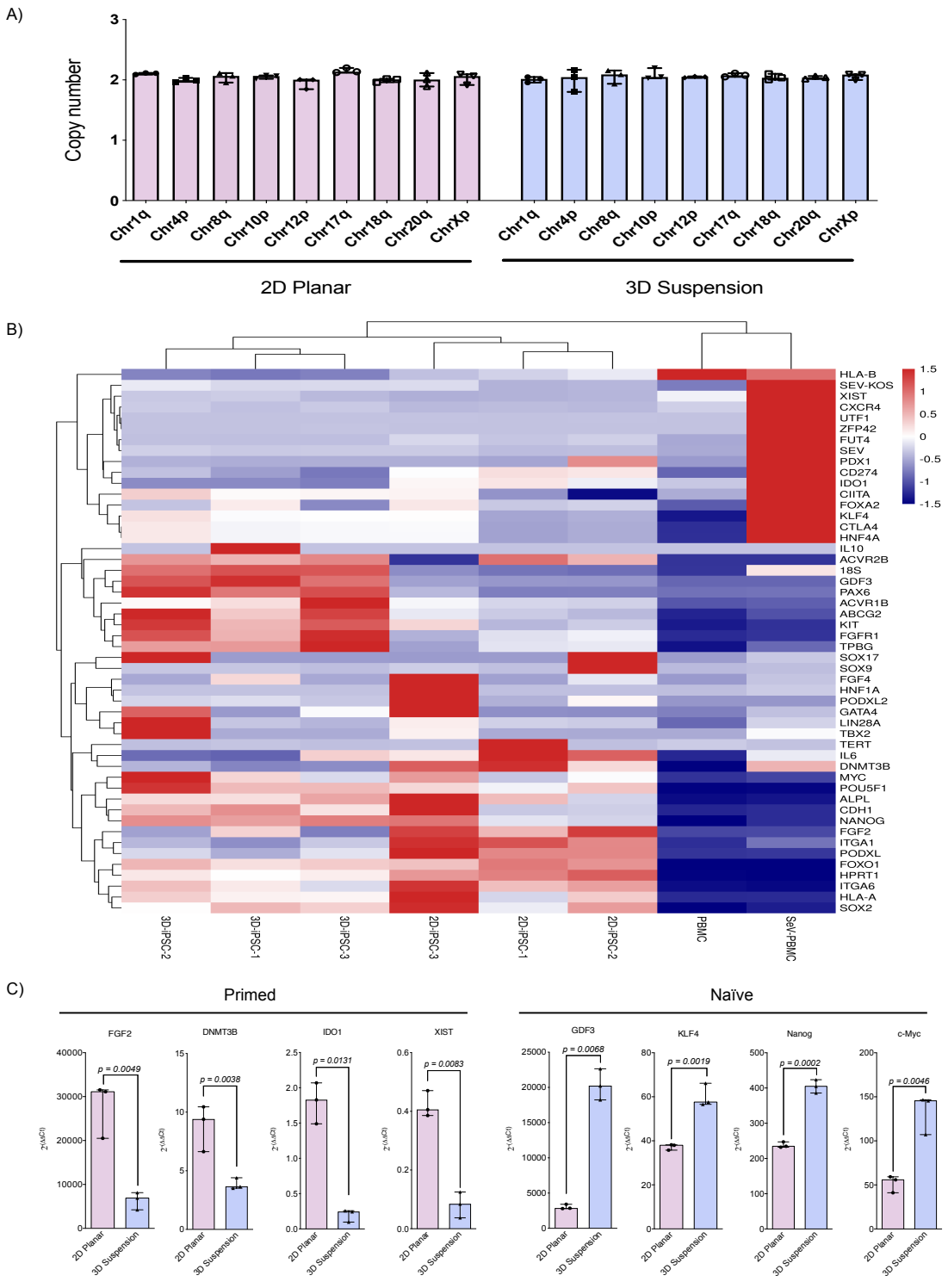

Figure 2.2.6 Comparative assessment of chromosomal stability and gene expression.

A) Quantitative PCR evaluation of the established iPSC line frequently for genetic abnormalities within cells expanded in 2D and 3D conditions (n = 3 per group). B) Heat map showcasing differential gene expression between cells expanded in 2D and 3D conditions. C) Differential expression in 2D and 3D of primed markers FGF2, DNMT3B, IDO1 and XIST, and naïve markers GDF3, KLF4, Nanog and c-Myc (all n = 3 per group).

2.2.4.6 iPSCs expanded using 3D suspension conditions generate more mature teratomas with fewer proliferative cells

To assess the impact of iPSC expansion conditions on *in vivo* pluripotency potential, cells from both conditions underwent renal subcapsular transplantation followed by graft harvest and assessment after 8 weeks of *in vivo* maturation (Figure 2.2.7A). Cells from both conditions produced teratomas of equal size (2D: 26.5 mm [IQR 7.5 mm] vs. 3D: 26.5 mm [IQR 6.5 mm], $p = 0.85$; Figure 2.2.7B). However, morphology of the grafts generated by 2D planar and 3D suspension conditions differed, with 2D planar cells generating solid grafts and 3D suspension cells producing fluid-filled cystic grafts (Figure 2.2.7C). Histological assessment following H&E staining of the recovered grafts showed that all grafts had representative tissues from the three germ layer lineages (i.e. teratomas) (Figure 2.2.7D). Graft characterization with immunohistochemistry demonstrated expression of PAX6 (ectoderm), SOX17 (endoderm), and CD31 (mesoderm) further confirming trilineage differentiation capacity (Figure 2.2.7D). Teratomas from 3D expanded iPSCs assessed by a trained pathologist represented more mature ectoderm, mesoderm and endoderm tissues identified as exoskeletal stratified epithelial, mature muscle fibers and duct-glandular regions compared to the less mature tissues found in teratomas from 2D expanded cells visualized as neural rosettes, chondrocytes, and glandular tissue. Immunohistochemistry labelling for Ki67 demonstrated statistically fewer proliferative cells

within the grafts generated from 3D suspension cells (2D: 45.3% [IQR 3.0%] vs. 3D: 16.7% [IQR 3.2%], $p = 0.002$; Figure 2.2.7E) regardless of the germ layer evaluated (Figure 2.2.7F).

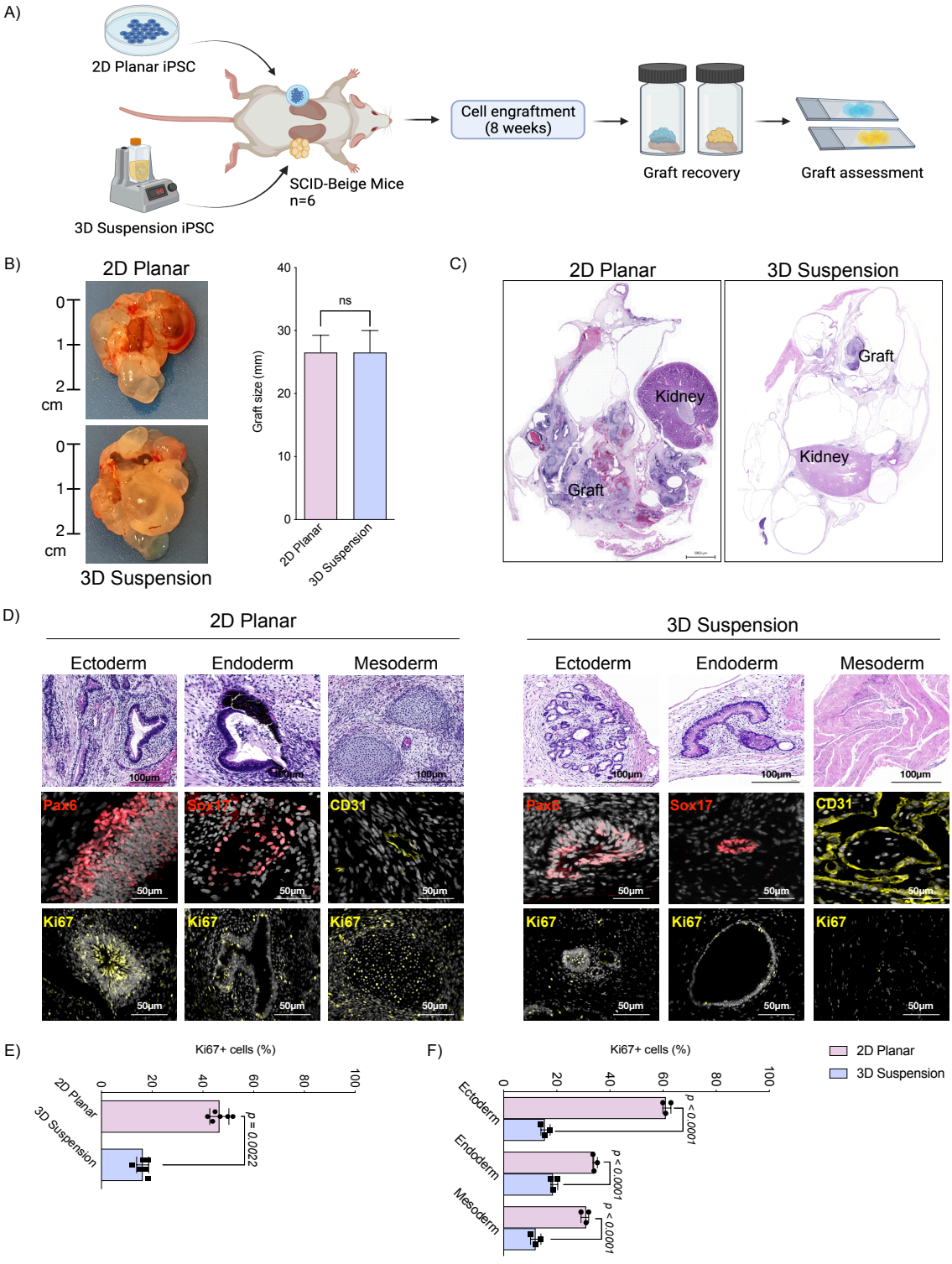

Figure 2.2.7 Teratoma formation assessment and comparison between iPSC expansion protocols.

A) Overview of process used for teratoma assay to characterize in vivo maturation of iPSCs (n = 6 per group). B) Teratomas excised from transplanted mice with size comparison of grafts achieved from cells expanded in 2D planar and 3D suspension conditions. C) Hematoxylin and eosin (H&E) staining of iPSC grafts transplanted into the renal subcapsular space following 2D and 3D cell expansion. D) Histological characterization of iPSC derived tumors demonstrating structures compatible with the three germ layers compatible with teratomas using H&E staining. Immunohistochemistry staining of iPSC grafts transplanted into the renal subcapsular space following 2D and 3D cell expansion with staining for PAX6 (ectoderm), SOX17 (endoderm), and CD31 (mesoderm) markers. Immunohistochemistry evaluation of Ki67 expression within 2D and 3D derived iPSCs with quantification of expression E) and F) All analyses represent n = 3 per group.

2.2.5 **Discussion**

This study presents a novel scalable iPSC expansion protocol using 3D suspension culture within Vertical-Wheel® bioreactors, achieving the greatest fold iPSC expansion in 5 days using these bioreactors reported to date. Cells expanded using this protocol acquire superior pluripotency phenotype compared to 2D planar expanded cells. Overall, as opposed to 2D planar culture, 3D suspension culture within Vertical-Wheel® bioreactors enables sufficient iPSC expansion for clinical implementation and offers a superior biomanufacturing process for economical, large volume generation of consistent, high quality cell products that advances clinical implementation of iPSC-derived cell therapies.

Compared to previous iPSC expansion protocols (Table 2.2.2), our protocol offers superior cell expansion with optimal cell cluster size consistency. Earlier studies by Nogueira et al. (2019) and Rodrigues et al. (2018) expanded cells using single-use Vertical-Wheel® bioreactors and achieved <10-fold expansion over 5-6 days [40,41]. More recently, two parallel studies by Borys et al. (2020) and Dang et al. (2021) demonstrated 30-fold iPSC expansion in 6 days and 62-fold iPSC expansion in 7 days, respectively, using Vertical-Wheel® bioreactors

[27,28]. Alternatively, Manstein et al. (2021) demonstrated 70-fold iPSC expansion after 7 days using automated stirred tank bioreactors [42]. However, evidence from Borys et al. (2021) demonstrates caveats to horizontal-blade bioreactors due to high fluid force heterogeneity resulting in significant variation in cluster size compared to Vertical-Wheel® bioreactors. Replication of the Dang et al. (2021) protocol using StemFlex media in our hands achieved 180.8-fold expansion but unfortunately generated large >600 μm clusters. Previous literature demonstrates that cell clusters <400 μm are optimal for differentiation and downstream cell product generation by limiting central core necrosis [27,43]. Therefore, although the Dang et al. (2021) protocol achieved substantial cell expansion, the updated 5-day protocol presented here has superior cell expansion at day 5 and achieves an optimal iPSC cluster size with a more consistent cluster size distribution that is better for subsequent differentiation [44]. Overall, our modified expansion protocol demonstrates increased expansion (93.8-fold) over 5 days while maintaining consistent cell cluster sizes, indicative of a homogenous cell population. Reasons for this substantial increase in expansion using this protocol include use of StemFlex growth media, early dilution of RockI on day 2 of expansion, and optimally timed media changes.

Table 2.2.2 Comparison of recent published 3D suspension iPSC expansions studies in different bioreactor geometries.

Reference	Year	iPSC Cell Lines Used	Media	Bioreactor Size and Type	Highest Fold Expansion/Days
Elanzew et al. [45]	2015	iLB-C-31f-r1	mTeSR1, E8	50 mL, Tube rotation (BioLevitator)	5-fold / 4 days
Haraguchi et al. [46]	2015	201B7, 253G1	mTeSR1	100 mL, Horizontal-blade (Integra Biosciences)	10-fold / 12 days

Badenes et al. [47]	2016	Gibco CD34+ derived	E8	50 mL, Horizontal-blade (StemSpan)	3.5-fold / 10 days
Kropp et al. [26]	2016	hCBiPS2, hHSC_F1285T_iPS2	mTeSR1, E8	250 mL, Eight blade impeller (DASbox)	6-fold / 7 days
Meng et al. [48]	2017	4YA, 4YF	mTeSR1	100 mL, Horizontal-blade (NDS)	12- to 13-fold / 5 days
Abecasis et al. [49]	2017	ChiPS C4, ChiPS C12, ChiPS C15, ChiPS C18, ChiPS C22	Cellartis DEF-CS Xeno-Free	200 mL, Trapezoid-paddle (DASGIP)	19-fold / 4 days
Kwok et al. [50]	2018	AFiPS, FSiPS	mTeSR1, StemMACs iPS-Brew	125 mL, Horizontal-blade (Corning) 1000 mL, Horizontal-blade (Mobius)	16-fold / 7 days
Rodrigues et al. [41]	2018	F002.1A.13, Gibco Human Episomal iPSC	E8	0.1 L, Vertical-Wheel® (PBS Biotech) 0.5 L, Vertical-Wheel® (PBS Biotech)	6.7-fold / 6 days
Noguiera et al. [51]	2019	F002.1A.13, Gibco Human Episomal iPSC	mTeSR1, mTeSR3D	0.1 L, Vertical-Wheel® (PBS Biotech)	9.3-fold / 5 days
Borys et al. [27]	2020	4YA	mTeSR1	0.1 L Vertical-Wheel® (PBS Biotech)	32-fold / 6 days
Borys et al. [20]	2021	4YA	mTeSR1	0.1 L, Vertical-Wheel® (PBS Biotech) 0.5 L, Vertical-Wheel® (PBS Biotech)	32-fold / 6 days
Manstein et al. [52]	2021	MHHi006-A, MHHi001-A, MHHi008-A	E8	250 mL, Eight blade impeller (DASbox)	70-fold / 7 days

Dang et al. [28]	2021	4YA	mTeSR1, Modified B8	0.1 L mL, Vertical-Wheel® (PBS Biotech) 0.5 L, Vertical-Wheel® (PBS Biotech)	62-fold / 7 days
Current protocol	2023	Healthy donor derived cell lines	StemFlex	0.1 L mL, Vertical-Wheel® (PBS Biotech) 0.5 L, Vertical-Wheel® (PBS Biotech)	93.8-fold / 5 days

(Table adapted from Borys et al. (2020))[27]

This protocol's modifications were based on several iterations and prior literature. First, StemFlex media was used as it contains thermostable FGF2; FGF2 has previously been described as a limiting factor for the expansion of human iPSCs [53]. FGF2 promotes phosphorylation of mitogen-activated protein kinases (MEK) and extracellular signal-regulated kinases (ERKs) to improve cell expansion, maintain cell pluripotency, reduce spontaneous differentiation, and direct cells towards a naïve pluripotency state [54-57]. Replication of the Dang et al. (2021) protocol using StemFlex resulted in higher fold expansion (180.8 vs. 62) than reported by their group using either mTeSR1 or modified B8 media [28], supporting the importance of thermostable FGF2 supplementation. However, considering that our protocol still achieves superior fold expansion at day 5 compared to the Dang (2021) protocol, it is likely that StemFlex alone is not the only factor contributing to this study's results.

In addition to StemFlex use, early RockI elimination and media change at day 3 also contributed to the increased expansion potential of our protocol. Despite the role of RockI in early single cell survival and clustering, we diluted RockI on day 1 because it has also been

described to reduce iPSC proliferation [58]. Similarly, media exchange on day 3 was utilized to ensure appropriate nutrient availability during the exponential growth phase. Assessment of the pH and metabolite concentration available in the culture supernatant during expansion ensured that reduced proliferation in 2D planar expanded iPSCs was not a result of unfavourable environment (pH or lactate) or decreased metabolite availability (glucose or glutamine). Interestingly, lactate dehydrogenase and ammonia concentration were increased in the 2D planar expansion media compared to the 3D suspension media, which suggests increased cell death and catabolism of amino acids in 2D planar conditions. Indeed, the combination of early RockI and ensuring adequate nutrient conditions for cells with only 2 media changes maximizes cell growth and economic benefits of our updated protocol.

Overall, it is likely that a combination of StemFlex media, early RockI removal, and optimally timed media exchanges promotes superior expansion demonstrated using this protocol. These changes optimized cell expansion and correlated with an increased percentage of proliferative ($Ki67^+$) iPSCs and increased population doubling level. Practically, this means that although our protocol requires expensive bioreactors and uses more media, the total cost to generate $100x10^6$ iPSCs using 3D suspension condition is only 46.9% of the total cost compared to 2D planar condition – an attractive advantage for scalability. Furthermore, expansion using 0.5 L bioreactors offers even greater opportunity for cell expansion with increased cost savings and a much more scalable, robust process with fewer user interventions and the option of future automation to further increase repeatability.

In addition to demonstrating improved iPSC expansion, this study shows that 3D suspension expanded cells better express pluripotency markers and transition to a naïve

pluripotency phenotype [59-63]. Stem cell culture with thermostable FGF2 has previously resulted in similar findings, potentially accounting for some of these results [54-57]. Notably, the requirement for exogenous FGF2 supplementation should not be confused with reduced endogenous *FGF2* expression by 3D suspension expanded cells, as this represents independence from the MEK-ERK pathway, an important marker of naïve pluripotency phenotype [14,64]. Regardless, both 2D planar and 3D suspension cells benefited from thermostable FGF2. Therefore, we hypothesize that phenotypic changes in 3D expanded cells occur due to growth within clusters that mimics epiblastic structures with supporting integrin microstructure provided by nearby cells [65-67]. This structural support allows akt1 activation downstream from FGF2, resulting in FGF2 independence [67]. Key advantages of the naïve pluripotency state have previously been well described including their improved capacity for differentiation [68,69], and expansion with cell doubling time half that of their primed counterparts [68].

While cells expanded using each method were capable of *in vitro* trilineage differentiation, more thorough *in vivo* evaluation using the teratoma assay further supports the findings of a naïve phenotype of 3D suspension expanded cells. Teratomas generated from 3D suspension expanded cells had increased tissue maturity and decreased proliferation; others have demonstrated that lineage potential is protected in naïve cells, whereby naïve pluripotent cells can better differentiate and form mature teratoma tissue [68]. Clinically, increasingly mature teratomas have reduced risk of malignant transformation [70,71]. Although limited studies exist evaluating the safety of iPSC therapies clinically, tumorigenic risk due to residual iPSCs remains a concern. As iPSC-derived cell therapies transition towards clinical applications, further optimization of expansion protocols is crucial to ensure scalability and safety of cell therapies.

Assessment of the *in vitro* and *in vivo* characterization of cells expanded in 2D planar and 3D suspension conditions suggests that 3D suspension condition offers a potentially safer cell product with reduced risk of malignant and proliferative off-target growth originating from remnant non-differentiated iPSCs [72,73].

These promising findings supporting iPSC expansion within Vertical-Wheel® bioreactors should be considered in the context of important limitations. First, while we provide one of the first direct comparisons of 2D planar to 3D suspension expansion techniques, both remain specific to the cell source origin, iPSC reprogramming techniques, matrices, bioreactors, and media used in this study. Previous studies have demonstrated that iPSC cell source does not affect subsequent expansion, however, limited studies have compared iPSC generation techniques (Sendai virus vs. other) [74]. It is possible that other 2D planar matrices could provide similarly high quality iPSCs during expansion as those achieved in 3D suspension conditions; however, these matrices would confer an even greater cost limiting their translational applicability. Similarly, while we have demonstrated identical expansion capacity and cost savings by using 5-fold larger 0.5 L bioreactors, further expansion using large commercial bioreactors (e.g., 3 L-15 L) remains untested and may not achieve similar efficacy due to differences in metabolism, hydrodynamic forces experienced by the cells, and the introduction of computer-controlled systems. Further evaluation of these factors during scale up remain of importance for future investigation. However, the ability to control pH and dissolved oxygen at precise levels in the liquid in larger bioreactors would most likely offset any additional complexities during scale up. Future studies evaluating larger bioreactors are needed, especially with regards to potential allogeneic cellular transplantation techniques whereby a single cell

source could be expanded for many patients. Additionally, the mice used for teratoma assay were all male since female mice were allocated for breeding at the time of these experiments; sex has previously been shown to not affect the outcome of teratoma assays [75] but could potentially impact our results. Finally, this study offer data from 4 healthy donor iPSC lines and it remains unclear if patient factors will affect iPSC generation or expansion.

Considering ongoing optimization of iPSC-derived cell products, the importance of generating and exponentially expanding a reliable iPSC starting product should not be overlooked and will continue to become increasingly valuable as we approach broader clinical implementation. Despite these limitations, this study offers an updated iPSC expansion protocol achieving the greatest fold growth over 5 days reported to date using Vertical-Wheel® bioreactors. Not only does this protocol achieve superior cell expansion to previously reported 2D planar, and 3D suspension protocols using Vertical-Wheel® reactors, but yields iPSCs with superior pluripotency marker expression and a naïve pluripotency phenotype.

2.2.6 **Conclusions**

This study demonstrates an improved iPSC expansion protocol using 3D Vertical-Wheel® bioreactors achieving almost 100-fold expansion over 5-days, representing the largest iPSC expansion reported to date. The ensuing 3D suspension expanded cell product appears to have improved expression of pluripotency markers with transition towards a naïve stem cell phenotype. Additionally, 3D suspension expanded cells are capable of trilineage differentiation and generate more mature and less proliferative teratomas. These results support application of

3D suspension techniques using Vertical-Wheel® bioreactors to efficiently produce high-quality iPSCs for subsequent differentiation into cell products for clinical implementation.

2.2.7 **Appendix: chapter 2 subsection 2**

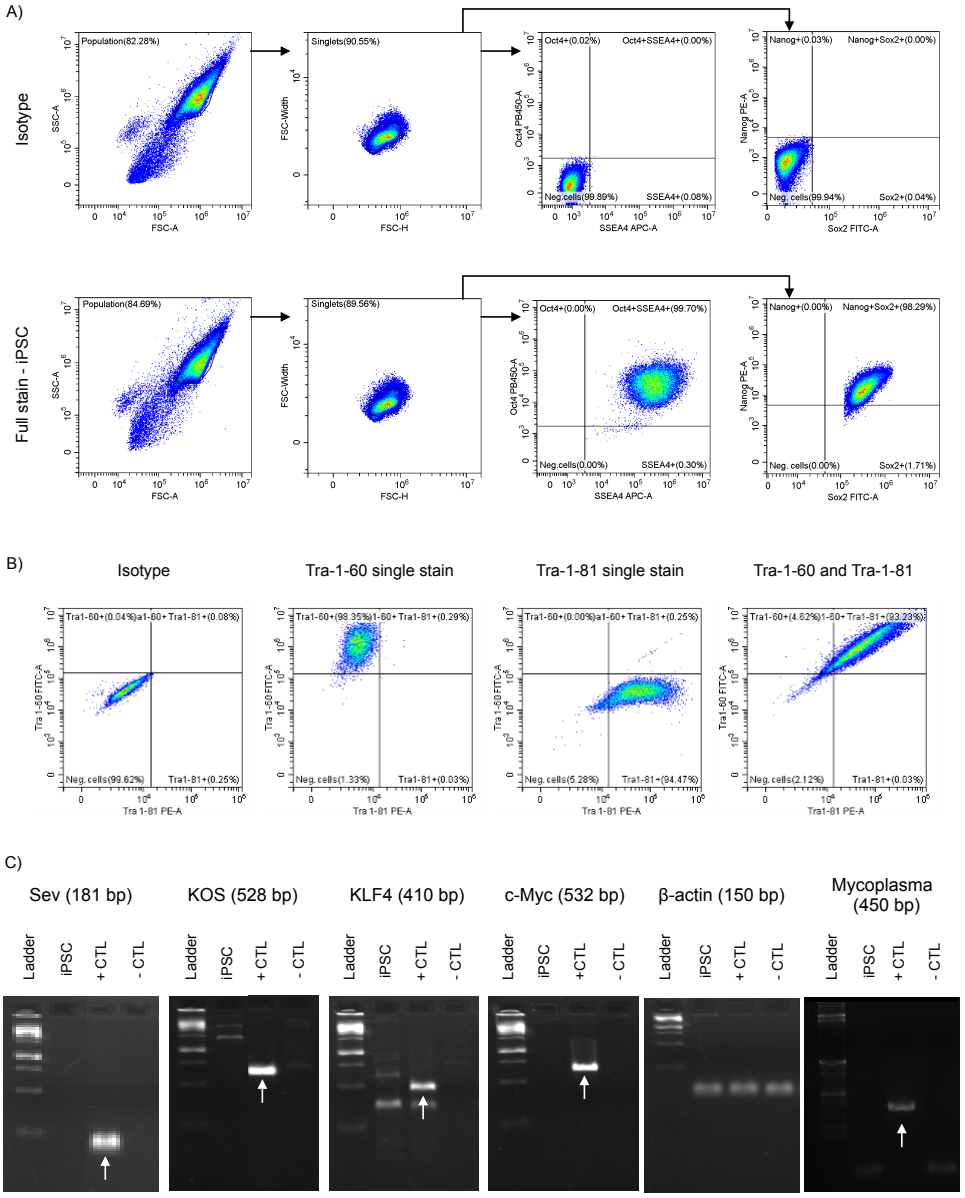

Figure S2.2.8 Extended quality control performed on reprogrammed iPSC lines.
A) Gating strategy used for flow cytometric analysis of the selected iPSC line with isotype control. Briefly, forward and side scatter was used to identify the cell population and remove debris and other events of non-interest based on size and complexity. Width and height of cells was used to exclude the double or multiple cells from single cells. Single cells were selected for further analysis and examined for the expression of Oct4, SSEA4, Nanog and Sox2. Isotype

controls were used to accurately gate positive staining and data were acquired using the CytoFLEX S flow cytometer and analysed using the CytExpert software (Beckman Coulter). B) Gating strategy for cytometric analysis of Tra-1-60 and Tra-1-81 with single stain results. C) Clearance of reprogramming vectors and lack of mycoplasma contamination. To test the absence of the Sendai reprogramming vectors a PCR that detects the Sendai virus genome and the transgenes, was used. PCR products were analysed by 1% agarose gel electrophoresis. iPSCs were tested for the expression of Sev, KOS, KLF4, and c-Myc with β-actin as an internal control. Infected PBMC were used as positive control for transgene presence while un-infected PBMC were used as negative control. F) Similarly, Mycoplasma PCR Detection Kit was used to detect contamination by 200+ strains of Mycoplasmas. This kit includes a positive Mycoplasma control and water was used as negative control. Full-length blots/gels are presented.

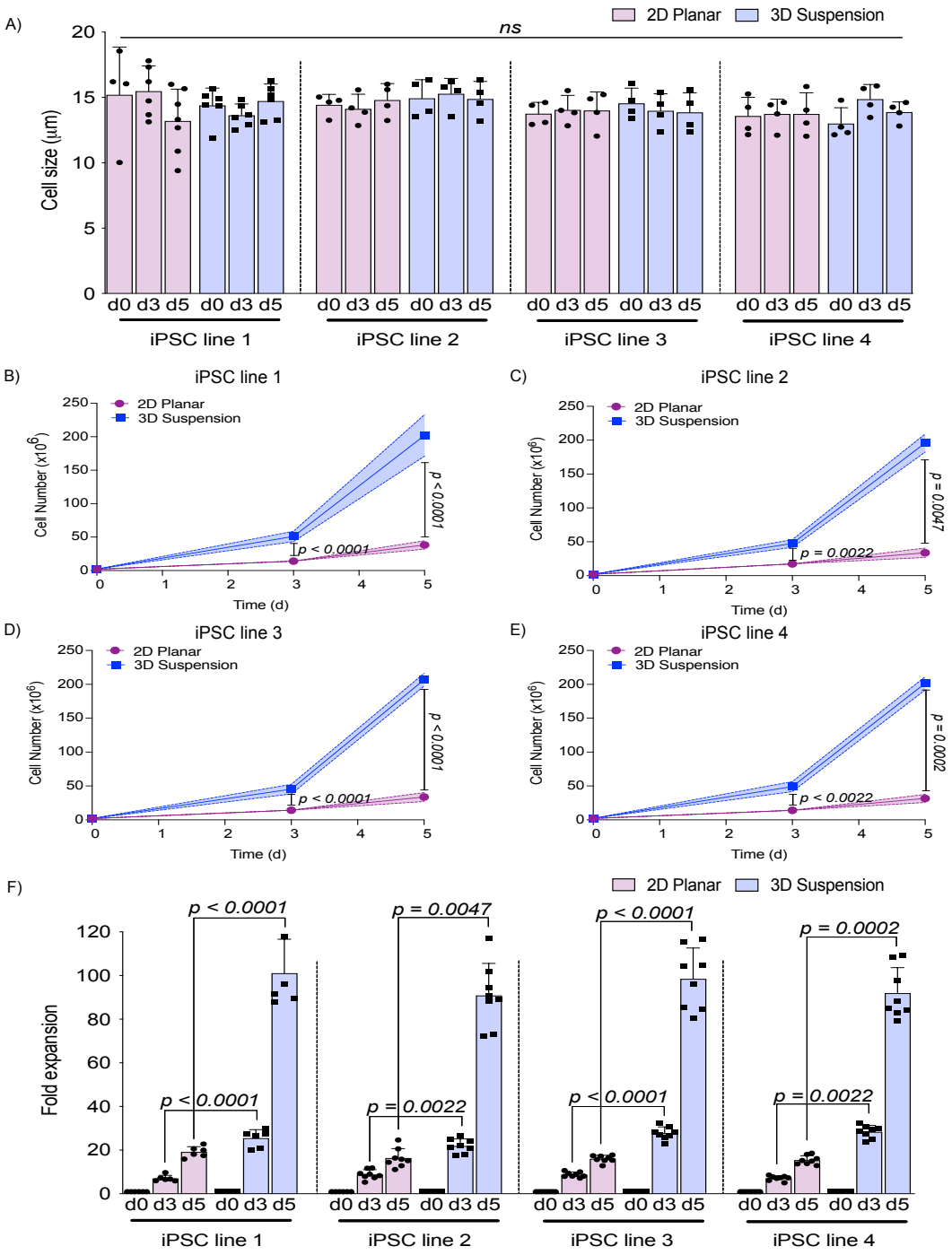

Figure S2.2.9 Expansion and evaluation of four iPSC lines expanded in 2D planar and 3D suspension cell culture.

A) Cell size following 3D suspension cluster dissociation and 2D cell passaging on days 0, 3, and 5 of expansion of three iPSC lines. B) Absolute cell number expansion using 2D planar and

3D suspension cell culture of three iPSC lines. G) Fold expansion following 3 and 5 days of cell expansion in 2D planar and 3D suspension cell culture of three iPSC lines.

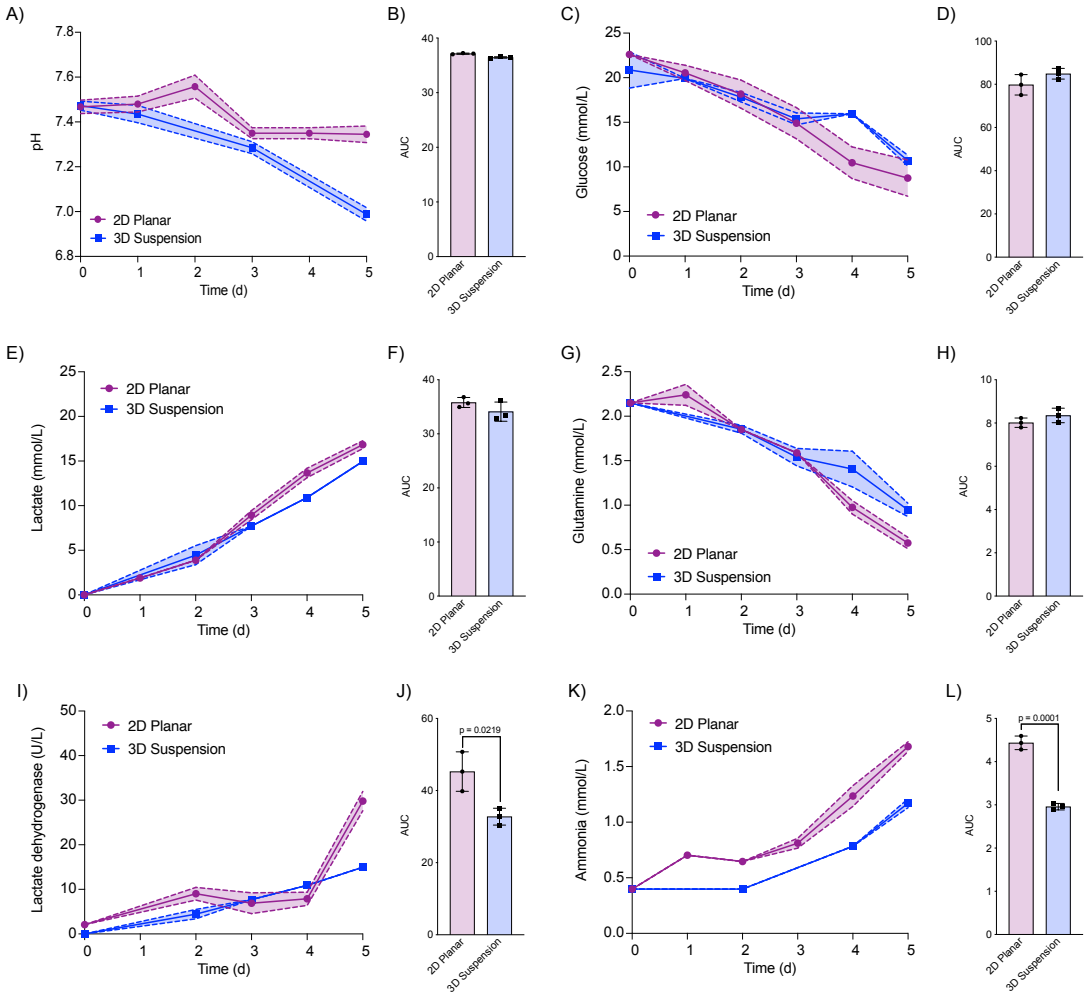

Figure S2.2.10 pH and metabolite concentration in media of induced pluripotent stem cells (iPSCs) expanded in two-dimensional planar (2D) and three-dimensional suspension (3D) cell culture conditions.

A) pH of cell culture media over time for expanded iPSCs in 2D and 3D conditions (n = 3 per group). C) Glucose concentration of cell culture media over time for expanded iPSCs in 2D and 3D conditions (n = 3 per group). E) Lactate concentration of cell culture media over time for expanded iPSCs in 2D and 3D conditions (n = 3 per group). G) Glutamine concentration of cell culture media over time for expanded iPSCs in 2D and 3D conditions (n = 3 per group). I) Lactate dehydrogenase concentration of cell culture media over time for expanded iPSCs in 2D

and 3D conditions (n = 3 per group). K) Ammonia concentration of cell culture media over time for expanded iPSCs in 2D and 3D conditions (n = 3 per group).

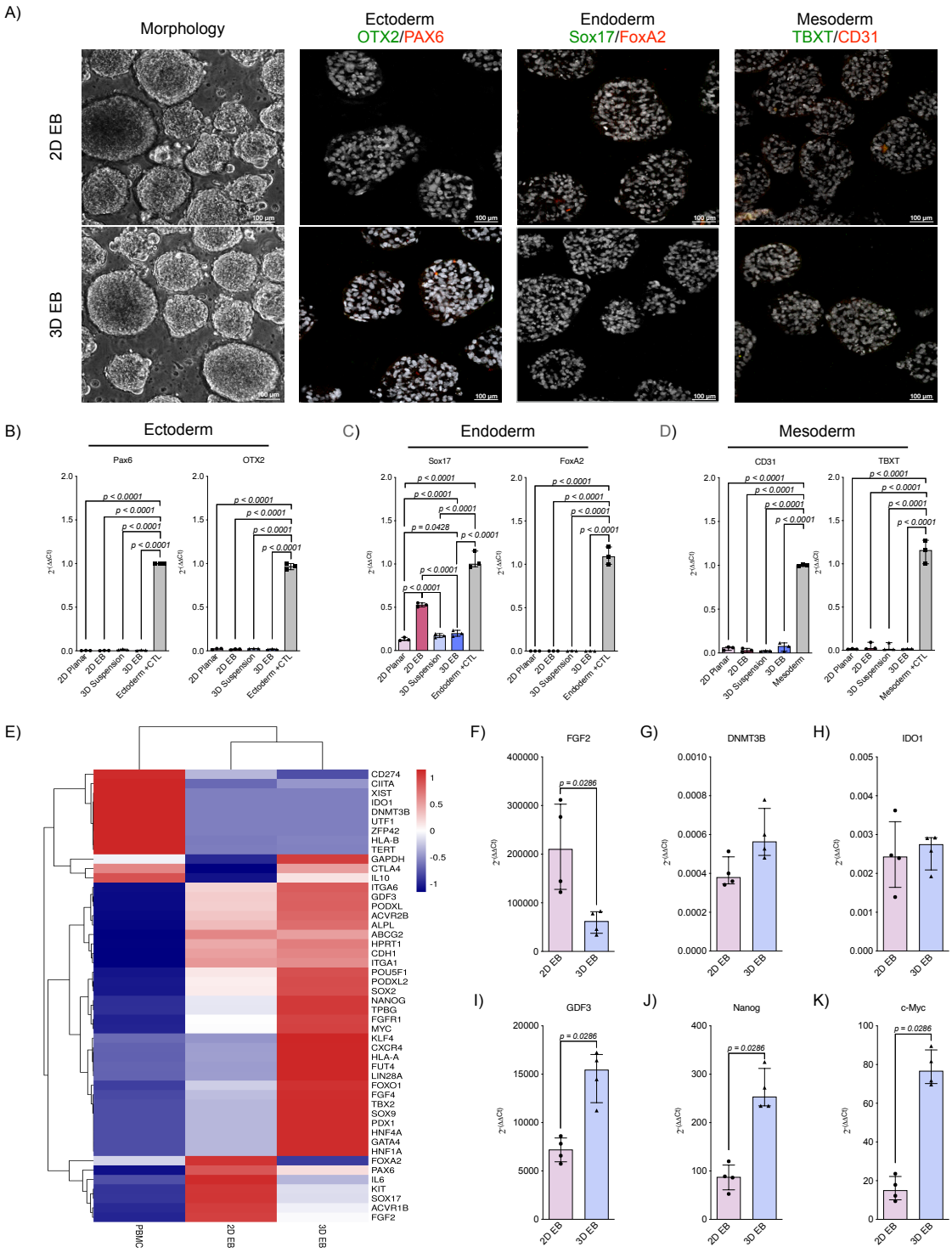

Figure S2.2.11 Comparison of embryoid bodies generated from iPSCs expanded through 2D planar and 3D suspension culture conditions.

A) Microscopy showing embryoid body morphology and immunohistochemistry of embryoid bodies evaluating ectoderm, mesoderm, and endoderm markers to assess spontaneous differentiation. B) Transcriptomic assessment of embryoid bodies generated from 2D planar and 3D suspension iPSCs with comparison to cells expanded in 2D planar and 3D suspension culture conditions. C) Transcriptomic assessment of ectoderm, mesoderm, and endoderm gene expression within embryoid bodies generated from 2D planar and 3D suspension conditions and iPSCs expanded using 2D planar and 3D suspension culture conditions.

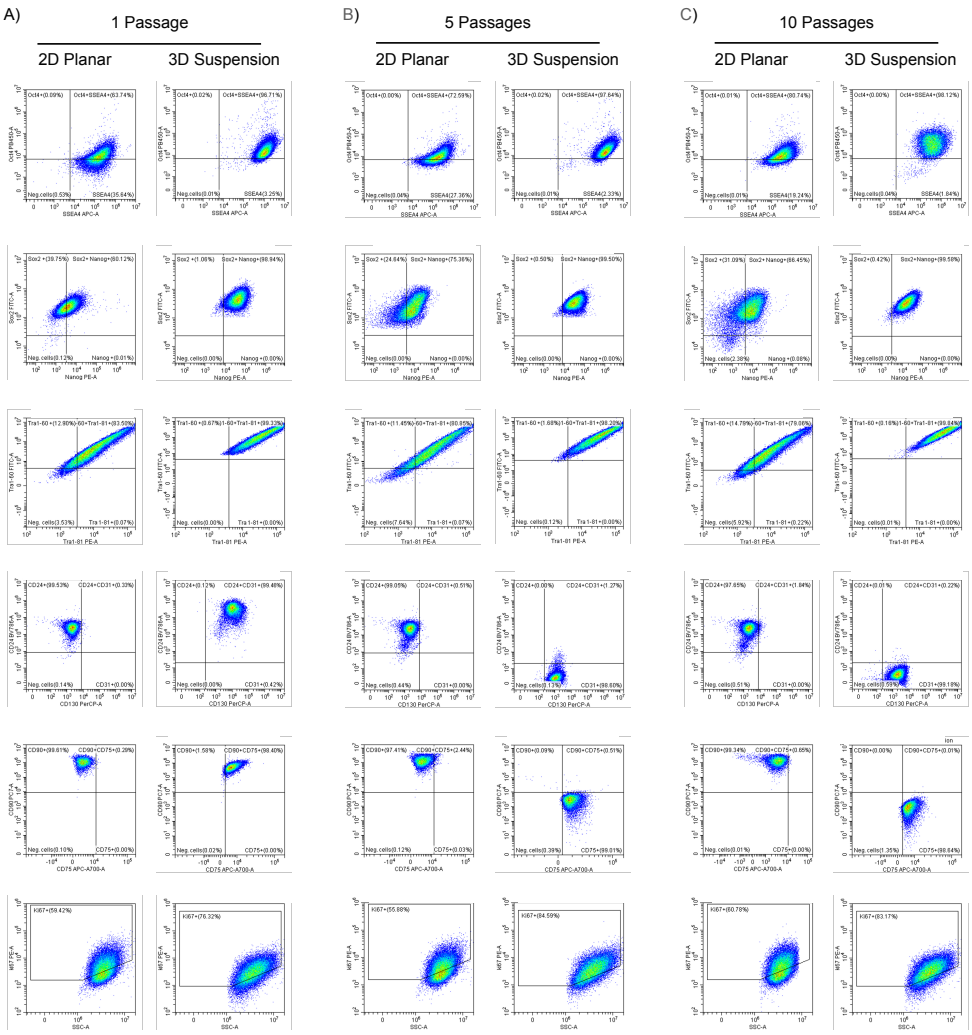

Figure S2.2.12 Flow cytometric cell characterization following 1, 5, and 10 passages using 2D planar and 3D suspension iPSC expansion.

Characterization of Oct4, SSEA4, Sox2, Nanog, Tra-1-60, and Tra-1-81 pluripotency markers, CD24, CD130, CD90, CD75, naïve/prime markers, and Ki67 during iPSC expansion using 2D planar and 3D suspension approaches following A) 1 passage, B) 5 passages, and C) 10 passages.

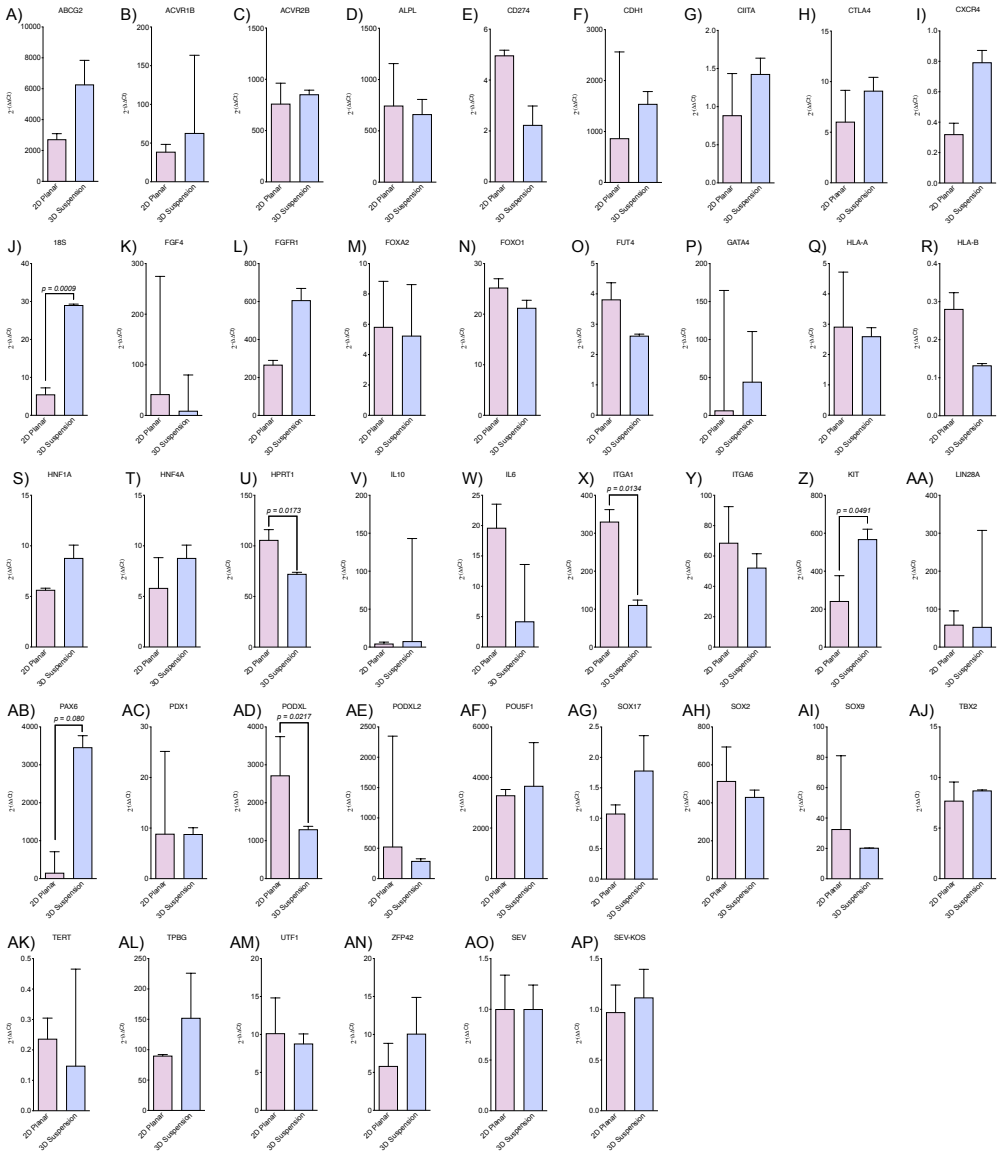

Figure S2.2.13 Transcript assessment of iPSCs expanded using 2D planar and 3D suspension protocols.
Only statistically significant differences are noted within graphs.

Table S2.2.3 Patient demographics used in this study.

iPSC line	Age	Sex	Gender	Health status
#1	53	Female	Female	Healthy
#2	43	Female	Female	Healthy
#3	28	Male	Male	Healthy
#4	29	Male	Male	Healthy

Table S2.2.4 Polymerase chain reaction mix used for assessment of viral clearance in iPSCs.

Reagent	10 µL total volume	x3.2 reactions
***Template DNA**	1.0 µL	-
Primers F & R	2.0 µL	6.4 µL
Master Mix	5.0 µL	16.0 µL
Nuclease free water	2.0 µL	6.4 µL
Total Volume:	10 uL	32 uL

*x3.2 reactions were prepared to allow for 1 tube containing the test sample one for the positive control (Beta actin) well and one for the negative control (nuclease free water).

Table S2.2.5 Forward and reverse primer sequences for polymerase chain reaction assessment of viral clearance in induced pluripotent stem cells.

Gene ID	Forward Primer	Reverse Primer	Product Size (base pair)
SEV	GGA TCA CTA GGT GAT ATC GAG C	ACC AGA CAA GAG TTT AAG AGA TAT GTA TC	181
KOS	ATG CAC CGC TAC GAC GTG AGC GC	ACC TTG ACA ATC CTG ATG TGG	528
CMYC	TAA CTG ACT AGC AGG CTT GTC G	TCC ACA TAC AGT CCT GGA TGA TGA TG	532
KLF	TTC CTG CAT GCC AGA GGA GCC C	AAT GTA TCG AAG GTG CTC AA	410
Human β-Actin	TGC CCA TTT ATG AGG GCT AC	GCC ATC TCG TTC TCG AAG TC	195

These sequences were adapted from CytoTune iPS 2.0 Sendai Reprogramming Kit (Thermo Fisher cat. A16517).

Table S2.2.6Thermocycler sequence for Viral Screening PCR

Temperature	Duration	Cycles
95°	5 minutes	1
95°	30 seconds	34
55°	30 seconds	
72°	30 seconds	
72°	5 minutes	1

Table S2.2.7. Quantitative Polymerase Chain Reaction Sequence for Karyotype Analysis

Stage	Cycles	Temperature (°C)	Cycling Time (min:sec)
Polymerase Activation	1	95.0	3:00
Denature	40	95.0	0:05
Anneal		60.0	0:30

Table S2.2.8 Thermo Fisher TaqMan Micro Array configuration.

Assay ID	Gene	Gene Name(s)	Species	Amplicon Length	Best Coverage	3' Most
Hs01053790_m1	ABCG2	ATP binding cassette subfamily G member 2 (Junior blood group)	Human	83	Yes	No
Hs00923299_m1	ACVR1B	activin A receptor type 1B	Human	74	Yes	No
Hs00609603_m1	ACVR2B	activin A receptor type 2B	Human	101	Yes	No
Hs01029144_m1	ALPL	alkaline phosphatase				
Hs00187842_m1	B2M	beta-2-microglobulin	Human	64	Yes	No
Hs00204257_m1	CD274	CD274 molecule	Human	77	Yes	Yes
Hs01023895_m1	CDH1	cadherin 1	Human	80	Yes	No
Hs00172106_m1	CIITA	class II				
Hs00175480_m1	CTLA4	cytotoxic T-lymphocyte associated protein 4	Human	93	Yes	No
Hs00607978_s1	CXCR4	C-X-C motif chemokine receptor 4	Human	153	Yes	Yes
Hs99999905_m1	GAPDH	-	Human	0	No	No
Hs00999691_m1	FGF4	fibroblast growth factor 4	Human	130	No	No
Hs00915142_m1	FGFR1	fibroblast growth factor receptor 1	Human	62	No	No
Hs00232764_m1	FOXA2	forkhead box A2	Human	66	No	No

Hs00231106_m1	FOXO1	forkhead box O1	Human	103	Yes	Yes
Hs01106466_s1	FUT4	fucosyltransferase 4	Human	152	Yes	Yes
Hs00171403_m1	GATA4	GATA binding protein 4	Human	68	Yes	No
Hs00220998_m1	GDF3	growth differentiation factor 3	Human	65	Yes	Yes
Hs01058806_g1	HLA-A	major histocompatibility complex				
Hs00818803_g1	HLA-B	major histocompatibility complex				
Hs00167041_m1	HNF1A	HNF1 homeobox A	Human	96	Yes	No
Hs00230853_m1	HNF4A	hepatocyte nuclear factor 4 alpha	Human	49	Yes	No
Hs99999909_m1	HPRT1	hypoxanthine phosphoribosyltransferase 1	Human	100	No	Yes
Hs00961622_m1	IL10	interleukin 10	Human	74	Yes	Yes
Hs00174131_m1	IL6	interleukin 6	Human	95	Yes	Yes
Hs00235006_m1	ITGA1	integrin subunit alpha 1	Human	87	Yes	No
Hs01041011_m1	ITGA6	integrin subunit alpha 6	Human	64	Yes	No
Hs00174029_m1	KIT	KIT proto-oncogene receptor tyrosine kinase	Human	64	Yes	No
Hs00358836_m1	KLF4	Kruppel like factor 4	Human	110	Yes	Yes
Hs00702808_s1	LIN28A	lin-28 homolog A	Human	143	Yes	Yes
Hs00153408_m1	MYC	v-myc avian myelocytomatosis viral oncogene homolog	Human	107	Yes	Yes
Hs04260366_g1	NANOG	Nanog homeobox	Human	99	No	Yes
Hs00240871_m1	PAX6	paired box 6	Human	76	No	No
Hs00236830_m1	PDX1	pancreatic and duodenal homeobox 1	Human	73	Yes	Yes
Hs01574644_m1	PODXL	podocalyxin like	Human	82	Yes	Yes
Hs00210532_m1	PODXL2	podocalyxin like 2	Human	73	Yes	No
Hs04260367_gH	POU5F1	POU class 5 homeobox 1	Human	77	Yes	Yes
Hs00751752_s1	SOX17	SRY-box 17	Human	149	Yes	Yes
Hs01053049_s1	SOX2	SRY-box 2	Human	91	Yes	No

Hs00165814_m1	SOX9	SRY-box 9	Human	102	Yes	Yes
Hs00266645_m1	FGF2	fibroblast growth factor 2	Human	82	Yes	No
Hs00911929_m1	TBX2	T-box 2	Human	60	Yes	No
Hs00972656_m1	TERT	telomerase reverse transcriptase	Human	79	No	No
Hs00907219_m1	TPBG	trophoblast glycoprotein	Human	100	No	No
Hs00864535_s1	UTF1	undifferentiated embryonic cell transcription factor 1	Human	102	Yes	Yes
Hs01938187_s1	ZFP42	ZFP42 zinc finger protein	Human	146	Yes	Yes
Mr04269880_mr	SEV	Sendai	Markers & Reporters	59	No	No
Mr04421257_mr	SEV-KOS	Sendai-KLF4-KOS	Markers & Reporters	80	No	No

Table S2.2.9 Sequences and amplicon length of primers used for RT-PCR assessment.

Gene	Forward Primer	Reverse Primer	Amplicon Length
XIST	GTTAGGGACAGTGAGTTAGAAATTGT	CTGGACTCAGTAACACCCCTTTC	512
DNMT3B	CTGGCGTCTGAGCCTTCG	ATTGAGATGCCTGGTGTCTCC	268
CD31	CTGAGGAATTGCTGTGTTCTGTG	CTGCTTTGCATTTTCTTTGAGAAGTG	274
TBXT	CCAGTGCGTTCAGCATCG	CTACCAAGAGCTGCCTCCAC	254
Pax6	CACTTAAAAGTGATGGGATTGACTGTCT	ACAGCCCTCACAAACACCTAC	244
NES	CACCCCTAAGTCCCCAGTG	GGAGCAGTCTGAGGAAGTGG	234
OTX2	CCCTCTAAGGCCCTTCGTTTT	GCTTGGATTATAAGGACCAAACTGC	266
IDO1	CCCTGTGATAAACTGTGGTCACT	CCACAGTTGTTCAGTAGAAGTTAACTTG	274

Table S2.2.10 Antibodies and concentrations used for flow cytometry and immunohistochemistry.

Antibody	Fluorophore	Primary Antibody Supplier (catalog number)	Secondary Antibody Supplier (catalog number)	Dilution for flow cytometry	Dilution for immunohistochemistry

Tra-1-60	FITC	Invitrogen (A25617)	-	1:100	1:100
Tra-1-81	Cy3	EMD Millipore (MAB4381C3)	-	1:100	1:100
Oct4	BV421	EMD Millipore (MAB4419A4)	-	1:100	1:100
Nanog	PE	Invitrogen (PA5-46891)	-	1:100	N/A
Nanog	FITC	EMD Millipore (MABBD24A4)	-	N/A	1:100
Sox2	FITC	Invitrogen (53-9811-82)	-	1:100	1:100
Ki-67	PerCP-Cy 5.5	BD (561284)	-	1:50	N/A
Ki-67	Secondary PE	Abcam (ab15580)	Sigma (A11036)	N/A	:50
SSEA4	Secondary APC	Invitrogen (MA1-021)	Jackson Immuno (115-135-164)	1:100	1:100
CD-24	BV-786	BD (740971)	-	1:100	1:100
CD130	BB700	BD (746079)	-	1:100	1:100
PAX6	Secondary: APC	Fisher (42-6600)	(Jackson Immuno) 115-135-164	N/A	1:200
CD184	BV421	BD (562448)	-	N/A	1:100
CD31	Secondary PE	Abcam (ab28364)	Sigma (A11036)	N/A	1:50
Sox17	Secondary FITC	R&D (963121)	Thermo (A16000)	N/A	1:10
FoxA2	Secondary PE	Abcam (108422)	Sigma (A11036)	N/A	1:50
Otx2	Secondary FITC	R&D (963273)	Thermo (A16000)	N/A	1:10
TBXT	Secondary FITC	R&D (963427)	Thermo (A16000)	N/A	1:10

*All secondaries for flow cytometry were used at a 1:500 concentration and all secondaries for immunohistochemistry were used at a 1:250 concentration.

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Chapter 3: Evaluation of Techniques for Efficient, Safe, and Reliable Stem Cell-Derived Islet Generation

- Chapter 3 subsection 1: Optimizing Generation of Stem Cell-Derived Islet Cells
- Chapter 3 subsection 2: Cell characterization, graft evaluation, and yield of islet-like cells differentiated from patient-derived iPSCs

Chapter Summary:

Chapter 3 includes a review and preclinical study both focused on optimizing generation of stem cell-derived islets. Subsection 3.1 first provides an overview of current differentiation protocols and the embryological pathway for islet generation. The review also discusses potential approaches to optimize differentiation but also to reduce off-target populations. This knowledge is then translated into a preclinical study (chapter 3.2) where 32 different protocols are evaluated to generate an optimized islet differentiation process. Subsequently, islet-like cells from this differentiation are characterized throughout their transition from induced pluripotent stem cells to islets with transplant into a mouse model and graft evaluation. Most importantly, we demonstrate that this differentiation protocol is applicable in three-dimensional suspension culture within Vertical-Wheel® bioreactors, demonstrating an approach to maximize cell product and reduce costs.

3.1 Chapter 3 subsection 1 – Optimizing Generation of Stem Cell-Derived Islet Cells

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Optimizing Generation of Stem Cell-Derived Islet Cells

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Abstract
Islet transplantation is a highly effective treatment for select patients with type 1 diabetes. Unfortunately, current use is limited to those with brittle disease due to donor limitations and immunosuppression requirements. Discovery of factors for induction of pluripotent stem cells from adult somatic cells into a malleable state has reinvigorated the possibility of autologous-based regenerative cell therapies. Similarly, recent progress in allogeneic human embryonic stem cell islet products is showing early success in clinical trials. Describing safe and standardized differentiation protocols with clear pathways to optimize yield and minimize off-target growth is needed to efficiently move the field forward. This review discusses current islet differentiation protocols with a detailed break-down of differentiation stages to guide step-wise controlled generation of functional islet products.

Keywords Islet cell transplant · Diabetes · Inducible pluripotent stem cells · Differentiation

Introduction

Islet transplantation (ITx) has shown to be a highly effective treatment for type 1 diabetes mellitus (T1D) in patients with hypoglycemia and brittle disease [85]. Recent long-term outcomes have demonstrated 10-year graft survival rates of 78% coupled with substantial improvements in insulin requirements, glycemic control, and mortality [71, 113, 133, 138]. Despite these promising outcomes, the primary barriers for ITx remain immunosuppression requirements and limited donor organ supply. Stem cell (SC)-derived islet cell production has long been considered as an option to surmount these barriers [18, 152]. Drs. Takahashi and Yamanaka's discovery of controllable factors for induction of pluripotent stem cells (iPSCs) from adult somatic cells, has reinvigorated the possibility of autologous-based cell regenerative therapies [137]. Similarly, recent progress in human allogeneic embryonic stem cell (hESC) islet products is showing early success in clinical testing [102, 114], while efforts are underway to make these cells less immunogenic through gene editing. Efficiently and safely differentiating SCs into functional islets could provide an unlimited supply of hypo- or non-immunogenic cells for β-cell replacement therapies [67, 137, 138].

Multiple groups have successfully generated mature and functional islets from SCs that are capable of reversing diabetes in preclinical models [51, 52, 92, 99, 134]. Despite success, differentiation protocols remain highly heterogenous [51, 52, 92, 99, 134]. Inadvertent production of off-target cells remains a major issue, with numerous techniques to ensure product safety by eliminating off-target growth described [9–12, 121, 136]. To move the field forward,

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3.1.1 **Abstract**

Islet transplantation is a highly effective treatment for select patients with type 1 diabetes. Unfortunately, current use is limited to those with brittle disease due to donor limitations and immunosuppression requirements. Discovery of factors for induction of pluripotent stem cells from adult somatic cells into a malleable state has reinvigorated the possibility of autologous-based regenerative cell therapies. Similarly, recent progress in allogeneic human embryonic stem cell islet products is showing early success in clinical trials. Describing safe and standardized differentiation protocols with clear pathways to optimize yield and minimize off-target growth is needed to efficiently move the field forward. This review discusses current islet differentiation protocols with a detailed break-down of differentiation stages to guide step-wise controlled generation of functional islet products.

3.1.2 **Introduction**

Islet transplantation (ITx) has shown to be a highly effective treatment for type 1 diabetes mellitus (T1D) in patients with hypoglycemia and brittle disease. Recent long-term outcomes have demonstrated 10-year graft survival rates of 78% coupled with substantial improvements in insulin requirements, glycemic control, and mortality [1-4]. Despite these promising outcomes, the primary barriers for ITx remain immunosuppression requirements and limited donor organ supply. Stem cell (SC)-derived islet cell production has long been considered as an option to surmount these barriers [5,6]. Drs. Takahashi and Yamanaka's, and concomitantly Dr. Thomson's, discovery of controllable factors for induction of pluripotent stem cells (iPSCs) from adult somatic cells, has reinvigorated the possibility of autologous-based cell regenerative therapies [7]. Similarly, recent progress in human allogeneic embryonic stem cell (ESC) islet products is showing early success in clinical testing [8,9], while efforts are underway to make these cells less immunogenic through gene editing. Efficiently and safely differentiating SCs into functional islets could provide an unlimited supply of hypo- or non-immunogenic cells for β-cell replacement therapies [3,7,10].

Multiple groups have successfully generated mature and functional islets from SCs that are capable of reversing diabetes in preclinical models [11-15]. Despite success, differentiation protocols remain highly heterogenous [11-15]. Inadvertent production of off-target cells remains a major issue, with numerous techniques to ensure product safety by eliminating off-target growth described [16-21]. To move the field forward, developing safe and standardized differentiation protocols capable of efficiently generating glucose responsive, insulin producing, highly specific

β-like cells free of off-target growth, while enabling scalability under good manufacturing practices (GMP) conditions, remain critical for clinical translation.

This review discusses SC-derived ITx and the two pathways, autologous and allogeneic, that may be taken to achieve a T1D cure. We also highlight their primary barrier, which remains optimization of differentiation protocols and off-target growth elimination. The review focuses on current islet differentiation protocols, with clear pathophysiological break-down of each differentiation stage, including stage-specific approaches to optimize islet generation. We aim to provide a clear experimental pathway for researchers to collectively improve islet differentiation protocols, and efficiently move in-human SC-derived islet cell therapy trials forward.

3.1.3 **Stem Cell-Derived ITx**

Allogeneic ITx has already provided proof-of-concept for a cell-based T1D treatment with substantial long-term benefits [1,2,8,9,22-26]. However, allogeneic ITx currently remains limited by immunosuppression requirements and organ supply, which SC-derived ITx aims to overcome. Currently, there are two approaches to generating SC-derived replacement therapies, autologous and allogeneic.

3.1.3.1 Allogeneic Stem Cell-Derived ITx

Two options for allogeneic SC-derived ITx exist. The first, which remains untested, involves creating a human leukocyte antigen (HLA) SC-derived islet bank to enable HLA-matched ITx. The more robustly tested approach, with clinical trials already in place (NCT03525444 and NCT04678557), involves generating a genetically-modified ESC line to

produce hypoimmunogenic ESC-derived islets. Both options enable scalability, but currently remain untested with regards to their potential for immunosuppression freedom.

Generating SC-derived islet cell banks to provide HLA-matched allogeneic ITx would enable scalability, but in our opinion will remain limited by immunosuppression requirements. Although immunosuppression requirements could theoretically be decreased using HLA-matched islets, the inability to match every HLA antigen (including minor antigens), and a growing recognition of non-HLA mediated allograft responses suggest that lifelong immunosuppression will almost certainly be required [27,28]. This approach markedly underestimates the destructive power of minor HLA antigen epitopes, and the assumption that this could be overcome by reduced need for immune suppression is likely false.

To potentially eliminate the need for immunosuppression, generating modified hypoimmunogenic SC-derived islets has been proposed. Genetically modifying ESCs to express immunotolerant molecules such as IL-10 or PD-L1 [29,30], or eliminating HLA class 1 molecule expression has been accomplished and shown reduced T-cell and macrophage reactivity, and minimal NK cell-mediated death [31-33]. Combinatorial approaches are also being actively tested, for example in ViaCyte's PEC-QT system [3,7,34], but outcomes remain unreported. More recently, Vertex Pharmaceuticals Inc. has initiated their first-in-human phase 1/2 clinical trial, with promising early phase results testing the VX-880 ESC-derived islet product in their first subject, but less so with two subsequent cases. With intraportal transplantation and immunosuppression, optimal glycemic control and near complete insulin independence has been shown in a patient living with T1D, demonstrating proof-of-concept for these approaches [35]. Despite promising early results, it remains uncertain whether immunosuppression will remain a barrier and whether

genetic manipulation will affect the ability to produce functional, safe β-cells following differentiation.

3.1.3.2 Autologous iPSC-Derived ITx

Autologous iPSC ITx offers a potential solution to both immunosuppression, and donor limitations [7,10]. It involves personalized regenerative medicine, with patients providing cells (likely blood) for iPSC generation, and subsequent differentiation into iPSC islets for autologous transplantation without immunosuppression [3,7]. This technique parallels autologous ITx, which has proven to be highly successful for patients undergoing pancreatectomy [36-38]. Drawbacks include the potential for recurrent autoimmunity, and scalability issues to generate autologous iPSCs for >8 million patients with T1D [7,39].

Work to eliminate substantial barriers for autologous iPSC ITx is ongoing. To combat potential recurrent autoimmunity, immune reset techniques to increase regulatory T-cells and combat autoimmune responses show promise [40-44], with a first in-human clinical trial to prevent T1D autoimmunity underway (NCT03182426). Regarding scalability, artificial intelligence, automated systems, commercial-sized bioreactors, and standardized efficient protocols, offer promise to enable cost-effective production of autologous iPSC islets, but remain to be delivered [7].

3.1.3.3 Commonalities of Optimized ESC and iPSC Differentiation Protocols

Currently, the major limitation to moving either technique further within clinical trials remains potential off-target growth risks [16,18,19]. Teratomas and off-target growth occur in these

cell products because of remnant pluripotent or proliferating cells [45,46]. To decrease that risk, elimination of off-target cells can be achieved through immunologic selection, genetic manipulation, and pharmacologic/chemical or mechanical techniques, as we discuss below. Developing an optimized, standard, and safe process without teratoma risk is crucial to moving the field forward, regardless of whether allogeneic or autologous approaches are applied [12,14,47,48].

3.1.4 **Current Differentiation Protocols for Stage 1-7**

Current differentiation protocols manipulate SCs through embryological stages to produce glucose-responsive islets. A month-long differentiation protocol transitions cells from iPSCs to definitive endoderm, primitive gut tube, posterior foregut, pancreatic endoderm, endocrine precursors, immature β-cells, and mature β-cells (Figure 3.1.1). Here, we discuss the generation of iPSCs, detail the embryologic pathways for islet cell differentiation, and summarize current protocols used to guide cells from iPSCs to mature β-cells.

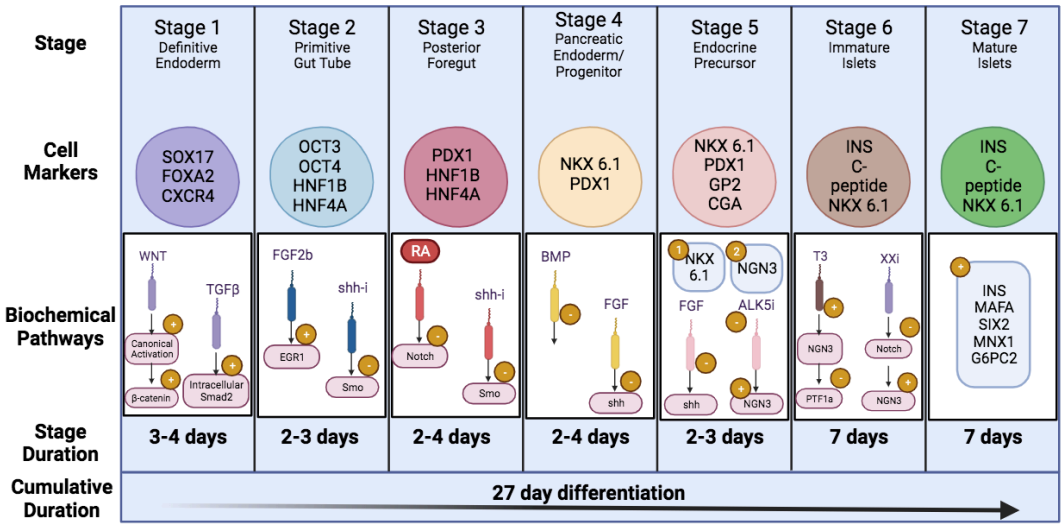

Figure 3.1.1 Seven stage differentiation of islet-like clusters from induced pluripotent stem cells.
*WNT: Wnt signaling pathway; TGFβ: transforming growth factor β; FGF2b: fibroblast growth factor receptor 2b; shh-i: sonic hedgehog inhibitor; Smo: Smoothened; RA: retinoic acid; BMP: bone morphogenic protein; FGF: fibroblast growth factor; shh: sonic hedgehog; NGN3: neurogenin 3; ALK5i: ALK5 inhibitor; T3: L-3,3′,5-Triiodothyronine; XXi: Gamma secretase XX inhibitor.

3.1.4.1 iPSC Generation

To reprogram somatic cells into iPSCs, reprogramming transcription factors (Oct3/4, Sox2, Klf4 and c-Myc) discovered by Yamanaka et al. and Thomson et al. are overexpressed to induce pluripotency [14,47,49]. Most frequently, peripheral blood mononuclear cells are used as somatic cells and a commercially available Sendai virus transduction kit efficiently produces GMP-compliant iPSCs [50]. Following iPSC generation, cells must be passaged approximately ten times to ensure the absence of Sendai viruses, followed by cell purity evaluation prior to differentiation [51]. Once iPSCs are generated and confirmed virus free, a seven-stage

differentiation protocol begins. Several protocols have been published (Table 3.1.1), with work to optimize these protocols ongoing.

Table 3.1.1 Key differentiation protocols and their reported efficiency of islet cell generation

Protocol	Stage 1	Stage 2	Stage 3	Stage 4	Stage 5	Stage 6	Stage 7	Protocol Efficiency*
Rezania et al. (2014) [48]	Duration: 3d GDF8 GSK3β inhibitor (only day 1)	Duration: 2d FGF7 Vitamin C	Duration: 2d FGF7 Vitamin C RA Sant-1 TPB LDN193189	Duration: 3d FGF7 Vitamin C RA Sant-1 TPB LDN193189	Duration: 3d Sant-1 RA ALK5 inhibitor T3 LDN193189	Duration: 7-15d ALK5 inhibitor T3 LDN193189 XXi	Duration: 7-15d ALK5 inhibitor T3 N-acetyl cysteine R428 (AXL inhibitor)	56.1% insulin
Yabe et al. (2017)[52]	Duration: 5d Bovine serum albumin Sodium pyruvate Activin A FGF2 BMP CHIR9902 (only days 1-2)	Duration: 2d FGF2 B27 EC23 Dorsomorphin SB431542 Sant-1	Duration: 4d FGF2 B27 Dorsomorphin Sant-1	Duration: 4d FGF10 B27 Dorsomorphin Sant-1 ALK5 inhibitor Indolactam V	Duration: 4d B27 Dorsomorphin Sant-1 ALK5 inhibitor Exendin-4	Duration: 6d B27 FGF2 BMP4 HGF IGF-1 ALK5 inhibitor Exendin-4 Nicotinamide forskolin	No stage 7	33.6% C-peptide
Sui et al. (2018) [53]	Duration: 4d Activin A Wnt3a	Duration: 2d FGF7 B27	Duration: 2d Cyclopamine RA B27 LDN193189	Duration: 4d EGF FGF7 B27	Duration: 1d B27 ALK5 inhibitor FGF7 Y-27632	Duration: 7d T3 B27 XXi ALK5 inhibitor Y-27632	Duration: 7d FBS	70% C-peptide
Nair et al. (2019) [54]	Duration: 2d Activin A Wnt3a	Duration: 3d KGF	Duration: 3d RA	Duration: 5d RA EGF	Duration: 5d T3 XXi	Cell sorting	Duration: 7d T3	59.7% C-peptide

	(only day 1)	TGFb inhibitor IV	Cyclopamine Noggin	KGF Noggin	LDN193189 TPB ALK5 inhibitor Noggin		ALK5 inhibitor	93% C-peptide after cell sorting
Velazco-Cruz et al. (2019)[13]	Duration: 3d Activin A CHIR99021 (only day 1)	Duration: 3d KGF	Duration: 1d KGF Sant-1 RA LDN193189 (only day 7) PdBU	Duration: 5d KGF Sant-1 RA Y27632 Activin A	Duration: 7d Sant-1 RA ALK5 inhibitor XXi T3 Betacellulin	Duration: >9d ESFM	No stage 7	75% C-peptide
Hogrebe et al (2021)[15]	Duration: 3d Activin A CHIR99021 (only day 1)	Duration: 3d KGF	Duration: 2d KGF LDN193189 TPB RA Sant-1	Duration: 4d KGF LDN193189 TPB RA (low dose) Sant-1	Duration: 7d ALK5 inhibitor XXi RA Sant-1 T3 latrunculin A (only day 1)	Duration: 7d ESFM Dispersed and reaggregated	Duration: 7d ESFM	60% C peptide

*Protocol efficiency was determined by flow cytometry evaluating the percent of cells expressing C-peptide or insulin.

3.1.4.2 Stage 1 (Definitive Endoderm Formation):

The first stage of differentiation involves transitioning SCs into definitive endoderm, a process that is comprehensively reviewed by D'Amour et al. (2005) [55]. During embryologic islet development, this occurs during gastrulation as epiblast cells undergo epithelial to mesenchymal transition [56,57]. Initial investigations demonstrated that WNT and TGF-β signaling are crucial, with transition failure observed when either pathway was disrupted [55,58,59]. Further work showed that the WNT pathway activator (Wnt-3a) provides canonical protein signaling that upregulates

intracellular β-catenin and initially directed cells to an endoderm fate [60-62]. Activation of the TGF-β pathway by Nodal with subsequent intracellular Smad2 induces the primitive streak and differentiation into definitive endoderm [63,64]. Therefore, together Wnt-3a and Nodal act to advance iPSCs into definitive endoderm that express the phenotypic markers SOX17, FOXA2, and CXCR4 [47,55,64].

In vitro, a commercially available endoderm differentiation kit using Wnt3a and activin-A produces definitive endoderm cells with 95% efficiency [53]. Using this approach, activin-A replaces Nodal as an alternative biochemical analogue to activate the TGF-β pathway because GMP-compliant, stable, bioactive Nodal currently does not exist [55,65,66]. Others have also reported success using CHIR99021, a selective glycogen synthase kinase-3β inhibitor, instead of WNT3a during the first 1-2 days of differentiation [12-15,48,52]. Overall, stage 1 involves transition of SCs into definitive endoderm over a 2-3 day period through exposure to activin-A and Wnt3a or CHIR99021.

3.1.4.3 Stage 2 (Primitive Gut Tube Formation):

Stage 2 involves anterior-posterior axis patterning to create the primitive gut tube. During this stage, elimination of activin-A/Nodal signaling is crucial [47,55,64]. In addition to Nodal downregulation, inhibition of Sonic hedgehog (shh) signaling is needed to support both primitive gut tube formation (stage 2) and posterior foregut differentiation (stage 3) [47,67,68]. Activation of fibroblast growth factor (FGF) receptor 2b, either with FGF2/KGF, FGF7 or FGF10, leads to early expression of growth response gene (EGR1) and ultimately induction of primitive gut tube formation [69].

In vitro, all studies report removal of activin-A, but several different FGFs and shh inhibitors are reported. For FGF, in order of oldest publication to most recent, D'Amour et al. (2006) and Kroon et al. (2008) use FGF10, Rezania et al. (2012) and Sui et al. (2018) use FGF7, Velazco-Cruz et al. (202) and Hogrebe et al. (2021) use keratinocyte growth factor (KGF) in their stage 2 media [12-15,47,53,70,71]. Similarly, for shh inhibition some authors use cyclopamine [47,53], while others describe use of Sant 1 molecules [12,14,72]; both Sant1 and cyclopamine act through smoothened (Smo) inhibition to prevent shh expression. In summary, stage 2 involves elimination of Nodal signaling, shh inhibition, and FGF2b activation to produce primitive gut tube cells expressing Hepatocyte nuclear factor (HNF) 1B and HNF4A [73-75].

3.1.4.4 Stage 3 (Posterior Foregut Formation):

Stage 3 encompasses generating posterior foregut cells capable of becoming pancreatic, hepatic, and duodenal tissues [70,71,76]. Continuous inhibition of shh with FGF is needed [68]. Additionally, retinoic acid (RA) signaling further inhibits shh in the dorsal prepancreatic endoderm and also inhibits Notch signaling, which is crucial for patterning of the posterior foregut that gives rise to liver and pancreas [77-79]. Once completed, cells express HNF1B, HNF4A, and pancreatic and duodenal homeobox 1 (PDX1).

During this stage, Sant1 or cyclopamine are used variably for shh inhibition. All protocols use RA during this stage for Notch inhibition and posterior foregut patterning [12,14,47,53,71]. Finally, while nearly all protocols incorporate neurotrophic factors to their media from stage 3 onwards, some authors report B27 supplementation [47,53,70,71], while others use nicotinamide [14], or (2S,5S)-(E,E)-8-(5-(4-(trifluoromethyl)phenyl)-2,4-pentadienoylamino)

benzolactam (TPB) [14,48]. Overall, stage 3 most commonly occurs over 2-4 days and requires ongoing shh inhibition and additional RA signaling to generate posterior foregut cells.

3.1.4.5 Stage 4 (Pancreatic Endoderm/Progenitor Formation):

Stage 4 encompasses differentiating posterior foregut cells into pancreatic progenitors (i.e pancreatic endoderm). During stage 4, posterior foregut cells can differentiate into hepatic or endocrine tissues. Hepatic tissues are favored through bone morphogenetic protein (BMP) signaling, while endocrine differentiation is blocked by FGF10 activation of shh [80,81]. Therefore, inhibition of both BMP and FGF10 signaling is required to generate pancreatic endoderm cells expressing NKX 6.1 and PDX1.

Current protocols use different approaches for FGF10 and BMP inhibition, with some authors using Noggin [47,70,71], and others LDN193189 [12,14,53]. Both molecules are BMP and FGF10 inhibitors and have successfully produced pancreatic endoderm cells [70,71,80]. In summary, stage 4 occurs over 2-4 days and directs cells from posterior foregut tissues towards pancreatic progenitors through inhibition of both BMP and FGF10.

3.1.4.6 Stage 5 (Endocrine Precursor Formation):

At this stage, cells can potentially become pancreatic epithelial progenitors or endocrine precursors. To generate endocrine precursors, ongoing inhibition of shh is continued. Next, for cells to become endocrine precursors they must first express NKX6.1 and subsequently express NEUROG3 (NGN3) [82]; expression of NGN3 prior to NKX6.1 produces non-functional polyhormonal cells [14,83]. Temporal control of expression is controlled by the cellular

microenvironment, with the actin cytoskeleton and cellular attachments directing NGN3 expression [14]. The impact of cellular microenvironment explains why stage 5 differentiation historically only occurred in 3D culture [12,14,48,53]. Alternatively, in 2D culture adhesion of stage 4 ($PDX1^+$) cells to Type-I collagen coated culture plates leads to NKX6.1 expression, which can be followed by stage 5 actin depolymerization using latrunculin A and YAP1 inhibition to increase NGN3 expression and direct differentiation into endocrine precursors [14,15,84]. Later in this stage, TGFβ receptor I (TβRI/ALK5) inhibition can further prevent β-cell de-differentiation and improve NGN3 expression [85-87].

Importantly, β-cells fail to mature from polyhormonal populations, which produce glucagon and somatostatin but not insulin [88]. Polyhormonal cells express a small amount of C-peptide (CPEP) (~10%), and also express PDX1 and NKX6.1 to a lesser extent. On the other hand, endocrine precursors differentiate into insulin-producing cell populations [14,88]; those cells express glycoprotein 2 (GP2), and isolation of only GP2 populations enables generation of mono-hormonal β-cells [88].

As previously mentioned, current protocols use variable techniques for shh inhibition, with most continuing the FGF inhibitor they use in stage 4 [12]. Because of the newly discovered utility of ALK5 inhibition, protocols have also added this to their stage 5 media [12,14,71]. Similarly, because of studies suggesting improved β-cell specificity with epidermal growth factor (EGF) activation [89,90], authors have begun adding betacellulin/EGF or heparin to their stage 5 media onwards [12,14,15,48]. D'amour et al. (2006) also reported the addition of DAPT and exendin-4 here, but noted limited benefits [55]. Finally, during stages 5-7, higher glucose media (~10mM) is required for cell survival, to improve cell maturation, and to improve glucose responsiveness of

the cell product [15,48,52,54]. In summary, stage 5 involves a key transition towards endocrine precursors, which occurs due to ongoing shh inhibition, EGF activation, and temporal control of NGN3 and PDX1 expression prior to NKX6.1.

3.1.4.7 Stage 6 (Immature Islet Cluster Formation):

Stages 6 and 7 offer endocrine cells time to mature and become functional with hormone expression. During stage 6, persistent NGN3 expression is needed to inhibit PTF1a exocrine differentiation and improve β-cell maturation [14,48,53,55,80,91]. Increased NGN3 expression occurs via inhibition of the Notch pathway through Gamma secretase XX inhibitors (XXi), which may also prevent apoptosis of islet cells [92,93]. Thyroid hormone L-3,3′,5-Triiodothyronine (T3) is also added because embryologically it increases following day 12.5 with substantial expression after day 17.5; T3 activates thyroid hormone receptors and increases NGN3 expression to promote endocrine lineages [94-97].

In vitro protocols first attempt to recreate these processes using XXi; D'amour et al. (2006) use DAPT as their XXi, while others typically use small molecule inhibitors [12,14,47]. More recently published protocols have also added T3 to their media during this stage [12,14,48,53]. D'amour et al. (2006) have also added exendin-4, and IGF-1 to this stage but noticed minimal benefit. Overall, stage 6 cells begin producing the five pancreatic endocrine hormones (insulin, glucagon, somatostatin, pancreatic polypeptide and ghrelin), with exposure to XXi and T3 allowing their maturation [47].

3.1.4.8 Stage 7 (Mature Islet Cluster Formation):

Stage 7 descriptions are heterogeneous, with many authors only reporting *in vitro* or *in vivo* cell maturation. A growing body of evidence suggests that, with time, cells mature and express *INS, MAFA, SIX2, MNX1*, and *G6PC2* that correlate with insulin secretion [98,99]. Similarly, studies have shown that cellular insulin content increases sixfold 3-weeks after transplantation as cells mature *in vivo* [98,100]. Despite maturation and transcriptional differences, the biochemical pathways driving these changes are poorly understood. However, similar post-natal islet maturation occurs *in vivo* with increased capacity for glucose-stimulated insulin secretion over time [101]. Most protocols report the continuation of all stage 6 supplements with removal of XXi and addition of enriched serum-free medium [14,47]. These culture conditions aim to provide amino acid, protein, vitamin, and growth factor to support cell maturation, with improved *in vitro* function recognized [15].

3.1.5 **Protocol Optimization and Selective Teratoma Elimination in Stage 1-7**

Although studies have comparatively evaluated, and often demonstrated benefits of specific techniques, no current study has optimized differentiation at every stage. In this section, we evaluate protocol differences and propose an optimal islet differentiation protocol. Because the primary barrier for iPSC ITx remains potential off-target growth, we also review stage-specific approaches to eliminate this risk [16]. The risk of teratoma and off-target growth occurs due to persistence of a small number of pluripotent cells following maturation [45,46]; therefore, achieving optimal differentiation efficiency using the techniques discussed should be the first approach. If needed, selectively eliminating any remaining pluripotent populations will enable

safe iPSC ITx. Three general approaches exist to eliminate off-target cells including immunologic selection, genetic manipulation, and chemical or mechanical strategies; each of these approaches offer specific benefits and drawbacks that are discussed below.

3.1.5.1 iPSC Generation

Generation of iPSCs can be achieved using numerous techniques that allow cells to express the reprogramming factors (Oct4, Sox2, Klf4, and c-myc)[49]. Briefly, this can be achieved by cell exposure to reprogramming vectors, non-integrating viruses, plasmids, mRNA transfection and other techniques [51]. More extensive review of these approaches is conducted by Malik et al. (2013) and Maherli et al. (2008) [51,102]. We suggest Sendai virus transduction be used, as it is the only GMP-compliant, well-defined, technique that can achieve adequate reprogramming efficiency without genomic integration [103].

Once an iPSC line is generated, genetic modification has been considered to eliminate off-target risks. This may be accomplished by modifying tumor-progression genes [104,105], or introducing drug-activated apoptosis genes to enable selective apoptosis in case of off-target growth [106-109]. Alternatively, genetically labelling proliferating cells for future immunologic separation has also been evaluated [19]. These techniques have not been evaluated for iPSC-derived islet generation; thus, it remains uncertain whether differentiation would still occur following genetic manipulation and whether cell functionality would be affected. The cost of genetically editing cells prior to autologous transplant would also likely be too high. However, if an immune-silenced iPSC line could be generated and undergo genetic manipulation to eliminate off-target risks, it may enable immunosuppression free allogeneic iPSC ITx [32,33].

3.1.5.2 Stage 1

The two points of contention for stage 1 are the use of Wnt3a versus CHIR99021 and the stage's duration. For Wnt3a versus CHIR99021, the only comparative evaluation has been completed by Yabe et al. (2017), who reported improved cell viability and efficiency of definitive endoderm formation with CHIR99021 compared to Wnt3a [52]. However, their cell product after stage 1 was >90% SOX17 and FOXA2 positive, which others have reported using commercially available kits [47,53]. In terms of duration, Toivonen et al. (2013) demonstrate that three days is optimal to generate tissues of pancreatic lineage, with Wnt3a or CHIR99021 exposure only during the first 24-hours [110].

Overall, stage 1 should likely occur over three days, with Wnt3a or CHIR99021 exposure limited to the first 24-hours. No evidence suggests better outcomes with Wnt3a compared to CHIR99021 and both are available under GMP-compliant presentation, meaning either product is likely acceptable.

3.1.5.3 Stage 2

In stage 2, authors agree that Nodal downregulation is required. However, it remains uncertain what type of FGF to use, and which chemicals to apply for shh inhibition. For FGF selection, KGF, FGF2, FGF7, and FGF10 have all been trialed. Kroon et al. (2008) improved pancreatic progenitor formation using FGF10 as compared to KGF [70]. Similarly, D'Amour et al. (2006) demonstrated that addition of FGF10 and hedgehog-signaling inhibitor cyclopamine increased efficiency of primitive gut tube formation by 160-fold [47]. Ye et al. (2005) also provide comparative results demonstrating improved differentiation efficiency with FGF10 compared to

FGF7 [69]. A recent comparative study evaluating FGF2, FGF7, and FGF10 supports these findings and demonstrates that FGF7 and FGF10 have similar capacity to produce PDX1 positive cells, but that both are significantly better than FGF2 [111]. They demonstrate that FGF2 acts through FGFR1c/3c and that culture using FGF7 and PD-173074, a small molecule inhibitor of this receptor, significantly improved differentiation efficacy [111]. Therefore, FGF10 and potentially the addition of PD-173074 may be the most efficient approach for stage 2 differentiation.

With regards to Smo inhibitor-directed shh inhibition, few studies have compared cyclopamine to Sant1. Evaluating their biochemical pathways, we see that they both bind directly to Smo for inhibition, but have variable effects [76]. Comparing Sant 1, to Sant 1, 2, and 3 and cyclopamine demonstrates that Sant1 inhibits shh signaling 60-times more [76]. Using these biochemical findings, it is possible that Sant1 may be ideal for islet differentiation efficacy in both stage 2 and 3, although this remains to be studied.

Stage 2 durations of 2-3 days have been reported, but optimal timing of stage 2 based on embryological studies in mice should be 2.5 days considering that anterior-posterior patterning occurs from days 4-5.5 [112-114]. For off-target cell elimination during stage 2, Nodal down-regulation appears crucial. If persistent Nodal exposure persists, Oct3 and Oct4 are expressed and development of teratomas occurs [47,64]. Additionally, including vitamin C from stages 2-4 may reduce premature NGN3 expression and potentially reduce teratogenicity [48,91].

3.1.5.4 Stage 3

Chemicals used variably in stage 3 include B27 versus nicotinamide. Cogger et al. (2017) have suggested that nicotinamide from stages 3 onwards increases the proportion of cells expressing GP2 [88]. Others have suggested nicotinamide coupled with phosphoinositide 3-kinase (PI3K) inhibition may yield the best results. This was originally suggested by Hori et al. (2002), who cultured stage 3-5 cells with nicotinamide and the phosphoinositide 3-kinase (PI3K) inhibitor LY294002 and developed ESC-derived islet cells without tumorigenicity [115]. More recently this has reliably been reproduced with PI3K inhibitors (LY294002 or TGX-221) added alongside nicotinamide, producing more mature and islet like cells compared to culture with B27 alone [116,117].

Activation of protein kinase C (PKC) also appears to increase expression of PDX1 in the posterior foregut and decrease development of other hepatic or duodenal cells [71,118]. Authors have reported use of PKC activators including Indolactam V, TPB, and phorbol 12, 13-dibutyrate (PBDu). Rezania et al. compared TPB to PBDu and reported similar differentiation efficacy but better safety profiles with TPB [71]. Similarly, Hogrebe et al. (2020) and others used TPB to improve induction of pancreatic progenitors from primitive gut tube (stages 3-4) [14,71,119-121]. Others have reported similar outcomes using indolactam V [120,122], but comparative evaluation has not been completed.

Durations ranging from 1-4 days have been reported for stage 3. Few studies have evaluated optimal timing of this stage but it appears that FGF and RA activation is crucial during days 8-9 of differentiation [123]. Therefore, we suggest a duration of at least 2.5 days for stage 3.

3.1.5.5 Stage 4

The primary difference during stage 4 is the use of Noggin or LDN-193189 for BMP inhibition. Rezania et al. (2014) provide preliminary evidence suggesting that LDN-193189 is optimal, but few other studies have provided comparative data [48]. Interestingly, when Noggin or LDN-193189 are compared to antibody-directed BMP inhibition, immunologic inhibition appears to be more efficient during stage 4 [124]. Comparatively evaluating antibody mediated BMP inhibition to LDN-193189 or Noggin during islet cell differentiation may be of interest in future studies [124].

For stage 4 duration, Jorgensen et al. (2007) provide a comprehensive review of the timing for embryonic pancreatic endoderm and endocrine precursor formation [125]. They show that PDX1 and NKX 6.1 increase substantially from days 8-11 and that HBlx9, a marker of the dorsal endoderm is lost at day 11 [125]. Considering these findings, stage 4 duration should be 3 days, and occur from days 8-11 of the differentiation process.

3.1.5.6 Stage 5

Protocols remain poorly defined following stage 5 of differentiation. For stage 5, the selection of FGF molecule for inhibition of shh is variable and typically carried forward from stage 4, but no comparative evaluation has been completed. Considering the potential for improved shh inhibition with FGF10, future studies evaluating its use as the FGF isoform from stages 4-5 may be of interest. Additionally, some authors have reported the addition of heparin or EGF/betacellulin to stage 5-6 media [14,15,48], which may activate EGF receptors to promote β-cell proliferation [126,127]. Rezania et al. (2014) noted improved cell viability with heparin at stage 5 [48],

and others have also reported improved PDX1 sustained expression with its use [128]. Biochemical evaluation suggests that they act through heparin-binding EGF to selectively increase β-cell differentiation and proliferation as opposed to pancreatic ductal cells [89,90]. Therefore, the most specific EGF activator is likely heparin, although no direct comparison to betacellulin exists.

Culture of stage 5-7 cells is also conducted in higher glucose media [15,48,52,54], which improves their glucose responsiveness and insulin secretion, with the potential added benefit of SC cytotoxicity [129,130]. Lower glucose levels prior to this stage are required to maintain cellular pluripotency and enable differentiation [131,132]. In terms of stage 5 duration, the optimal timing corresponds to increased NGN3 expression during embryonic days 11.5 to 12.5 [125]. Thus, we suggest achieving ≥ 2-day stage 5 duration to ensure NGN3 expression [125].

Several chemical approaches to eliminate off-target growth have been considered during stage 5. These methods act by selectively killing proliferating or pluripotent cells and include PluriSIn1, aphidicolin, querceptin or YM155, clostridium perfringens exotoxin, AT7867, and MitoBloCK-6 [17-19,21,133-135]. For PluriSIn1, high throughput screening of 52,000 molecules identified, PluriSIn1 as the most efficient pluripotent cell inhibitor [18]; further studies demonstrated that it induces apoptosis through inhibition of stearoyl-coA desaturase (SCD1), the key enzyme in oleic acid biosynthesis for pluripotent cells [17,18]. Similarly, the DNA polymerase inhibitor aphidicolin inhibits G1 to S phase transition and can selectively eliminate iPSCs [17,21]. Inhibition of the pro-oncogene *survivin* with quercetin or YM155 has also demonstrated targeted iPSC cell death [134]. Alternatively, evaluation of cell pathways of undifferentiated pluripotent cells has shown that the tight-junction protein Claudin-6 is specific to pluripotent cells and can be targeted by selective antibodies or *Clostridium perfringens* exotoxin to prevent tumorigenicity

of iPSCs [19]. Application of these drugs during stage 5, once cells have reached adequate maturation, may enable targeted elimination of any remnant off-target cells.

Alternatively, mechanical approaches during stage 5 to eliminate teratogenicity also exist. The most evaluated approach involves disaggregation and reaggregation of islet cell clusters at the conclusion of stage 5 [20]. Similar techniques have been described to purify islets during differentiation from ESCs [136], and during porcine pancreas islet isolation to achieve ~98% endocrine cells specificity [137].

3.1.5.7 Stage 6

To optimize *in vitro* islet maturation most protocols incorporate ALK5 inhibition, XXi, and T3 to increase NGN3 expression. Numerous studies also demonstrate that islet cell survival is dependent on zinc, with culture concentration of 0.02 mM leading to optimized insulin secretion [138-140]. Therefore, authors added zinc sulfate ($ZnSO_4$) to stage 6-7 media to improve cell's glucose-stimulated insulin secretion [48]. However, maturation has also been reported *in vivo* following transplantation of stage 5 cells [12,70,71], which could improve scalability of iPSC-derived products if equal results were shown.

In terms of stage 6 and 7 duration, Hogrebe et al. (2021) recently reported that optimal *in vitro* glucose responsive insulin release following static stimulation testing occurs after 14 days of culture and deteriorated thereafter [15]. Therefore, we suggest a 7-day stage 6, and 7-day stage 7 duration.

3.1.5.8 Stage 7

Stage 7 also remains poorly described and primarily involves maturation. However, several techniques have been considered to reduce tumorigenicity. The first method involves immunologically selecting iPSCs from mixed colonies during maturation. Selectively eliminating iPSCs immunologically has been trialed using cell sorting techniques [19,141-143], and antibody mediated cytotoxicity [19,144,145]. Immunologic selection techniques are achievable for cells grown in 2D culture where antibodies can access the individual cells [14,15], but remain limited for cells grown in 3D culture due to their growth within cell clusters. Disaggregation of cell clusters during 3D differentiation to expose antigenic targets is possible, but leads to substantially reduced cell yield as a result of apoptosis during the dissociation process [141]. If used during 3D culture, immunologic selection or elimination would be required for each islet preparation thereby reducing efficiency and increasing costs substantially, which could significantly hamper efficient clinical translation [10,39].

3.1.6 **Optimal Cell Stage and Location for Transplantation**

Beyond to optimization of each stage, evaluation of the ideal cell maturity and transplant location also remains largely unaddressed. In terms of the optimal stage for SC-derived ITx, experimental results have demonstrated success for SC-derived ITx with diabetes reversal in animal models following transplantation of stage 3, 4, 5, and 6 cells that mature into islets *in vivo* [20,52,70,146]. In fact, promising first in-human data from ViaCyte clinical trials was achieved by transplanting stage 5 pancreatic progenitors into patients, with detectable meal-regulated C-peptide secretion in a subset of recipients [8,9]. Transplantation of earlier stage cells could potentially

improve scalability, and a more rapid delivery of these cell therapies. However, as we have discussed, elimination of any risks related to off-target growth remains a priority for the field; we hypothesize that these off-target risks would increase if earlier stage, less mature cells that include progenitors are used. Although capable of reversing diabetes in animal models, reports have also suggested a substantial rate of teratoma formation if proliferative cell populations are not eliminated [20,21,53,70]. Therefore, we hypothesize that stage 7, mature β-like cells would be the optimal cell product to be evaluated, particularly during early proof-of-concept clinical trials. However, the potential to further optimize differentiation and enable a scalable, rapid, and safe cell product requiring shorter *in vitro* differentiation may further enable expanded islet cell therapies and may supplant longer differentiation protocols once safety has been demonstrated.

Further to optimizing the cell stage for transplantation, ongoing studies continue to evaluate the site of transplant for SC-derived islets. While data from allogeneic ITx suggests that, with current techniques, the intraportal route remains superior to extrahepatic sites such as the omentum, gastric submucosa, subcutaneous space, and within devices [147-149], optimization of extrahepatic sites is ongoing [150]. These novel sites may enable SC-derived ITx transplant within retrievable sites that reduce any risk of off-target growth. Once more, ViaCyte's recent clinical trial where cells were transplanted within macroencapsulation devices provides evidence for such endeavors. Optimizing and evaluating transplant in these retrievable extrahepatic sites, or implementing them alongside potential islet organoids may enable alternative transplant sites that optimize SC-derived islet survival [151-153], and offer retrievability as a safety measure for islet cell therapies. Organoid environments may also offer potential immunoprotection to islets and may serve as a barrier to recurrent autoimmunity for recipients [152]. Overall, extrahepatic sites may offer

safe options during phase I-II clinical trials and with ongoing optimization of both extrahepatic sites and organoids may further enhance applicability of SC-derived islets.

3.1.7 **Concluding Remarks and Future Directions**

SC-derived ITx offers a potentially curative option for T1D; however, ongoing optimization of differentiation protocols remains crucial as we embark into clinical trials. Optimized protocols must generate functional cells without teratogenic risk in a scalable, cost-efficient way. Considering this, we believe that genetically modifying patient-specific iPSCs to allow optimal cell selection is likely not economically feasible using current techniques. Similarly, immunologic selection, cell sorting, or antibody-mediated cytotoxicity requires cells to be disaggregated, resulting in substantial product loss, which likely precludes clinically relevant cell yields for in-human use in its current state.

We hypothesize that optimization of each stage, with chemical ablation of any remnant teratogenic cells, remains the most feasible technique to generate a cell product without off-target growth risk. Herein, we provide a review of each differentiation stage, and from that, propose the following hypothetical SC-derived islet cell differentiation protocol (Figure 3.1.2). Future studies should evaluate this hypothesized differentiation protocol with comparison to the three most efficient protocols previously reported [13,15,53]. Furthermore, evaluation of chemical methods to eliminate teratogenicity during differentiation is required. If chemical elimination methods are unsuccessful, secondary evaluation of immunologic or genetic manipulation may be required to ensure SC-islet safety without off-target growth. Certainly, demonstrating product safety

following differentiation remains crucial regardless of whether allogeneic or autologous SC-derived ITx is to be pursued.

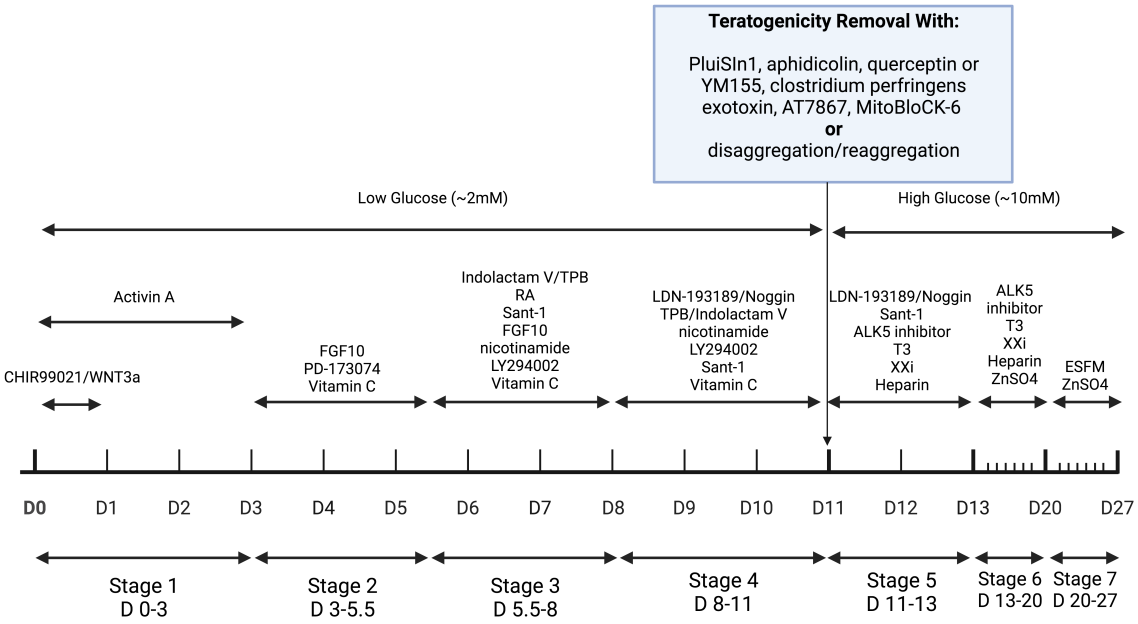

Figure 3.1.2 Proposed optimized islet cell differentiation schedule.

3.1.8 **References**

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3.2 Chapter 3 subsection 2 – Cell characterization, graft evaluation, and yield of islet-like cells differentiated from patient-derived iPSCs

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3.2.1 **Summary**

Induced pluripotent stem cells (iPSCs) offer the potential to generate autologous iPSC-derived islets (iPSC islets). Differentiation protocol optimization with stage-wise characterization, off-target evaluation, and cell yield assessment are necessary to inform clinical implementation. Herein, we report stage-wise characterization of cells generated following an improved differentiation protocol capable of generating 90.4% $PDX1^+/NKX6.1^+$ pancreatic progenitors and 100% $C\text{-}peptide^+/NKX6.1^+$ iPSC islet cells. However, 82.1%, 49.6% and 0.9% of the cells expressed SOX9 (duct), SLC18A1 (enterochromaffin cells) and CDX2 (gut cells), respectively. Explanted grafts contained mature monohormonal islet-like cells, however, $CK19^+$ ductal tissues persist. Importantly, planar differentiation achieved $8.3x10^6$ cells, whereas complete suspension differentiation within Vertical-Wheel® bioreactors significantly increased cell yield to $105.0x10^6$ cells, reducing costs by 88.8%. This study offers improved stage-wise characterization of iPSC islet cells that will enable future protocol comparison and evaluation of approaches for off-target cell elimination. Proof-of-concept for complete suspension-based differentiation highlights an important advancement to facilitate clinical implementation.

3.2.2 **Introduction**

Islet transplantation (ITx) provides clear proof-of-concept for a cell-based regenerative diabetes therapy[1-3]. Allogeneic ITx from deceased donors remains limited to patients with severe glycemic lability and recurrent hypoglycemia due to inadequate organ supply and requirement for lifelong immunosuppression; however, the advent of stem cell-derived ITx (SC-ITx) could generate unlimited cells for transplant to expand application[4]. Recent clinical trials have demonstrated meal-stimulated C-peptide secretion following in-human subcutaneous embryonic SC-ITx, with studies underway evaluating genetically modified stem cell-derived islets (SC islets) to eliminate immunosuppression required to combat allorejection[5-7]. Alternatively, induced pluripotent stem cells (iPSCs) offer the possibility to generate autologous SC islets, which would inherently circumvent the need for immunosuppression, but are far more complex to manufacture in bulk. Clinical translation of iPSC ITx relies on protocols to reliably generate large numbers of high quality iPSC islets without risk of off-target growth[8]. While numerous differentiation protocols have been published[9-14], thorough stage-wise characterization, product yield, and detailed post-transplant graft evaluation remain underreported.

Early first-generation SC islet differentiation protocols directed cells into $PDX1^{+}/NKX6.1^{+}$ expressing pancreatic progenitors (PPs) that were transplanted and underwent further differentiation into glucose responsive SC islets *in vivo*[15-18]. To eliminate off-target populations and improve cell product safety, recent protocols have further differentiated PPs into SC islets *in vitro* prior to transplant[11,19]. In doing so, newer protocols have successfully generated SC islets that exhibit immature glucose-stimulated insulin release and metabolism but that can

mature either *in vitro* or *in vivo*[9,12-14,20,21]. However, review of previous studies reporting islet differentiation highlights uncertainty regarding the optimal protocol to direct cells from their pluripotent state towards SC islets[22]. Furthermore, previous studies lack cell characterization at each Stage of differentiation, fail to evaluate or report cell yield, and often do not adequately report post-transplant evaluation of off-target tissues within grafts (Table 3.2.1), making it difficult to compare results. In depth characterization of cells during each Stage of differentiation will facilitate ongoing stage-wise protocol optimization[8], while understanding the yield of SC islet differentiation protocols and the safety of the final cell product remains paramount for clinical translation.

Table 3.2.1 Evaluation of Stage wise characterization, yield assessment, functional characterization, off-target graft evaluation, and electrophysiological testing of SC islets from key studies since 2010

Protocol	Reported Within Study					
	Stage-Wise Characterization	Final Product Yield	Off-Target Graft Evaluation	Functional Characterization	Electrophysiologic Evaluation	Unique Data
Schulz et al. (2012)[18]	Stage 1-4 transcriptomic evaluation	No	Yes	*In vivo* only	No	Suspension culture of PPs with transplant showing *in vivo* maturation
Pagliuca et al. (2014)[11]	No	No	No	Yes	No	Production of iPSC and ESC islets capable of GSIS using suspension culture within spinner flasks
Rezania et al. (2014)	Stages 4-7	Minimal reporting: 1 SC islet per 2 SCs	Yes, 10 week evaluation	Yes	No	Added an additional Stage 7 maturation phase and characterized Stages 4-7
Russ et al. (2015) or	No	No	Yes, aggregated	Yes	No	Thorough evaluation of SC islet

Nair et al. (2019)			cells did not have masses			metabolic maturation
Velazco-Cruz et al. (2019)	Stage 5-6	No	No	Yes	No	Update from Pagliuca (2014) with Stage 5-6 optimization
Hogrebe et al. (2020) and (2021)	Yes	0.5–0.75x10^6 cells per cm^2	No, only included evaluation of endocrine markers 3 weeks post-transplant. Stated that "no overgrowths were observed"	Yes	No	Entirely two-dimensional planar culture due to use of latrunculin-A.
Aghazadeh et al. (2022)	No	Unclear overall yield. Reported 84% and 76% cell loss following cell sorting	Yes, reported teratoma elimination with $GP2^+$ cell sorting	*In vivo* evaluation only	No	Magnetic-activated cell sorting of $GP2^+$ PPs to eliminate teratoma formation
Balboa et al. (2022)	No	No	No	Yes, thorough characterization throughout *in vitro* maturation.	Yes	Characterization following prolonged *in vitro* maturation

*Pagliuca et al. (2014), Velazco-Cruz et al. (2019) and Hogrebe et al. (2020) and (2021) represent protocols established in the same lab.

This study aims to comparatively evaluate protocols for iPSC islet generation using patient-derived iPSC lines. More critically, we provide stage-wise characterization of the cell product achieved from an updated and optimized protocol with determination of product yield, function, and safety through graft characterization after *in vivo* maturation. Understanding these

aspects of SC islet differentiation protocols will enable ongoing advancement of efforts towards a definitive stem cell-based cure for diabetes.

3.2.3 **Methods**

3.2.3.1 Experimental model and subject details

Blood sample donors for this study provided written consent for use of tissue, cell reprogramming and differentiation, and result disclosure. This study and its methods have been approved by the Stem Cell Oversight Committee of Canada (SCOC), and the University of Alberta Institutional Health Research Ethics Board (PRO00084032). Animal protocols were conducted in accordance with the Canadian Council on Animal Care Guidelines and Policies and have been approved by the Animal Care and Use Committee (Health Sciences) at the University of Alberta. Euthanasia was performed by filling the euthanasia chamber with 25% CO_2. All experiments were planned *a priori* and intended to complete technical and biological triplicates as a minimum for all experiments based on standard experimental procedures. Contaminated iPSC islet preparations and intraperitoneal glucose tolerance tests at 8-weeks for one cohort of mice (n =7 due to a suspected protocol failure) were excluded from analysis. Randomization and blinding were not performed.

3.2.3.2 Cell culture

Cell culture was completed using good manufacturing practice (GMP) compliant materials, where available, to ensure clinical applicability of these protocols[23]. Cells were cultured at 37°C with 5% CO_2 within humidified incubators. Cell processing was performed in a

Class-II biocontainment compliant lab with the manipulation of cells taking place in a sterile environment with high efficiency particulate air filtration.

3.2.3.2.1 Generation, maintenance and expansion of induced pluripotent stem cell lines

Human iPSC lines (n = 3) generated from peripheral blood mononuclear cells (PBMCs) of three healthy donors (patient demographics in **Appendix** Table S3.2.2) were used in this study. iPSC lines were generated using Sendai virus transfection of PBMCs, clone selection, and culture according to previously published protocols[24]. Detailed iPSC line establishment, maintenance, and quality control were completed according to previously reported protocols[24]. iPSCs were cultured on 60 mm tissue culture plates (Thermo Fisher Scientific cat. 130181) covered with recombinant human vitronectin (Thermo Fisher Scientific cat. A27940) in StemFlex media (Stem Cell Technologies, cat. A3349401) and passaged using CTS EDTA Versene Solution (Fisher Scientific, cat. A4239101) supplemented with 10 μM Rho-kinase inhibitor (RockI; Y-27632 STEMCELL Technologies, cat. 72304). To prepare cells prior to differentiation experiments, iPSCs were passaged and seeded on 150-mm Geltrex (Fisher Scientific cat. A1413301) coated plates at a density of ~0.06 million cells/cm^2, and grown for 3-4 days to achieve 80-90% confluency prior to differentiation.

3.2.3.2.2 Differentiation of induced pluripotent stem cell lines

For protocol optimization, 32 different media and additive compositions were tested from Stages 1-4, modified from key publications[14,25,26], and are described in **Appendix** Table S3.2.3. Protocol evaluation was based on the proposed quality control standards to assess the efficiency

and safety of differentiation protocols established by Cuesta-Gomez et al. (2022)[8]; upon identical quality control results, yield and cost were also considered. For a pathophysiological insight of each differentiation stage, including stage-specific approaches to optimize islet generation refer to Verhoeff et al. (2022)[22].

Upon establishment of the optimized differentiation protocol described in **Appendix** Table S3.2.3, iPSCs were expanded and differentiated in 150 mm plates. To determine protocol yield and for stage-wise characterization, independent differentiations were carried out with discontinuation and cell collection at each Stage to allow cell counting and sample collection for flow cytometry and RNA assessment. Briefly, cells on 150 mm plates were lifted using 6 minutes (min) TrypLE treatment (10 mL; Thermo Fisher Scientific, cat. 12605010) supplemented with 10 μM ROCKi followed by enzyme dilution with 10 mL of ROCKi supplemented (10 μM) DMEM (Sigma cat. D0822) and cell lifting using a cell scraper. Single cells were centrifuged for 2 min at 2000 rotations per min (rpm) and resuspended in PBS. Cells were counted and viability was assessed using the Thermo Fisher Scientific Invitrogen Countess II AMQAX1000 Cell Counter. Live cell numbers were used to calculate cell requirements for all processes. Following pancreatic progenitor differentiation, cells on 150 mm plates were lifted as described above and seeded into microwells (6-well AggreWell® 400 plates, Stem Cell Technologies, cat. 34460) at a density of 800-1,000 cells per microwell using the protocol described by Barsby et al. (2022)[27]. After 48 hours within microwell plates, the clusters were gently resuspended using the media within each well and transferred to a 50 mL conical. Two additional washes of the microwell plates with 2 mL/well of Stage 5 media ensured maximal cluster transfer. Clusters were allowed to settle by gravity in the 50 mL conical followed by

media removal, cluster resuspension in Stage 5 media, and transfer to suspension culture in 0.1L Vertical-Wheel® Bioreactors that were set to rotate at 60 rpm. Media changes were performed according to the differentiation schedule (typically every two days, as described in **Appendix** Table S3.2.3). Within Vertical-Wheel® bioreactors, cells were allowed to gravity settle with supernatant removed and media replacement. Cell number and samples for RNA and flow cytometry were also collected at the end of Stages 5 and 6. Clusters in suspension culture were dissociated by transferring cells into a 1.5 mL Eppendorf tube with 1 mL of accutase (Thermo Fisher Scientific cat. A11105-01) for 10 min followed by mechanical disruption using a pipette; single cells and viability were measured as above.

We also report differentiation occurring entirely (i.e. from Stages 1-6) within Vertical-Wheel® bioreactors. To achieve this, $2x10^6$ iPSCs were seeded within Vertical-Wheel® bioreactors with 55 mL of media, followed by cell expansion for 5 days according to our previously reported protocol[24]. Following expansion, a cell sample was collected for counting and assessment of pluripotency markers prior to differentiation. Throughout differentiation 100 mL of media was used and Vertical-Wheel® bioreactors were set at 60 rpm. Media changes involved allowing cell clusters to gravity settle followed by 100 mL media exchange according to the same schedule and media composition as completed in planar conditions (**Appendix** Table S3.2.3).

3.2.3.2.3 Isolation of primary adult islets

S6 iPSC islet morphology and function were compared to data from human islets isolated from deceased donor pancreata for research by The Alberta Diabetes Institute IsletCore

(University of Alberta, Canada) using previously described methods[28]. All human islet studies were approved by the Human Research Ethics Board (Pro00013094; Pro00001754) at the University of Alberta and all families of organ donors provided written informed consent. Islet donor characteristics are listed in **Appendix** Table S3.2.2.

3.2.3.3 Immunohistochemistry and image analysis

Differentiation in planar conditions was carried out on Geltrex coated coverslips with wells washed with PBS and fixed in 4% PFA for 20 min at room temperature (RT). Samples differentiated in suspension conditions and explanted iPSC islet grafts were fixed overnight in 4% PFA and embedded in paraffin. Paraffin embedded samples were sectioned at 8 μm and were deparaffinized, rehydrated, and subjected to antigen retrieval using hot citrate buffer (0.0126 M citric acid, Sigma cat. C-0759; 0.0874 M sodium citrate, Sigma, cat. S-4641; pH 6.0) for a total of 20 min prior to staining. Coverslips and slides were then blocked and permeabilized with 5% normal donkey serum (Sigma, cat. S30-M) in FoxP3 permeabilization buffer (Biolegend, cat. 421402) for 1 hour (hr) at RT and incubated with primary antibodies diluted in permeabilization buffer overnight at 4°C. Secondary antibodies were diluted similarly and incubated for 1 hr at RT followed by DAPI (Sigma cat. D1306) staining for 4 min at RT. Antibodies and concentrations used are listed in **Appendix** Table S3.2.4. Slides were visualized using the Leica DMI 6000 inverted fluorescence microscope and images were processed using the LAS X Life Science Microscope Software.

3.2.3.4 Flow cytometry

Upon lifting of cells from plates or dissociation of the clusters, $5x10^6$ single cells were filtered through a 40 μm strainer and then fixed with 4% PFA for 20 min at RT and stored at 4°C until staining. Prior to staining, cells were permeabilized using Cytofix/Cytoperm (BD Biosciences, cat. 554714) for 20 min on ice followed by 2 washes with 1x Perm/Wash buffer (BD Biosciences, cat. 554714). Primary antibodies were incubated for 1 hr hour on ice, or overnight at 4 °C for NKX6.1, and secondary antibodies for 30 min according to the dilutions in **Appendix** Table S3.2.4. Cells were resuspended in fluorescence-activated cell sorting buffer (2% FCS, 2 mM EDTA in DPBS). Data were acquired using the CytoFLEX S flow cytometer and analysed using the CytExpert software (Beckman Coulter).

3.2.3.5 Quantitative reverse transcription PCR (qRT-PCR)

Cells were lysed in 350 μL RLT buffer (Qiagen, cat. 79216) and frozen at -80°C until RNA extraction. Suspension of lysed cells in RLT buffer was defrosted and cells were homogenized using the QIAshredder system (Qiagen, cat. 79656) and total RNA was then extracted with the RNeasy Mini Kit (Qiagen, cat. 74104) according to the manufacturer's instructions. Concentration and purity of the isolated RNA samples were evaluated using spectrophotometry with the Multiskan SkyHigh Microplate Spectrophotometer and μdrop plate (Thermo Fisher, cat. A51119600DPC) by assessing the 260/280 nm and 260/230 nm absorption of samples. Samples were then stored at -80ºC until needed; RNA was quantified after each defrost. RNA was reverse-transcribed using the RevertAid First Strand cDNA Synthesis Kit as

per manufacturer guidelines (Thermo Fisher, cat. K1621). Complement DNA (cDNA) was stored at -20ºC until required for PCR.

Custom designed gene TaqMan Low Density Array Cards were used as per manufacturer instructions (Thermo Fisher Scientific cat. 4342253); gene array set up is described in **Appendix** Table S3.2.5. Briefly, 500 ng of cDNA was diluted in 55 µL of nuclease free water and combined with 55 µL TaqMan Universal PCR Master Mix (Thermo Fisher Scientific cat. 4305719). The combined solution was loaded into the gene array cards, centrifuged, and processed using the FAST-384 well array program via the QuantStudio 12K Flex Real-Time PCR system. Separately, pairs of primers were designed (sequences detailed in **Appendix** Table S3.2.6) to quantify the amount of specific cDNA by SYBR Green qRT-PCR (Thermo Fisher Scientific cat. 4385612). qRT-PCR assay was performed using the Applied Biosystems 7900HC Fast Real-Time PCR Systems detection system (Applied Biosystems). Samples were analysed using *GAPDH* as reference for data normalization. Data was then analyzed as above and represented as a heat map, 2(-ΔΔCT), or volcano plots.

3.2.3.6 Glucose-stimulated insulin secretion

Static tests of insulin secretion were carried out in 1.5 mL Eppendorf tubes. A total of 50 iPSC islets or human islets were handpicked and equilibrated in Krebs-Ringer buffer with 2.8 mM glucose (G3) for 120 min, and then subjected to sequential 30-min incubations of G3, 16.8 mM glucose (G17), 100 nM Exendin-4 (Sigma, cat. E7144) and 30 mM KCl in G3. Following incubations, the clusters were lysed with Triton X-100 buffer (Sigma, cat. 9002-93-1) with total insulin content analyzed.

Dynamic tests of insulin secretion were carried out using a perifusion apparatus (BioRep Technologies Perifusion System) with a flow rate of 0.1 mL/min, and sampling every 2 min. A total of 50 handpicked iPSC islets or human islets were used for each test. The islets were equilibrated in G3 for 120 min prior to sample collection. Samples were then exposed to 16 min G3, 16 min G17, and 12 min KCl-G3. iPSC islets were also exposed to 100 mM Exendin-4 in G3 for 16 min after G17 and before KCl-G3. Insulin content of secretion fractions and iPSC islet lysates were analyzed with enzyme-linked immunosorbent assay (ELISA) (Alpco, cat. 80-CPTHU-CH01).

3.2.3.7 Electrophysiology

iPSC islets and human islets were hand-picked and dissociated using enzyme-free Hanks'-based Cell Dissociation Buffer (Thermo Fisher Scientific, cat. 13150-016) and cultured in DMEM (Fisher Scientific cat. 11885092) supplemented with 10% FBS, and 100 U/mL penicillin/streptomycin and 10 uM Y-27632, (Stem technologies cat. 72302) for 1-2 days at a glucose concentration of 5.0 mmol/L on 35-mm tissue culture dishes (Thermo Fisher Scientific cat. 430165).

For whole-cell patch-clamping, fire polished thin wall borosilicate pipettes coated with Sylgard (3-5 MOhm) were filled with an intracellular solution containing (in mM): 125 Cs-glutamate, 10 CsCl, 10 NaCl, 1 MgCl2·6H2O, 0.05 EGTA, 5 HEPES, 0.1 cAMP, and 3 MgATP (pH 7.15 with CsOH) solution. Patch-clamp measurement of voltage-dependent Ca^{2+} currents and exocytotic responses in dispersed islet-like cells were performed at 32-35°C as described previously[29], in bath solution containing (in mM): 118 NaCl, 20 TEA, 5.6 KCl, 1.2

MgCl2·6H2O, 2.6 CaCl2, 5 HEPES, and 5 glucose (pH 7.4 with NaOH). Electrophysiological measures were collected using a HEKA EPC10 amplifier and PatchMaster Software (HEKA Instruments, Lambrecht/Pfalz, Germany) within 5 min of break-in. Data were analyzed using FitMaster (HEKA Instruments).

3.2.3.8 Oxygen consumption

Oxygen consumption was assayed using the Agilent Seahorse XFe24 analyzer. To prepare calibrant plates, 1 mL of calibrant solution was placed into wells of the extracellular flux assay kit and incubated at 37°C overnight without CO_2. On the day of the experiment, 70 iPSC islets or human islets were hand-picked and placed within DMEM (Agilent 103575-100) supplemented with 1% FBS, 2.8mM D-glucose (Sigma G8270), 2mM sodium pyruvate (Gibco 11360-070) and 2mM L-glutamine (Gibco 25030-081). iPSC islets and human islets were then placed within the depression of the islet capture microplates with the protective mesh positioned overtop. Supplemented DMEM was then topped off to a volume of 500 µL per well and incubated at 37°C with no CO^2 for one hour. Cells were sequentially exposed to glucose 16.7 mM, oligomycin 5 µm, carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP) 3 µm, and rotenone/antimycin A 5 µm. Data was standardized to DNA content.

3.2.3.9 iPSC islet transplantation

2,500 IEQs were transplanted under the kidney capsule of immunocompromised SCID beige mice (12-16 weeks old) balanced for sex [30]. IEQs were described according to the

Integrated Islet Distribution Program[31]. Institutional guidelines for perioperative care, anesthesia, and pain management were followed.

3.2.3.10 Evaluation of iPSC islet graft function

In vivo iPSC islet function was evaluated with intraperitoneal glucose tolerance tests at 8, 12 and 16 weeks after transplant. Animals were fasted overnight for 12-15 hr before receiving an intraperitoneal glucose bolus (3 g/kg). Blood was collected and it's C-peptide content measured at 0 and 60 min after glucose injection using enzyme-linked immunosorbent assay (ELISA) (Mercodia, cat. 10-1132-01). Blood glucose levels were monitored at 0, 15, 30, 60, 90 and 120 min after glucose administration.

iPSC islet grafts were retrieved by nephrectomy 16 weeks after transplantation[30]. Retrieved grafts were characterized using IHC. For IHC, complete or partial grafts were fixed overnight in 4%PFA and embedded in paraffin.

3.2.3.11 Cost Calculation

Cost evaluation and comparison in this study were completed solely for the purpose of comparing the bioeconomics of iPSC islet generation and should not be extrapolated to the true cost of clinically translating these findings. All costs are represented using 2023 Canadian Dollars ($CAD). Costs included media supplements, media, materials, and cost of technician time, as further described below. Cost of media supplements and media included the supplement costs, taxes, and shipping costs for all supplements needed for differentiation detailed in **Appendix** Table S3.2.3. Material costs included the price of plates, reactors, AggreWell® plates,

TryPLE, cell scrapers, and media needed during AggreWell® plate rinsing with costs including taxes and shipping. Material costs did not include the cost of pipettes, or any materials needed during evaluation of cell products. Technician time was calculated by timing media changes at all Stages for at least three different differentiations for two different personnel. Technician cost was calculated by assuming a technician wage of $28/hr according to the average wage for cell culture technicians in North America. Technician cost did not include the time needed to pre-prepare and freeze media or supplement aliquots nor did it include time for quality control and cell evaluation. Notably, no costs included materials or time needed for cell quality assessment, clinical costs of ITx, instrument cost or depreciation, or costs associated with clinical biomanufacturing, as it was assumed these would be similar regardless of the differentiation technique.

3.2.3.12 Data collection and statistical methods

Normality testing was performed with the D'Agostino-Pearson normality test to determine the need for non-parametric testing, which was utilized for all subsequent analyses. Between group comparisons of data were carried out using the non-parametric Mann–Whitney U test or Kruskal–Wallis tests with the alpha value set at 0.05. The alpha was modified *post hoc* to 0.01 for volcano plot evaluation of transcriptomic data to better display key gene expression changes. In the text and figures, continuous values are presented as medians with interquartile range (IQR), with discrete values presented as absolute values with percentages. All statistical analysis was completed using GraphPad Prism version 9.3.1for Mac, GraphPad Software, San Diego, California USA, www.graphpad.com.

Flow cytometry, transcriptomics, and functional assessments represent the median of technical triplicates from independent iPSC islet differentiations. Gross pathology and immunohistochemistry display representative images from grafts and iPSC differentiations. Electrophysiology and measurements of Ca^{2+} and exocytosis represent recordings from individual cells from n = 7 differentiations. *In vivo* data is derived from independent animals.

3.2.4 **Results**

3.2.4.1 Increased efficiency of pancreatic progenitor differentiation with alternative media composition

Following review of previously published islet differentiation protocols several media and additive variations were compared from Stages 1-4 to generate PPs (Figure 3.2.1A). Specifically, use of MCDB, RPMI, DES, and MCDB supplemented with 1x insulin-transferrin-selenium-ethanolamine (ITS-X), 1x non-essential amino acids (NEAA), and 1% human serum albumin (HSA) (referred to as MCDB+) as a basal media were compared during Stages 1-2. Additionally, addition of nicotinamide was tested at Stage 3, and/or Stage 4.

Morphological comparison of cells following differentiation using different media at Stage 1 showed cytoplasmic enlargement and cell spacing compared to iPSCs, with more fibroblastic-appearing cells when using RPMI and MCDB+ and reduced cell confluency with MCDB+ (Figure 3.2.1B). Flow cytometric evaluation showed that MCDB (91.2%; IQR 90.2-95.9%) and DES (90.4%; IQR 83.5-94.1%) produced a similar ($p = 0.23$) proportion of $CD117^+/SOX17^+$ cells, which was superior to RPMI (64.9%; IQR 46.6-71.0%), and MCDB+ (34.0%; IQR 18.6-48.7%) (all $p < 0.05$; Figure 3.2.1B).

When cells from the best Stage 1 conditions (DES and MCDB) underwent primitive gut tube differentiation (Stage 2) using RPMI or MCDB, those grown in RPMI were densely confluent but retained cell spacing, while plate confluency was reduced when using MCDB, especially when MCDB was also used for Stage 1 (Figure 3.2.1C). In keeping with this, when using RPMI in Stage 2, 97.6% (IQR 95.7-99.2%) and 95.2% (IQR 93.9-96.9%) of cells were $SOX17^+/FOXA2^+$ for cells grown in MCDB and DES, respectively, during Stage 1 ($p = 0.17$). This was significantly more than when MCDB was used in Stage 2 ($p = 0.008$ for both). Notably, no differences in cell morphology or proportion of $PDX1^+/FOXA2^+$ or $PDX1^+/NKX6.1^+$ cells were observed regardless of nicotinamide supplementation in Stage 3, and/or Stage 4 (Figure 3.2.1D-E). Morphology and efficiency of differentiation, measured as percentage of positive cells for key markers from Stages 1-4, was similar regardless of MCDB or DES at Stage 1. However, we elected to proceed with MCDB due to higher costs associated with DES ($567 DES vs. $81.25 MCDB for 100 mL of Stage 1 media and supplements). The optimized protocol for PP generation used MCDB for Stage 1, RPMI for Stage 2, and did not include nicotinamide. Of the PP cells generated after Stage 4 using this protocol 90.4% (IQR 83.9-92.0%) were $PDX1^+/NKX6.1^+$.

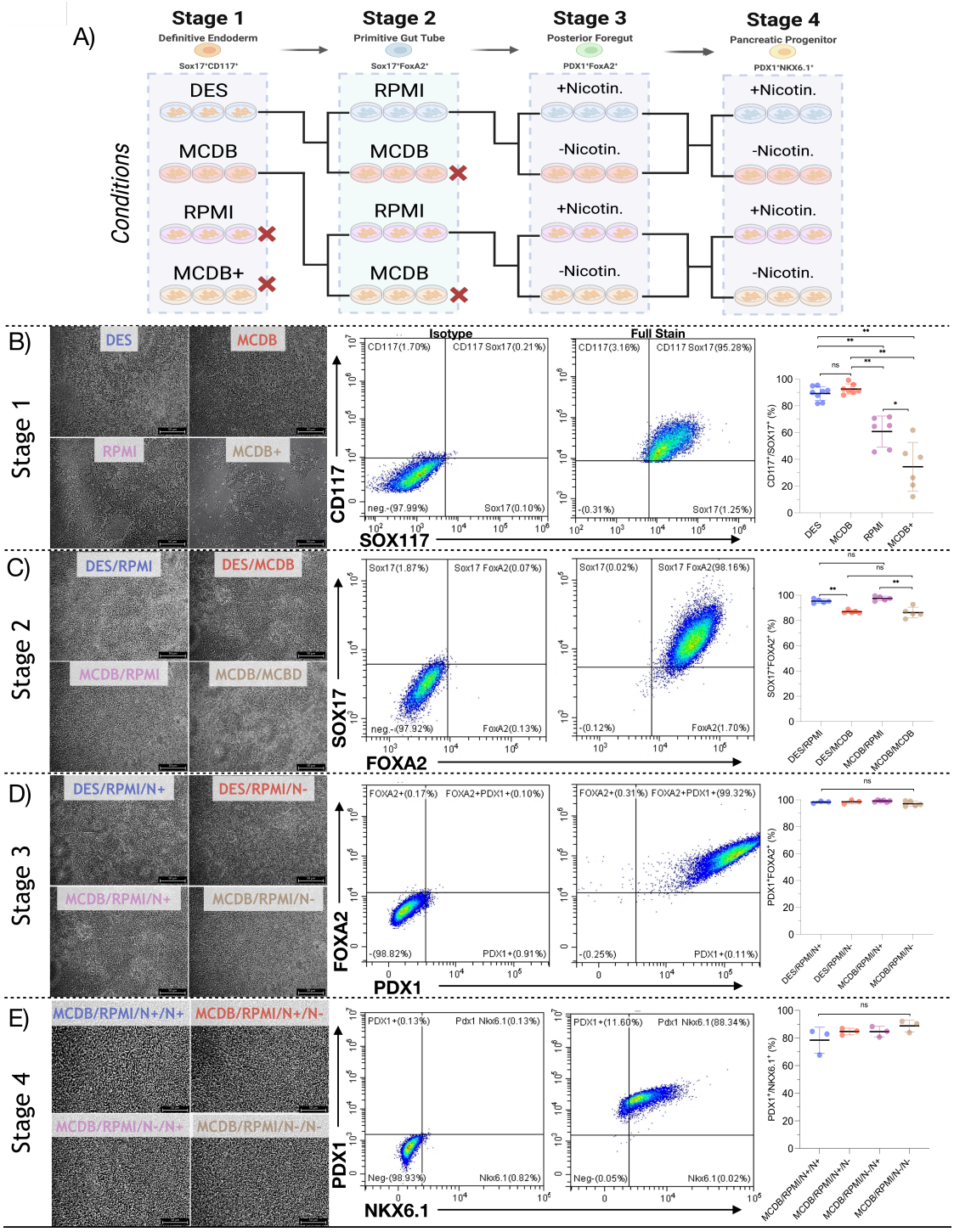

Figure 3.2.1 Protocol optimization to maximize pancreatic progenitor generation during Stages 1-4 of differentiation using different media compositions.

A) Graphical representation of the media and supplements evaluated from Stages 1-4. Red "X" represents protocols that did not achieve > 90% positive cells for stage-wise markers evaluated with flow cytometry. B) Microscopy and $CD117^+/SOX17^+$ flow cytometry results for different media conditions evaluated during Stage 1. C) Microscopy and $SOX17^+/FOXA2^+$ flow cytometry results for different media conditions evaluated during Stage 2. D) Microscopy and $FOXA2^+/PDX1^+$ flow cytometry results for different media conditions evaluated during Stage 3. E) Microscopy and $PDX1^+/NKX6.1^+$ flow cytometry results for different media conditions evaluated during Stage 4.
ns represents $p \geq 0.05$, * represents $p < 0.05$, and **represents $p < 0.001$. †MCDB+ represents MCDB media supplemented with 1x insulin-transferrin-selenium-ethanolamine (ITS-X), 1x non-essential amino acids (NEAA), and 0.5% human serum albumin (HSA). Nicotin in A and "N" in D-E represents either the addition (+) or not (-) of Nicotinamide. Full media composition and supplements are presented in **Appendix** Table S3.2.3.

3.2.4.2 Stage-wise characterization throughout differentiation highlights transition from iPSC to islet-like cells using an optimized protocol

Stage-wise proteomic characterization of the optimized protocol (Figure 3.2.2A) demonstrates promising progression of important islet differentiation markers (Figure 3.2.2). Flow cytometry quantification of Stage 1 cells demonstrates that in addition to 96.2% (IQR 94.45-98.2%) of cells being $CD184^+/SOX17^+$, 91.2% (IQR 90.2-95.9%) of cells were also $CD117^+/SOX17^+$, and 99.1% (IQR 95.4-99.4%) were $SOX17^+/CD55^+$ (Figure 3.2.2B). Interestingly, 95.2% (IQR 95.1-98.3%) of cells stained positive for pluripotency markers NANOG and SSEA4. Of Stage 1 cells, 75.9% (IQR 75.3-79.5%) of cells were proliferative as determined by $Ki67^+$ staining. Flow cytometric gating and immunohistochemistry supporting findings for Stages 1-5 can be found in **Appendix** Figure S3.2.8.

At Stage 2, 95.2% (IQR 93.9-96.9%) of cells were $SOX17^+/FOXA2^+$ and 39.4% were $Ki67^+$ (IQR 36.7-42.3%; Figure 3.2.2C). A proportion of cells also expressed PDX1 (48.7% [IQR 45.7-53.1%]) but <2% of cells were $NKX6.1^+$ or $SOX2^+$. At Stage 3, 97.9% (IQR 96.7-

99.1%) of cells expressed $PDX1^+/FOXA2^+$, and 92.4% (IQR 90.8-92.4%) expressed $SOX2^+/FOXA2^+$ (Figure 3.2.2D). Many of these Stage 3 cells were $Ki67^+$ (93.3% [IQR 92.4-98.0%]), and 92.3% (IQR 91.4-92.5%) were $PDX1^+/Ki67^+$. Only 0.8% (IQR 0.7-0.9%) of Stage 3 cells were $CDX2^+$.

Stage 4 PPs were 90.4% (IQR 83.9-92.0%) $PDX1^+/NKX6.1^+$ and 97.1% (IQR 94.9-97.3%) $PDX1^+/GP2^+$ (Figure 3.2.2E). Interestingly, 88.0% (IQR 83.3-89.5%) of Stage 4 cells were $NKX6.1^+/ChgA^+$, while 98.5% (IQR 98.1-99.9%) and 89.3% (IQR 88.2-98.7%) of the cells were positive for the non-endocrine markers $SOX9^+$ and $SOX2^+$, respectively. Few $CDX2^+$ cells existed at Stage 4 (1.2% [IQR 0.8-1.3%]). The proportion of $Ki67^+$ cells was 39.3% (IQR 34.4-46.3%), yet notably most of these cells were $Ki67^+/PDX1^+$ (34.4% [IQR 24.3-46.3%]).

Following clustering using AggreWell® plates and Stage 5 differentiation, PPs generated, round pancreatic endocrine progenitor aggregates that were $PDX1^+/NKX6.1^+/ChgA^+$ on immunohistochemistry (Figure 3.2.2F). Flow cytometry further confirmed that 97.4% (IQR 96.8-98.3%) of cells were $PDX1^+/NKX6.1^+$, 94.9% (IQR 93.5-95.4%) were $PDX1^+/ChgA^+$, and 94.3% (IQR 93.8-95.6%) were $NKX6.1^+/ChgA^+$ (Figure 3.2.2G). The percentage of $Ki67^+$ cells was 8.2% (IQR 7.7-13.8%) at the end of Stage 5. Similarly, the percentage of cells positive for non-endocrine markers including SOX9 (89.7% [IQR 87.6-91.5%]), and SOX2 (43.4% [IQR 41.3-47.2%]) decreased, and the percentage of $CDX2^+$ (1.2% [IQR 0.8-1.3%]) cells remained low.

At Stage 6, cell clusters (S6 iPSC islets) co-expressed INS^+ and GCG^+ on immunohistochemistry (Figure 3.2.2H). Flow cytometric evaluation demonstrated that nearly all cells were INS^+/GCG^+ (99.0% [IQR 98.3-99.2%]) and 39.2% (IQR 36.0-42.0%) were

INS$^+$/STT$^+$. Additionally, most cells were also C-peptide$^+$/ISL-1$^+$ (99.4% [99.3-99.9%]), C-peptide$^+$/NKX6.1$^+$ (100.0% [IQR 99.9-100.0%]), C-peptide$^+$/UCN3$^+$ (97.8% [IQR 96.2-99.1%]), and C-peptide$^+$/ChgA$^+$ (79.7% [IQR 78.7-80.5%]). In addition to being C-peptide$^+$, a proportion of S6 iPSC islet cells were also GP2$^+$ (56.7% [IQR 48.0-59.4%]) and PDX1$^+$ (89.5% [IQR 86.4-94.5%]). Additionally, a proportion of cells stained positive for non-endocrine markers such as SOX9 (82.1% [IQR 76.2-84.7%]), SLC18A1 (49.6% [IQR 47.1-53.7%]), SOX2 (13.8% [IQR 11.2-17.7%]), NANOG/SSEA4 (9.5% [IQR 5.5-10.8%]), and CDX2 (0.9% [IQR 0.2-1.6%]). Few cells were Ki67$^+$ (5.9% [IQR 4.7-7.4%]), with 3.9% (IQR 3.3-6.1%) being SOX9$^+$/Ki67$^+$ and few cells being SLC18A1$^+$/Ki67$^+$ (1.8%), SOX2$^+$/Ki67$^+$(1.3%), SSEA4/Ki67$^+$ (0.8%), or CDX2$^+$/Ki67$^+$ (0.07%) (Figure 3.2.2I). Overall, S6 iPSC islets were comprised by polyhormonal endocrine cells that expressed islet maturation markers. Non-endocrine populations were SOX9$^+$ and SLC18A1$^+$ and had limited proliferation based on Ki67$^+$ expression. Flow cytometric gating and supporting immunohistochemistry findings for S6 iPSC islets characterization can be found in **Appendix** Figure S3.2.9A. Results were similar in three independent S6 iPSC islet cell lines (**Appendix** Figure S3.2.9B-D).

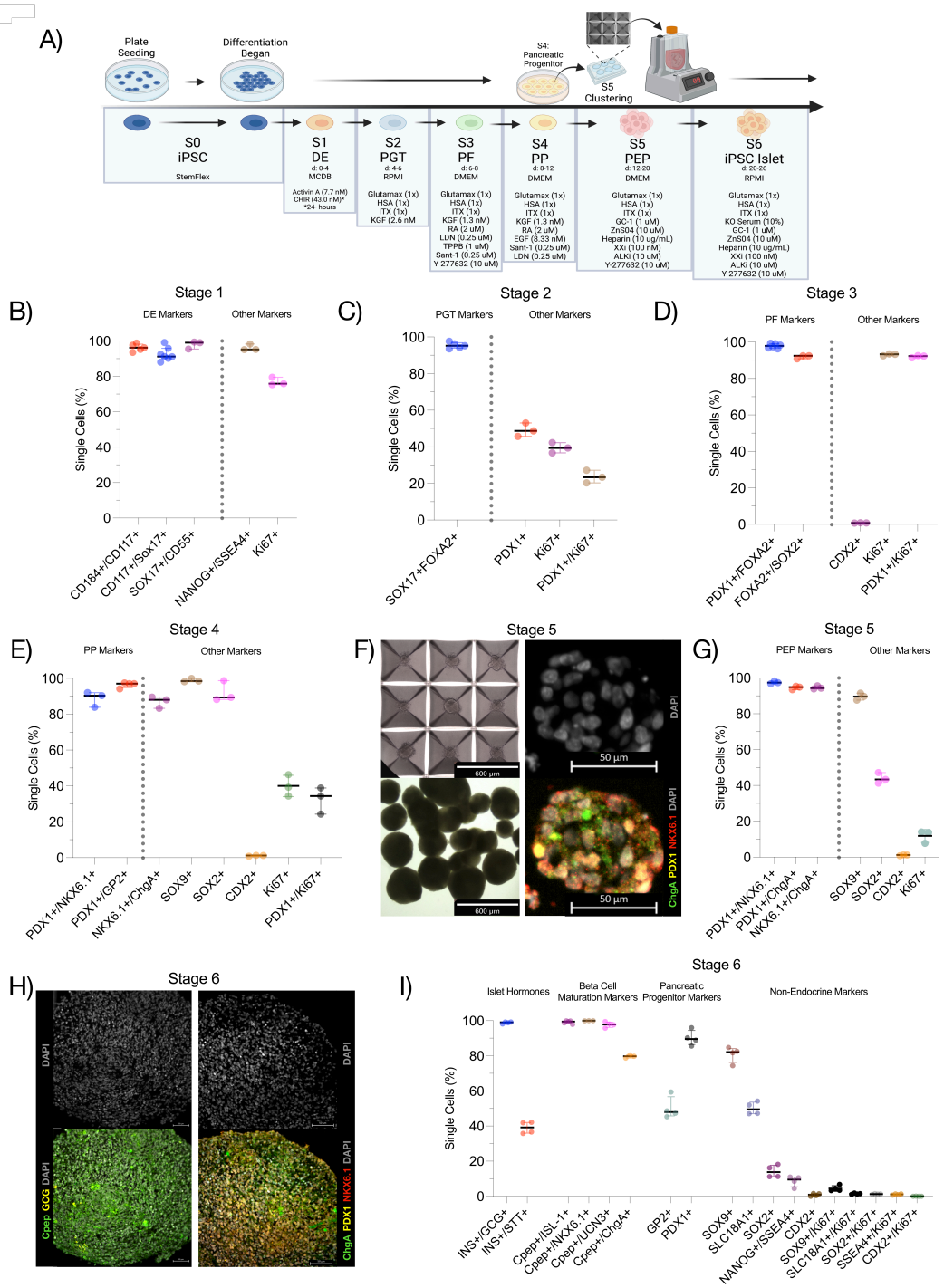

Figure 3.2.2 Stage-wise proteomic characterization using flow cytometry of the optimized induced pluripotent stem cell islet differentiation protocol.

A) Complete optimized protocol showing media and concentration of supplements. Detailed manufacturer and catalog information for differentiation reagents can be found in Appendix Table S3.2.3 Differentiation media and supplements. B) Percent of single cells after Stage 1 expressing definitive endoderm (DE) markers, pluripotency markers, and Ki67 as a proliferation marker. C) Percent of single cells after Stage 2 expressing primitive gut tube (PGT) markers, PDX1, and Ki67. D) Percent of single cells after Stage 3 expressing posterior foregut (PF) markers, CDX2, and Ki67 as a proliferation marker. E) Percent of single cells after Stage 4 expressing pancreatic progenitor (PP) markers, SOX9, SOX2, CDX2, and Ki67 as a proliferation marker. F) PP cells within AggreWell plates (top left) and following aggregation with immunohistochemistry demonstrating cell co-expression of ChgA/PDX1/NKX6.1. G) Percent of single cells after Stage 5 expressing pancreatic endocrine progenitor (PEP) markers, SOX9, SOX2, and CDX2. H) Immunohistochemistry of cell clusters following Stage 6 evaluated for Cpep/GCG (left) and ChgA/PDX1/NKX6.1 (right). I) Percent of single cells after Stage 6 expressing islet hormones, beta cell maturation markers, pancreatic progenitor markers, and non-endocrine markers including SOX9, SLC18A1, SOX2, NANOG/SSEA4, CDX2 and their co-expression with Ki67.
†Cpep: C-peptide; INS: Insulin; GCG: Glucagon; STT: Somatostatin; ChgA: Chromogranin A.

3.2.4.3 Transcriptomic analysis demonstrates transition of iPSCs towards immature islet-like clusters

Transcriptomic analysis throughout differentiation of iPSCs into iPSC islets supported proteomic cell characteristics described at each Stage of differentiation. During Stage 1 *CXCR4* ($p = 0.0065$), *SOX17* ($p = 0.0011$), and *FOXA2* ($p = 0.011$) were significantly upregulated compared to iPSCs (Figure 3.2.3A). At Stage 4, cells had upregulated *NEUROG3* ($p < 0.001$), *PDX1* ($p < 0.001$), *NKX6.1* ($p < 0.001$), and *GP2* ($p < 0.001$) compared to iPSCs (Figure 3.2.3A). Notably, *NEUROG3* expression initiated at Stage 3 followed by *PDX1* and *NKX6.1* in Stage 4 (Figure 3.2.3A). *STT* expression initiated at Stage 4 then decreased over time, while other endocrine-associated hormonal genes including *INS* and *GCG* did not increase until Stages 5 and 6 (Figure 3.2.3A). Assessment of 33 genes that are known to be associated with pancreatic differentiation showed expression that was progressively more similar to human islet expression

from Stage 1 through Stage 6 (Figure 3.2.3B). Stage-wise trends in gene expression compared to iPSCs for all 95 genes is demonstrated in **Appendix** Figure S3.2.10 to Figure S3.2.12.

Comparison of Stage 4 cells to iPSCs showed that PP genes including *PDX1, NKX6.1, ONECUT1, GP2*, and *NEUROG3* were significantly upregulated (Figure 3.2.3C). Genes associated to endocrine lineage commitment such as *CHGA, NEUROD1*, *UCN3, HNF4A, ISL1, TSPAN1,* and STT were also significantly upregulated in PPs compared to iPSCs (Figure 3.2.3C). Additionally, genes associated with Stages 2-3 (*SOX17, FOXA2, CXCR4*), and genes associated with enterochromaffin cells (*SLC18A1*) were significantly upregulated at the Stage 4 PP Stage compared to iPSCs (Figure 3.2.3C).

Comparing Stage 6 iPSC islet cells to Stage 4 PPs there was significant induction of endocrine hormone genes including *INS, GCG,* and *CHGA* (Figure 3.2.3D). Additionally, pancreatic endocrine associated genes including *SYP, ARX, GP2, GLP1R, TSPAN1, HNF4A, NKX6.1, PCSK1, CHGB,* and *ABCC8* were significantly upregulated at Stage 6 compared to Stage 4. Similar to flow cytometric assessment, *SLC18A1*, associated with enterochromaffin cells, was upregulated in Stage 6 iPSC islets (Figure 3.2.3D).

Despite upregulation of endocrine genes, significant differences between Stage 6 iPSC islets and human islet transcriptomics existed (Figure 3.2.3E-F). Importantly, hormonal genes including *INS, STT,* and *GCG*, and islet maturation genes including *UCN3, ISL-1, GLP1R,* and *MAFB* remain significantly lower in Stage 6 iPSC islets compared to human islets (Figure 3.2.3E). Additionally, *G6PC2, GP2, PDX1*, and *GLP1R* were significantly less expressed, while *NKX6.1*, *ABCG2, GATA4*, and *SLC18A1* were upregulated significantly more in Stage 6 iPSC islets compared to human islets (Figure 3.2.3F).

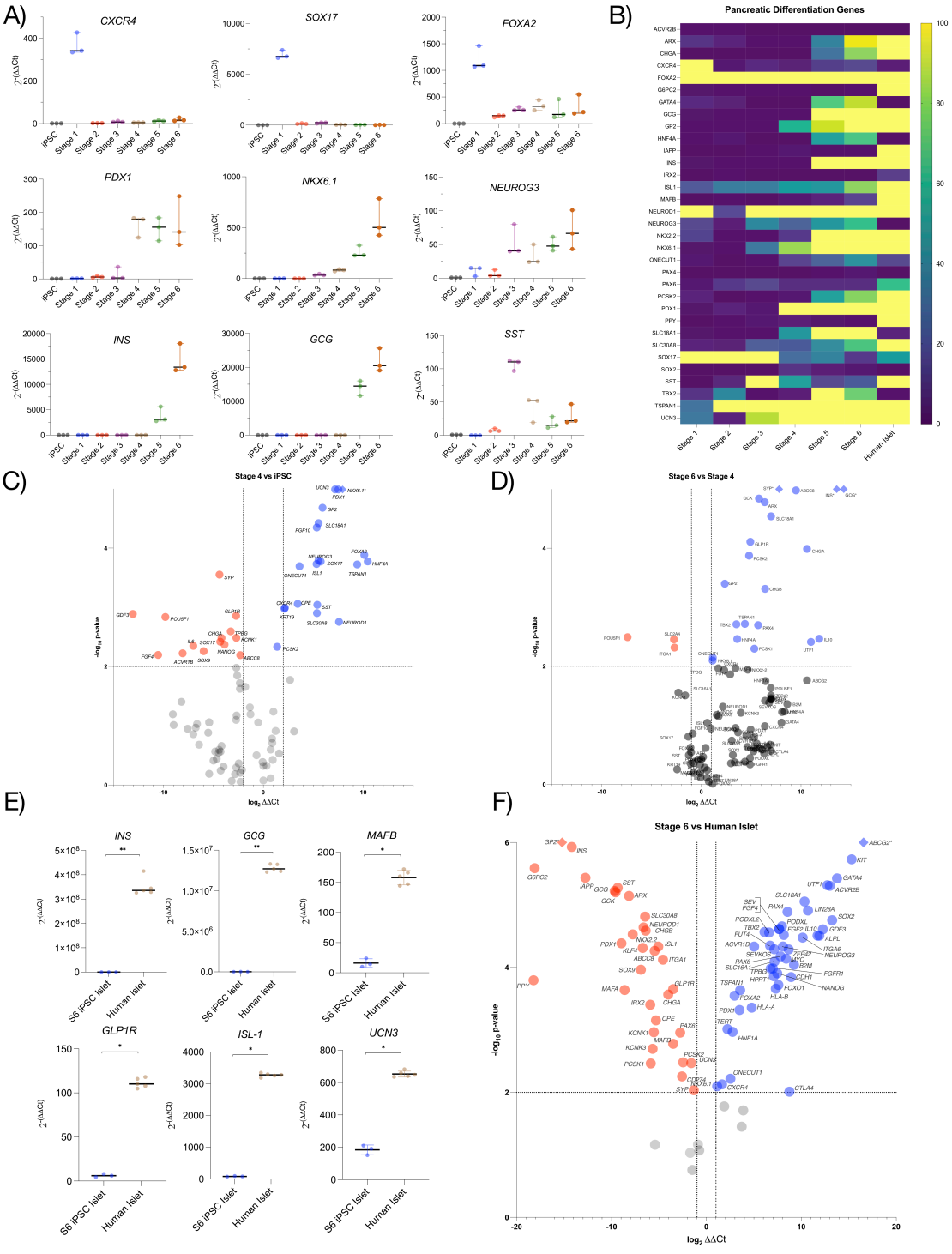

Figure 3.2.3 Stage-wise transcriptomic characterization of the optimized induced pluripotent stem cell islet differentiation protocol.

A) Temporal expression trends of key genes associated with early (top row), middle (middle row), and late (bottom row) islet differentiation throughout the six Stages of islet differentiation. B) Heat map of 33 genes previously associated with pancreatic islet differentiation demonstrating expression trends from Stage 1 to 6 during iPSC islet differentiation. C) Volcano plot demonstrating relative gene expression between cells at the end of Stage 4 compared to iPSCs. D) Volcano plot demonstrating relative gene expression between cells at the end of Stage 6 compared to cells at the end of Stage 4. E) Comparison of expression for key endocrine and islet maturation genes in Stage 6 iPSC islets and human islets. F) Volcano plot demonstrating relative gene expression between cells at the end of Stage 6 compared to human islets. ns represents $p \geq 0.05$, * represents $p < 0.05$, and **represents $p < 0.001$. Diamond (as opposed to square) symbols within the volcano plots with names that are starred* represent genes where the p-value was beyond the y-axis maximum. †significance is set at $p < 0.01$ for all volcano plots to highlight key genes. Cpep: C-peptide; INS: Insulin; GCG: Glucagon; STT: Somatostatin; ChgA: Chromogranin A.

Fold expression of all 84 genes and p-value of genes at Stage 4 compared to iPSCs, Stage 6 compared to Stage 4, and Stage 6 compared to human islets is provided in **Appendix** Table S3.2.7.

3.2.4.4 iPSC islets demonstrate in vitro glucose stimulated insulin secretion inferior to human islets with immature metabolic profile

Following aggregation in AggreWell® plates cell clusters continue to grow from (118.0 μm; (IQR 104.5-124.0 μm), forming 196 μm (IQR 146.8-254.0 μm) clusters at Stage 5 and 326.6 μm (IQR 235.5-409.3 μm) clusters at Stage 6, which were significantly larger than human islets (223.5 μm [IQR 175.0-266.0 μm]; $p < 0.001$; Figure 3.2.4A). However, S6 iPSC islets were more homogeneous in size than human islets and lack contaminating acinar tissue that is present in human islets as determined by lack of dithizone staining (Figure 3.2.4B). Insulin content was similar between S6 iPSC islets and human islets (12.0 ng/IEQ vs. 11.6 ng/IEQ, $p > 0.99$; Figure 3.2.4C), and S6 iPSC islets were capable of static glucose and GLP1 agonist stimulated insulin

secretion, producing 0.48 ng (IQR 0.31-0.56 ng) of C-peptide per IEQ in G3, 0.75 ng/IEQ (IQR 0.68 -0.89 ng/IEQ) in G17, 0.89 ng/IEQ (IQR 0.73-1.46 ng/IEQ) following exendin exposure, and 2.49 ng/IEQ (IQR 1.65-3.09 ng/IEQ) after cell depolarization with KCl (Figure 3.2.4D). GSIS results were similar from S6 iPSC islets generated using three independent cell lines. The stimulation index of S6 iPSC islets was 1.69 (IQR 1.47-1.80) in G17, 1.82 (IQR 1.38-2.52) in exendin, and 4.83 (IQR 3.71-5.45) in KCL (Figure 3.2.4E). Despite glucose stimulated insulin secretion, the stimulation index of S6 iPSC islets to G17 was significantly lower than human islets (5.08 [IQR 2.04-12.6]; $p = 0.026$; Figure 3.2.4F). Dynamic perifusion of S6 iPSC islets showed a biphasic response to G17 glucose (Figure 3.2.4G), which was again lower than human islets (22.4 AUC S6 iPSC islet vs. 60.8 AUC human islet; $p < 0.05$; Figure 3.2.4H). S6 iPSC islets responded to exendin during dynamic perifusion (stimulation index: 3.49), and S6 iPSC islets had similar insulin secretion in response to KCl as human islets (29.67 AUC S6 iPSC islet vs. 37.61 AUC human islet; $p = 0.07$).

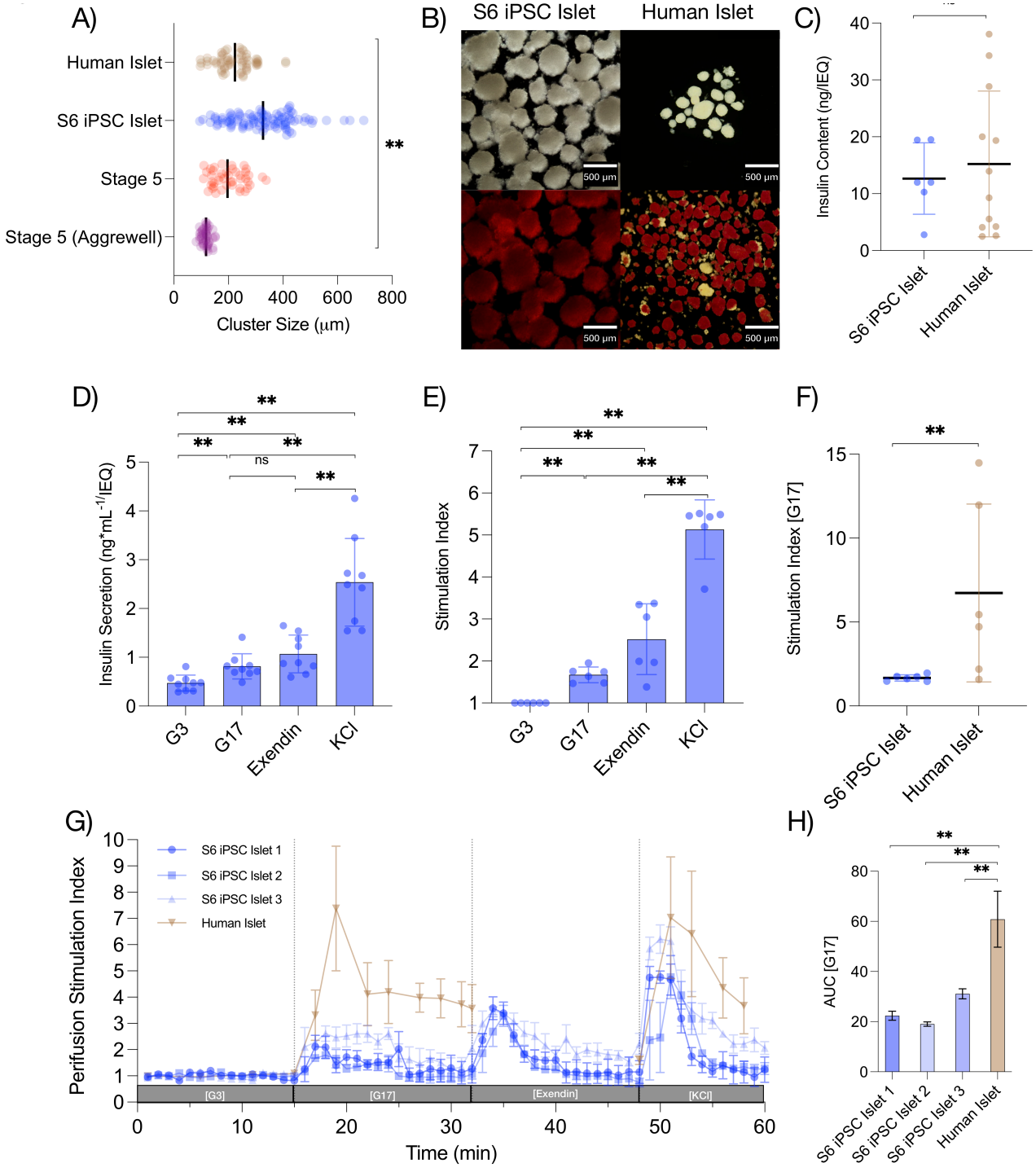

Figure 3.2.4 *In vitro* characterization of Stage 6 induced pluripotent stem cell-derived islets (S6 iPSC islet) morphometry and function compared to human islets.

A) Cell cluster size distribution for cell clusters following AggreWell® plate aggregation, after Stage 5, and following Stage 6 (S6 iPSC islet) compared to human islet cluster sizes. B) Microscopy of S6 iPSC islets and human islets without (top row) and with (bottom row) dithizone staining. C) Total insulin content of S6 iPSC islets and human islets following glucose stimulated insulin secretion (GSIS) assays. D) Absolute insulin secretion (ng/mL) per islet equivalent (IEQ) in response to low (G3), high (G17), Exendin, and KCl during static GSIS of S6 iPSC islets. E) Stimulation index of S6 iPSC islets during static GSIS assay. F) Comparison of the stimulation index of S6 iPSC islets and human islets in response to glucose.
ns represents $p \geq 0.05$, * represents $p < 0.05$, and ** represents $p < 0.001$.

Electrophysiologic evaluation demonstrated S6 iPSC islet cells to have larger surface area based on capacitance recordings than human primary (1°) β cells (10.49 pF [IQR 9.19-13.83 pF] vs. 6.37 pF [IQR 5.23-7.85 pF]; $p < 0.001$; Figure 3.2.5A-B). In response to series of depolarization in 5.0 mM glucose, S6 iPSC islets had significantly more exocytosis compared to human 1° β cells (32.62 fF/pF [IQR 16.7-61.47 fF/pF] vs. 6.75 fF/pF [IQR 1.85-15.88 fF/pF]; $p < 0.001$; Figure 3.2.5C). Upstream to exocytosis, depolarization induced Na^+ influx (-32.2 pA/pF [IQR -5.4 to -103.3 pA/pF] vs. -10.8 pA/pF [IQR -6.3 to -18.8 pA/pF]; $p < 0.001$; Figure 3.2.5D) and early Ca^{2+} influx (-5.5 pA/pF [IQR -2.6 to -10.0 pA/pF] vs. -3.5 pA/pF [IQR -2.1 to -5.2 pA/pF]; $p < 0.001$; Figure 3.2.5E) into cells that was larger for S6 iPSC islet cells compared to human 1° β cells respectively. Electrophysiological results signify that the machinery required to elicit exocytosis, which is downstream from their metabolic and glucose sensing capacity, is present and highly functional within S6 iPSC islets.

Evaluation of S6 iPSC islet oxygen consumption demonstrated significant differences across all conditions (Figure 3.2.5F). S6 iPSC islets had a higher basal metabolism (82.3 $pmol^{-min}/\mu g$ [IQR 77.1-86.9 $pmol^{-min}/\mu g$] vs. 28.8 $pmol^{-min}/\mu g$ [IQR 21.3-50.9 $pmol^{-min}/\mu g$]; $p < 0.001$; Figure 3.2.5G) and had higher non-mitochondrial oxygen consumption (21.7 $pmol^{-min}/\mu g$ [IQR

21.0-29.3 pmol^{-min}/μg] vs. 10.1 pmol^{-min}/μg [IQR 6.5-15.2 pmol^{-min}/μg]; p = 0.01; Figure 3.2.5H) compared to human islets. Despite overall higher metabolic activity, S6 iPSC islets had a significantly lower glucose stimulated oxygen consumption index (1.06 [IQR 1.0-1.1] vs. 1.71 [IQR 1.4-1.8]; p = 0.04; Figure 3.2.5I) even though S6 iPSC islets had a higher spared capacity than human islets (22.4 pmol^{-min}/μg [IQR 17.2-29.2 pmol^{-min}/μg] vs. 8.8 pmol^{-min}/μg 6.4-13.8 pmol^{-min}/μg]; p < 0.001; Figure 3.2.5J). Results were confirmed in three independent iPSC lines. Overall, these results suggest that S6 iPSC islets have comparatively higher metabolic function and capacity, yet fail to sense and respond metabolically to glucose stimulation.

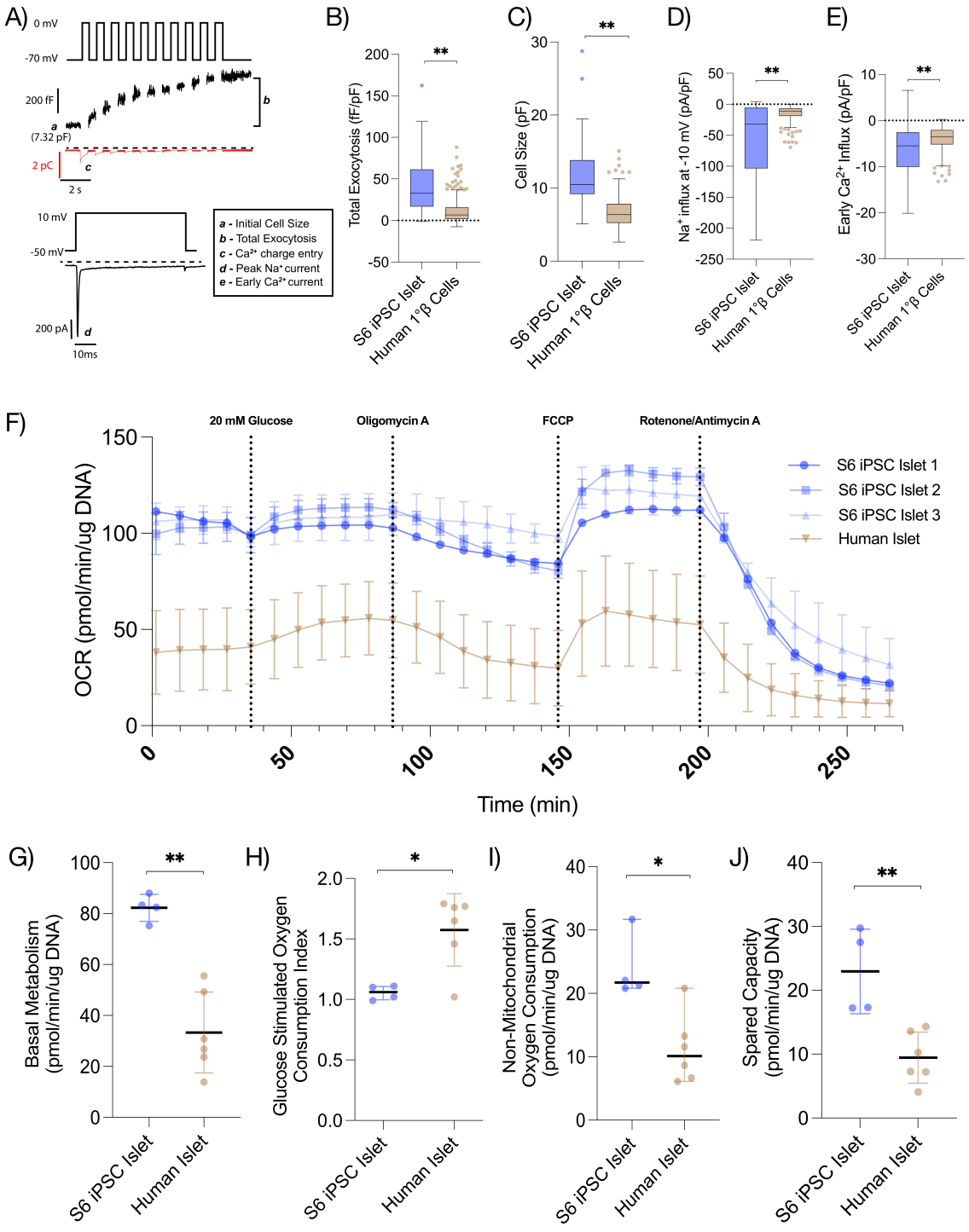

Figure 3.2.5 Electrophysiological and metabolic evaluation of Stage 6 induced pluripotent stem cell-derived islets (S6 iPSC islets) with comparison to human islets.

A) Representative patch clamp traces including an outline of calculations to determine cell size, exocytosis, Na+ charge entry, and Ca2+ charge entry. B) Cell size surface area of S6 iPSC islets based on capacitance recordings compared to human primary (1°) β cells. C) Cell exocytosis of S6 iPSC islets in response to depolarization based on capacitance recordings compared to human primary (1°) β cells. D-E) Na+ and Ca2+ influx into S6 iPSC islets in response to depolarization compared to human primary (1°) β cells. F) Oxygen consumption ratio of S6 iPSC islets and human islets in response to glucose, oligomycin A, FCCP, and Antimycin A. G-J) Evaluation of basal metabolism, glucose stimulated oxygen consumption index, non-mitochondrial oxygen consumption, and spared capacity of S6 iPSC islets and human islets calculated from seahorse oxygen consumption ratio evaluation.
ns represents $p \geq 0.05$, * represents $p < 0.05$, and **represents $p < 0.001$.

3.2.4.5 Transplantation of S6 iPSC islet like clusters results in stimulated human C-peptide secretion that augments over time

Following renal subcapsular transplantation in SCID-beige mice, intraperitoneal glucose tolerance tests were completed on post-transplant weeks 8, 12, and 16 followed by non-recovery nephrectomy and evaluation of transplanted cells immunohistochemistry. *In vivo* glucose tolerance tests demonstrated glucose responsive C-peptide production by week 8, which was significantly higher than sham mice (5.95 pM [IQR 2.5-10.2 pM] 8 weeks vs. 1.86 pM [IQR 0.8-2.1 pM] sham; $p = 0.028$; Figure 3.2.6A). Compared to week 8, grafts produced significantly more stimulated C-peptide after 12 weeks of *in vivo* maturation (5.95 pM [IQR 2.5-10.2 pM] 8 weeks vs. 17.1 pM [IQR 13.7-24.2 pM] 12 weeks; $p < 0.001$). Similar C-peptide production was demonstrated from weeks 12 to 16 (17.1 pM [IQR 13.7-24.2 pM] 12 weeks vs. 23.9 pM [IQR 17.2-30.0 pM] 16 weeks; $p = 0.42$; Figure 3.2.6A). Despite increased overall glucose stimulated C-peptide secretion, the stimulation index remained similar (1.8 [IQR 1.6-5.2] at 8 weeks, 1.8 [IQR 1.4-2.6] at 12 weeks, and 1.9 [IQR 1.7-2.0] at 16 weeks; $p = 0.71$; Figure 3.2.6B).

3.2.4.6 Explanted grafts demonstrate monohormonal endocrine cells within multiloculated cysts

Gross pathological assessment of grafted kidneys demonstrated them to weigh significantly more than non-grafted kidneys (1.24 g [IQR 1.2-1.3 g] grafted vs. 0.36 g [IQR 0.31-0.40 g] non-grafted; p=0.008) and to be approximately 2 times larger in size (1.98 cm [IQR 1.83-2.04 cm] grafted vs. 1.00 cm [IQR 0.91-1.04 cm] non grafted; $p < 0.001$; Figure 3.2.6C). Kidney enlargement was primarily composed of multiloculated polycystic masses contained within the kidney capsule. Haematoxylin and eosin staining of kidney sections further confirmed cystic duct-like masses within the non-invaded kidney capsule (Figure 3.2.6D). Immunohistochemistry staining demonstrated that grafts were composed of $CK19^+$ lined cysts with $SOX9^+$ interductal tissues (Figure 3.2.6E). Between cysts, and frequently pushed to the outside of grafts there were INS^+ cell clusters. Evaluation of the endocrine structures within grafts demonstrated islet-like structures composed primarily of monohormonal INS^+ cells with fewer monohormonal GCG^+ and STT^+ cells on the periphery of endocrine tissues (Figure 3.2.6F).

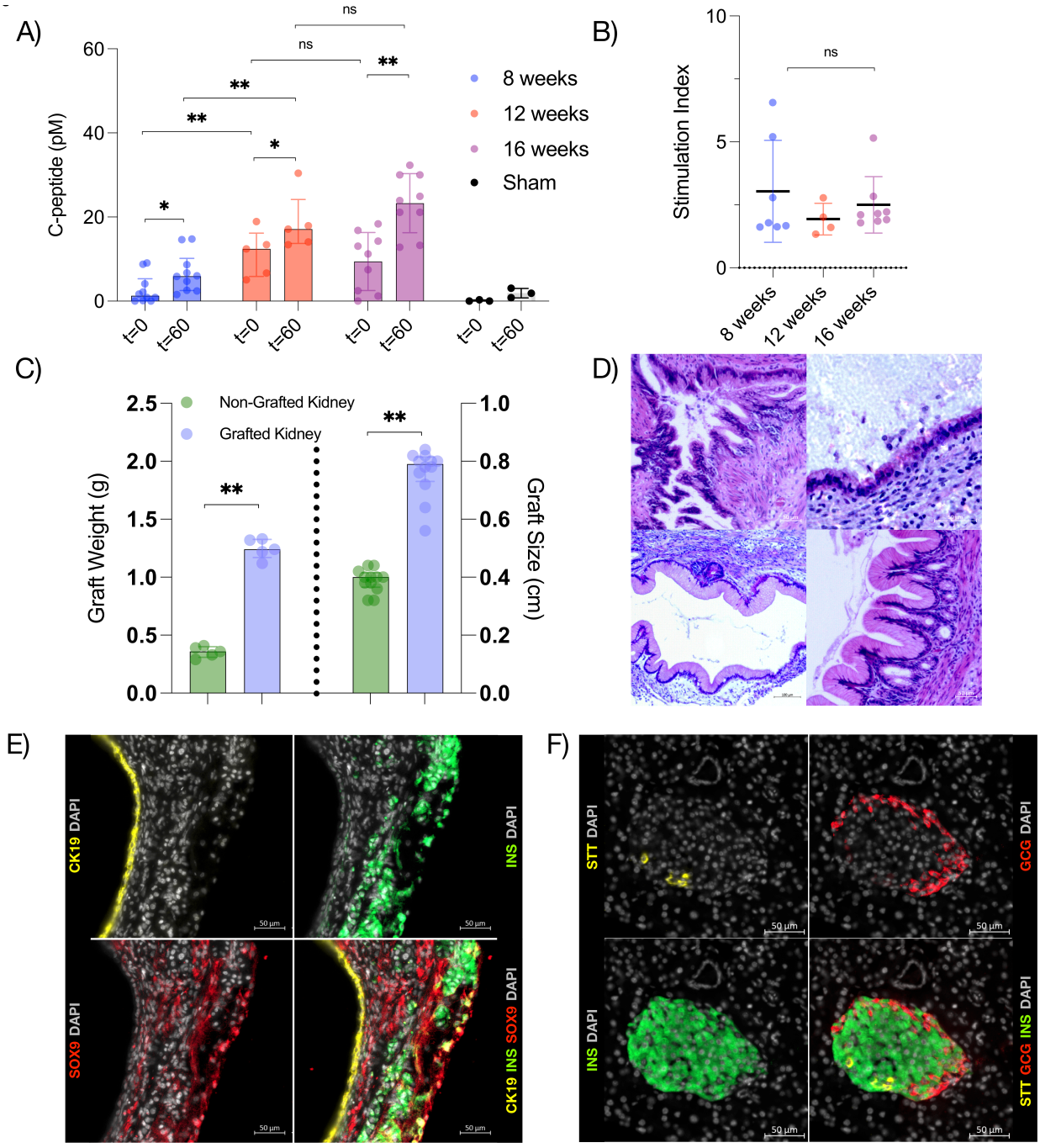

Figure 3.2.6 *In vivo* function and post-transplant graft evaluation of Stage 6 induced pluripotent stem cell-derived islets (S6 iPSC islets).
A) *In vivo* glucose stimulated insulin secretion (GSIS) of human insulin from transplanted S6 iPSC islets following intraperitoneal glucose tolerance testing at 8, 12, and 16 weeks after transplant. B) *In vivo* glucose stimulation index of S6 iPSC islets at 8, 12, and 16 weeks after

transplant evaluated using intraperitoneal glucose tolerance testing. C) Representative graft gross pathology of S6 iPSC islets following 16 weeks of *in vivo* maturation. D) Representative hematoxylin and eosin stained microscopy of grafts transplanted with S6 iPSC islets following 16 weeks of *in vivo* maturation. E) Representative immunohistochemistry of grafts transplanted with S6 iPSC islets following 16 weeks of *in vivo* maturation.
ns represents $p \geq 0.05$, * represents $p < 0.05$, and **represents $p < 0.001$. †Significance is set at $p < 0.01$ for all volcano plots to highlight key genes.

3.2.4.7 Anti-aging glycopeptide or complete suspension differentiation within differentiation Vertical-Wheel® bioreactors enables improved S6 iPSC islet yield

To evaluate the applicability of the proposed protocol for clinical ITx we estimated the number of cells required to hypothetically achieve insulin independence. Based on previous literature suggesting a minimum ITx mass of 10,000-15,000 IEQ/kg to achieve insulin independence, approximately 1750 cells per IEQ, and an average weight of 62 kg from worldwide data[1,2,32,33], we estimated that approximately $1{,}250 \times 10^6$ iPSC islet cells would be required to manufacture an effective ITx mass.

Following differentiation in 150 mm culture plates we generated 8.29×10^6 (IQR 7.12×10^6-9.13×10^6) cells per 150 mm plate. Considering the estimated number of cells required to achieve an effective ITx mass, 152.81 large 150 mm plates would be required per patient using the current protocol. Using this protocol, differentiation started with 121.3×10^6 (IQR 114.3×10^6-142.6×10^6) cells with notable cell loss at Stage 1 (43.7% loss from Stage 0) and during the transition to 3D culture in Stage 5 (78.2% loss from Stage 4) (Figure 3.2.7A). Despite substantial cell loss in Stage 1, plates were nearly 100% confluent from Stages 2-4 and cell loss was attributed primarily to significantly larger cells in Stage 2 onwards compared to Stage 1 (12.0 μm [IQR 11.1-13.5 μm] Stage 1 vs. 21.1 μm [IQR 18.3-24.9 μm] Stage 2; $p = 0.03$).

Because cell confluency was nearly 100% during Stages 1-4, it is unlikely that further optimization could be achieved from Stages 1-4 in 2D conditions. Therefore, our efforts to improve yield focused on optimizing Stages 5-6, with particular focus on Stage 5 due to the 78.2% cell loss that occurred compared to Stage 4.

Application of the small molecule antiaging glycopeptide (AAGP) during cell clustering within AggreWell® plates led to significantly increased cell death within 100 mL of media ($36.0x10^6$ [IQR $35.1x10^6$-$37.7x10^6$] control vs. $42.2x10^6$ [IQR $39.1x10^6$-$44.0x10^6$] AAGP; $p = 0.029$) and reduced yield following aggregation ($20.3x10^6$ [IQR $16.1x10^6$-$21.6x10^6$] control vs. $14.6x10^6$ [IQR $11.7x10^6$-$16.8x10^6$] AAGP; $p = 0.026$). However, application of AAGP following aggregation from days 14-18 within Vertical-Wheel® bioreactors led to significantly more cells at the end of Stage 5 ($5.44x10^6$ [IQR $4.22x10^6$-$7.48x10^6$] control vs. $10.7x10^6$ [IQR $8.59x10^6$-$14.4x10^6$] AAGP; $p = 0.004$) and subsequently Stage 6 ($8.15x10^6$ [IQR $6.83x10^6$-$8.53x10^6$] control vs. $20.0x10^6$ [IQR $18.5x10^6$-$21.1x10^6$] AAGP; $p = 0.004$; Figure 3.2.7B). Quality assessment of cells treated with AAGP demonstrated no significant effect on final cell composition or *in vitro* function (**Appendix** Figure S3.2.13A-D). When applying AAGP after aggregation during Stage 5, 62.5 large 150 mm plates would be required to achieve our hypothesized effective ITx mass.

Due to substantial limitations of 2D differentiation, including the need to pool multiple differentiation preparations to achieve sufficient transplant mass, limited scalability, and the substantial technical and time constraints associated with aggregation, we performed this optimized differentiation protocol completely within Vertical-Wheel® bioreactors (hereafter referred to as suspension differentiation protocol and labelled VWB within the text results). With

this, we achieved $105.0x10^6$ (IQR $103.0x10^6$-$113.0x10^6$) S6 iPSC islet cells in a single 0.1 L Vertical-Wheel® bioreactor (Figure 3.2.7A-B). Cell quality assessed by flow cytometry and function were not significantly affected (**Appendix** Figure S3.2.13A-D). Considering these results, 11.9 Vertical-Wheel® bioreactors of this size (0.1 L) would be required to achieve our hypothesized effective ITx mass.

3.2.4.8 Suspension differentiation within Vertical-Wheel® bioreactors reduces cost of S6 iPSC islet generation

Cost evaluation, including technician time, supplements, media, and materials needed to generate the hypothesized effective ITx mass demonstrated that suspension differentiation within Vertical-Wheel® bioreactors reduced costs compared to the control protocol ($248,135 [IQR $224,735-$271,749] control vs. $27,718 [IQR $26,352-$27,987] VWB; $p < 0.001$; Figure 3.2.7C). However, during our differentiation, we typically pooled 10 large 150 mm plates together following Stage 4 into a single Vertical-Wheel® bioreactor, significantly reducing the material cost of differentiation in planar conditions ($248,135 [IQR $224,735-$271,749] control vs. $79,635 [IQR $72,715-$84,923] pooled; $p < 0.001$). Despite pooling 10 large 150 mm plates together, the suspension differentiation within Vertical-Wheel® bioreactors continued to cost significantly less ($79,635 [IQR $72,715-$84,923] pooled vs. $27,718 [IQR $26,352-$27,987] VWB; $p < 0.001$; Figure 3.2.7C-D). Additionally, even when AAGP was added after aggregation and 10 plates were pooled together, the cost per $1,200x10^6$ cells remained significantly lower in Vertical-Wheel® bioreactors ($42,500 [IQR $40,784-$34,757] AAGP pooled vs. $27,718 [IQR $26,352-$27,987] VWB; $p < 0.001$; Figure 3.2.7C-D). A higher proportion of costs were

attributed to media supplements using Vertical-Wheel® bioreactors (61.3% control vs. 72.5% VWB), while a higher proportion of costs were due to materials using planar differentiation (27.6% control vs 14.4% VWB). We highlight that these results were achieved with limited optimization within Vertical-Wheel® bioreactors and do not include the cost of AAGP. Notably, differentiation using only Vertical-Wheel® bioreactors also significantly reduced technician time compared to pooling 10 plates (7.03 hr [IQR 6.95-7.10 hr] control vs. 4.11 hr [IQR 4.05-4.20 hr] VWB; $p < 0.001$; Figure 3.2.7E). There were no differences in technician time regardless of AAGP addition in planar differentiation.

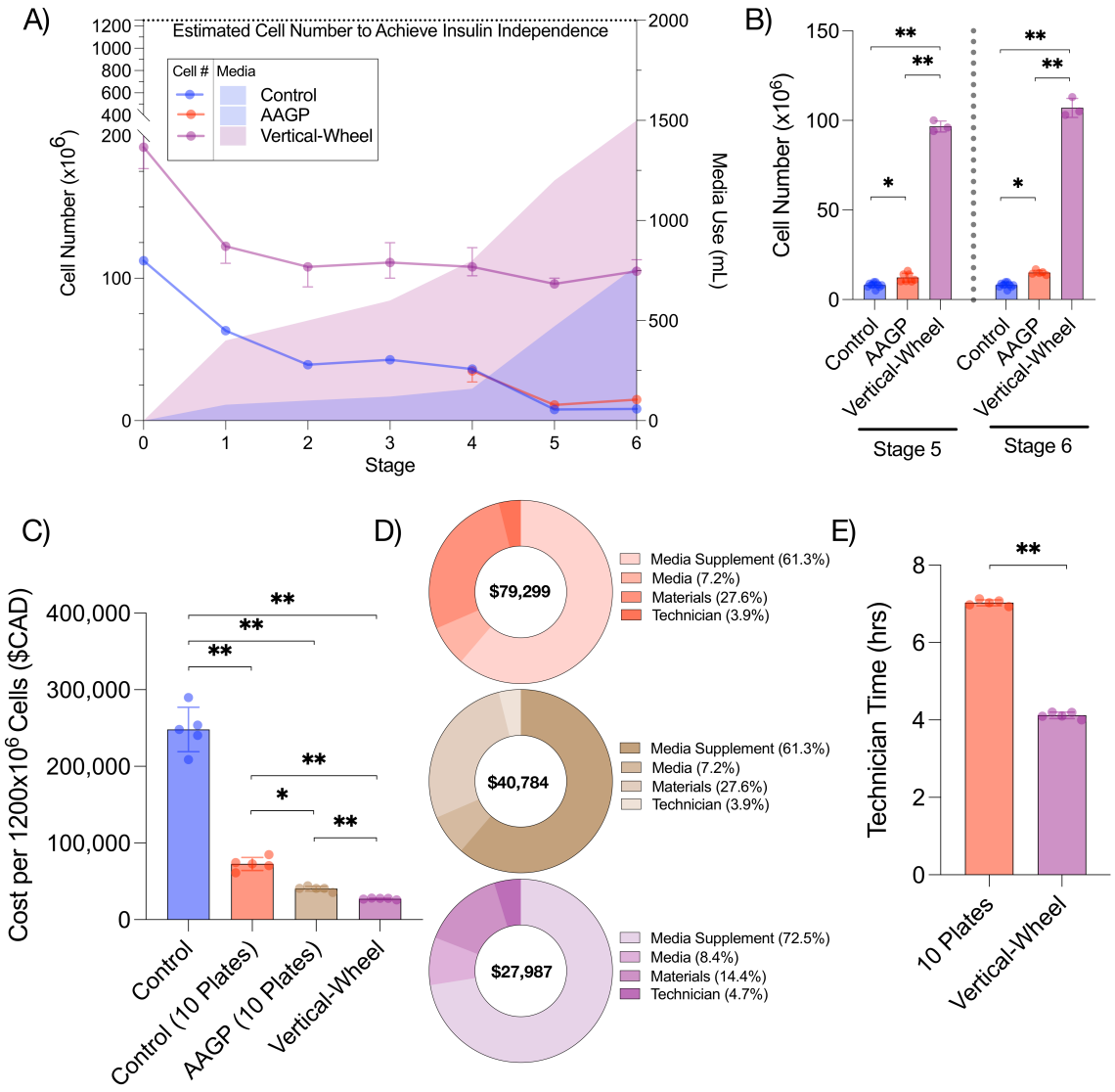

Figure 3.2.7 Yield assessment and cost evaluation to generate Stage 6 induced pluripotent stem cell-derived islets (S6 iPSC islets) using anti-aging glycopeptide and differentiation completed entirely within Vertical-Wheel® bioreactors.

A) Cell number following each stage of differentiation using the optimized protocol, with the addition of AAGP after aggregation during Stage 5, and with differentiation completely within Vertical-Wheel® bioreactors. B) Comparison of cell yield after Stage 5 and Stage 6 using the optimized control protocol, following addition of AAGP after aggregation during Stage 5, and with differentiation completely within Vertical-Wheel® bioreactors. C) Cost of generating 1,200x106 cells (i.e. the estimated number of cells to achieve insulin independence) using the optimized differentiation protocol (control), with pooling of 10 plates into one bioreactor at Stage 5 using the optimized differentiation protocol (Control 10 plates), with pooling of 10 plates into one bioreactor and the addition of AAGP after aggregation during Stage 5 (AAGP 10

plates), and following differentiation completely within 0.1 L Vertical-Wheel® bioreactors (Vertical-Wheel). D) Total cost to generate 1,200x106 cells and proportion of cost spent on materials, media, media supplements, and technicians for the optimized differentiation protocol and pooling of 10 plates (red), with pooling of 10 plates into one bioreactor and the addition of AAGP after aggregation during Stage 5 (brown), and following differentiation completely within 0.1 L Vertical-Wheel® bioreactors (purple). E) Technician time required to complete a single differentiation protocol from Stage 1-6 including using 10 plates compared to differentiation completed entirely within a Vertical-Wheel® bioreactor.
ns represents $p \geq 0.05$, * represents $p < 0.05$, and **represents $p < 0.001$.

3.2.5 **Discussion**

This study offers an optimized scalable protocol with stage-wise characterization capable of generating glucose responsive iPSC-derived islet-like clusters. Key aspects of the protocol optimization include targeting better basal media for pancreatic progenitor generation using sequential MCDB (Stage 1), RPMI (Stage 2) and DMEM without nicotinamide (Stages 3 and 4). Stage-wise proteomic and transcriptomic evaluation with functional characterization of the iPSC islet product demonstrates effective transition towards human islet-like insulin producing cell populations, yet still highlights the relative immaturity of these iPSC-derived cells. While off-target proliferation leading to cystic growth persists following this protocol, we describe a comprehensive stage-wise characterization to enable ongoing protocol optimization. Such detailed characterization is absent from much of the existing literature (Table 3.2.1) and we suggest that such characterization will facilitate better comparison of protocols in the field and thereby accelerate progress. Additionally, assessment of yield, preliminary proof-of-concept for a fully suspension-based differentiation protocol within Vertical-Wheel® bioreactors, and cost evaluation highlight a novel approach to enable scalability for clinical translation.

Current protocols report heterogeneous cell populations following differentiation with potential risks associated with proliferative non-endocrine populations. While a well-defined homogeneous population remains the goal, stage-wise characterization intends to inform future protocol comparisons and enable optimization throughout differentiation to reduce off-target cell populations to improve safety of cell products. In this study, differentiation efficiency was evaluated by assessing $SOX17^+/CD117^+$ co-expression and $CD55^+$ for definitive endoderm (DE), $SOX17^+/FOXA2^+$ for primitive gut tube (PGT), $FOXA2^+/PDX1^+$ for posterior foregut (PF), and $PDX1^+/NKX6.1^+$ for PP Stages. Previous literature supports these as differentiation markers in keeping with the transition from stem cells to pancreatic progenitors that are capable of further islet differentiation[8,26,34-38]. Stage-to-stage comparisons are currently limited due to a lack of reporting in prior studies. Certainly, we agree with others that highly efficient DE induction with >90% $SOX17^+/CD117^+$ is critical for successful downstream differentiation[35,36]. Notably, while others reported DE induction in 3 days, in our experience some cell lines may require 4 days to achieve >90% $SOX17^+/CD117^+$, leading us to use a 4-day Stage 1 to allow applicability across cell lines. Considering the nearly 100% induction of DE, PGT, and PF markers, it is unlikely that further protocol optimization could improve cell quality from Stages 1-3. Additionally, this study demonstrates generation of cells that are >90% $PDX1^+/NKX6.1^+$ and >95% $PDX1^+/GP2^+$, key markers associated with PPs that mature into functional endocrine cells[16,26]. This is the highest proportion of $PDX1^+/NKX6.1^+$ PP co-expression achieved at Stage 4 reported to date, including in comparison to Balboa et al. (2022) and Aghazadeh et al. (2022) who showed approximately 80% and 85%, respectively, with application of nicotinamide[25,26]. Despite these promising findings, the majority of Stage 4 PPs were $ChgA^+$ and transcriptomic analysis showed early

NEUROG3 induction, followed by *NKX6.1* expression, which some have suggested as being associated with non-functional polyhormonal cells that degranulate into alpha cells following *in vivo* maturation[19,39]. However, more updated studies evaluating single cell sequencing, along with comparison to cells generated using previous protocols, demonstrate that cells expressing *NKX6.1*, regardless of its acquisition before or after *NEUROG3*, can become monohormonal cells following maturation[40]. While our protocol led to polyhormonal iPSC islets at Stage 6, C-peptide^{+} cells also co-expressed NKX6.1, the key marker associated with islet function, suggesting a β-cell like phenotype[41]. In keeping with single cell data from Petersen et al (2017), the polyhormonal cells from this study likely represent early immature β-like cells with lower *PCSK1, ISL1, MAFA*, and *PCSK2* compared to human β cells[40]. This likely accounts for the low glucose responsiveness of our Stage 6 iPSC islets and maturation into monohormonal cells, including β-cells, following transplant. Regardless, in addition to demonstrating the stage-wise transition of cells during this study, it is our hope that data presented here will allow inter-protocol comparisons and most importantly, will inform stage-specific release criteria to enable consistent and safe clinical translation of iPSC islet therapies[8].

Functional, electrophysiological, and metabolic evaluation of the S6 iPSC islets generated with this protocol further support their immature phenotype. Similar to Nair et al. (2019) and Balboa et al. (2022), S6 iPSC islets had similar insulin content but had significantly reduced GSIS compared to human islets[20,25]. While lower than human islets, the GSIS response of our S6 iPSC islets is comparable to the blunted response seen in neonatal mouse islets and fetal human islets[42]. Additionally, patch clamp evaluation of S6 iPSC islets demonstrate that the functional machinery needed for excitability and exocytosis is present, and in fact the

depolarization induced currents and exocytotic responses were larger than in adult β-cells. Considering the capacity of iPSC islets to exocytose, the insulin secretion deficit in response to glucose likely relates to the cell's immaturity and decreased glucose sensing capacity and metabolism[42,43]. Like others, we have demonstrated that the immature β-cells generated following differentiation have significantly higher oxygen consumption with limited metabolic response to glucose stimulation[20,25]. Metabolite tracing analysis completed by Balboa et al. (2022) highlights the immature β-cell metabolism of SC islets with a primarily glycolytic metabolism that limits glucose responsiveness[25,44]. Like others, we demonstrate comparatively lower *G6PC2* expression in SC islets, as a likely contributor to this immature glycolytic metabolism[25]. Despite these consistent metabolic findings across numerous studies[20,25,44], considering the capacity of immature SC islets to mature *in vitro* and *in vivo* and improve their GSIS capacity, the clinical importance of this finding remains uncertain. While *in vitro* maturation may allow manipulation to further purify SC islets, a more likely clinical product involves transplant of immature cells with *in vivo* maturation that would reduce technician time and costs. Overall, SC islets appear to have the machinery required for exocytosis and with maturation achieve improved *in vivo* GSIS. Their relative immaturity at this stage of differentiation should therefore, in our opinion, not restrict clinical implementation.

Despite demonstrating an optimized protocol with *in vivo* maturation of iPSC islets, the presence of cystic growth following transplant remains a substantial translational barrier. Similar to our results, Rezania et al. (2014) and Schulz et al. (2012) demonstrated substantial $CK19^{+}$ ductal tissues in grafts transplanted with PPs, while Aghazadeh et al. (2022) demonstrate trilineage off-target tissue after transplanting heterogeneous PPs[19,26]. Unfortunately, despite

substantial discussion regarding approaches to eliminate off-target growth, including differentiating PPs into SC islets, few authors reporting differentiation protocols have described post-transplant graft evaluation. It is difficult to assess whether this is due to lack of off-target cells or lack of reporting; however, we believe this should be a priority for the field to enable clinical implementation and suggest that all future studies include graft reporting. Notably, within this study, a substantial proportion of cells from Stages 4-6 also express SOX9, SOX2, and SLC18A1, markers of ductal, intestinal, and enterochromaffin cells, respectively, that likely led to the off-target cystic growths following transplant.

Moving towards clinical translation will require better strategies to mitigate risk of cyst formation. Ongoing studies in our lab are looking at factors such as mouse strain, site of implantation, local paracrine stimulators of growth and differentiation, and use of targeted anti-proliferative agents to ensure the safety of these products. Additionally, others including Balboa *et al* have recognized the challenge of these vastly accelerated differentiation protocols (27-days compared to 9 months in a human infant), and have found that an additional period of 6-12 weeks *in vitro* in bioreactors may be a promising approach to improve maturation with less risk of off-target contaminating $SOX9^{+}$ and other components. Alternatively, considering the presence of off-target markers from Stage 4 onwards, reducing their induction earlier during differentiation may be a useful approach. Future work to apply previously presented chemical approaches for off-target cell elimination within recently published protocols are needed[45-51]. Alternatively, cell cluster disaggregation and reaggregation have also shown promise to eliminate off-target growth[52 53], but evaluation of their effect on yield are unclear. Application of

these methods in this differentiation protocol will certainly be interesting to determine their effect on cell quality, yield, and off-target cell populations.

Potentially most important to the field are the results of yield and cost analysis from this study. The only previous studies reporting yield are Rezania et al. (2014), who reported a 50% yield of embryonic stem cells to islets, and Hogrebe et al. (2020) who reported 0.5 - 0.75x10^6 SC islet cells per cm^2 corresponding to 108x10^6 islets per 150 mm plate. The Hogrebe et al. yield would be substantially higher than reported in this study, while the Rezania et al. (2014) yield would correspond with findings using Vertical-Wheel® reported here; unfortunately, the method to determine yield, details on cell number at each Stage, and absolute cell counts were not well discussed in these studies, making it difficult to accurately compare to those results. More so, the lack of yield evaluation and reporting in previous studies highlights the need to include these results in future work to evaluate the utility of protocols for clinical implementation. Our results demonstrate significant cell loss at Stage 1 and with aggregation at the beginning of Stage 5, resulting in increased costs with planar differentiation. Addition of AAGP following aggregation at Stage 5 achieved 2.5 times more cells and reduced costs significantly. Despite this promising finding, we highlight the potential of this protocol to generate iPSC islets using entirely suspension differentiation within Vertical-Wheel® bioreactors. While others have previously used Vertical-Wheel® bioreactors for specific stages, use from iPSC expansion through to iPSC islet generation has never been reported. This eliminates highly complex aggregation stages where substantial cell loss has previously been reported and where significant inter-user variability exists with pooling of cells from numerous plates to generate a final cell product. The variability, time, cost, and pooling of heterogeneous cells likely precludes widespread clinical

application of two-dimensional differentiation techniques. We have previously shown the potential of Vertical-Wheel® bioreactors to generate a superior iPSC starting product, but more importantly to be scalable[24]. We demonstrate that a single mini 0.1 L Vertical-Wheel® bioreactor offers 31.4% cost savings and 7.1-fold yield increase compared to planar conditions with AAGP and requires less technician time. Theoretically, assuming scalability of the suspension differentiation protocol within Vertical-Wheel® bioreactors, a single 3 L bioreactor would be capable of generating an adequate number of iPSC islets to achieve our hypothesized clinically meaningful islet mass. However, substantial cell loss remains an issue at Stage 1 of differentiation and ongoing optimization of differentiation within Vertical-Wheel® bioreactors is still needed. Optimization of these protocols to allow scalability of these approaches to larger 3 L, or 15 L bioreactors will be important to further expand cost savings and applicability of these technologies. Other studies have evaluated the optimal biophysical properties and rotational speed to maximize iPSC viability and expansion, and study of these properties at each stage is of interest. Due to the optimal conditions created within Vertical-Wheel® bioreactors, with superior oxygen and nutrient mixing whilst reducing shear stress compared to other bioreactor configurations, we believe these reactors will provide a superior format for ongoing optimization and scale up[54-56]. Considering the nearly 400 million patients with diabetes[57], we suspect that fully suspension-based protocols are the most likely approach to generate the islet mass needed for clinical implementation and should be of focus for future study.

The findings of this study should be contextualized within specific limitations. Most importantly, this protocol has been replicated in only three patient-derived iPSC lines generated with Sendai virus transfection of PBMCs and results may vary based on origin cell source, iPSC

generation technique, and patient factors including age, sex, or health conditions. Considering the ultimate goal of autologous iPSC ITx to treat patients with diabetes, evaluation of the efficiency of this protocol using iPSC lines from patients with diabetes (or other comorbidities) will be of importance. Additionally, while others, including Petersen et al. (2017)[40], Balboa et al. (2022)[25], Veres et al. (2019)[52], and Augsornworawat et al. (2020)[58] have reported single cell RNA sequencing of SC islet cells, this study offers data on whole cell populations. Future evaluation of the SC islets generated using this protocol with RNA sequencing, with post-transplant maturation sequencing would be of interest. Following transplant, our protocol also required 16-weeks to generate significant insulin, and evaluation of protocols that generate $ChgA^-$ cells at Stage 4 that may produce more mature endocrine cells is of interest. Of course, our results and others in the field remain limited to mouse models and in-human safety and efficacy data is needed to confirm promising results. Costs presented here should only be interpreted as a comparison between techniques and should not be extrapolated directly to clinical application; costs do not include the substantial resource requirement of clean rooms, cost recuperation of innovation and discovery, building maintenance, cell quality control, or clinical costs associated with ITx. Finally, this study presents only preliminary data on differentiation within Vertical-Wheel® bioreactors with limited optimization using this format and it is likely that superior cell yield will be achievable with ongoing improvements of this approach. However, it is worth noting that the scalability of Vertical-Wheel® bioreactors to 0.5 L for iPSC expansion has been demonstrated, and that 3 L and 15 L versions allow dynamic temperature, mixing, gas, and metabolite control that should allow scalability of these approaches. Optimization of our Vertical-Wheel® bioreactor protocol followed by proof of

scalability in larger 0.5 L, 3 L, or 15 L Vertical-Wheel® bioreactors will certainly be valuable for future clinical implementation.

Despite these limitations, we present a modified and updated iPSC islet differentiation protocol with stage-wise characterization, graft evaluation, and yield assessment. We highlight that few previous studies have reported yield or graft evaluation and suggest that all future studies should include this key data necessary for clinical implementation of SC islet therapies. Considering the field's advancements, it is no longer sufficient to simply report protocols that generate insulin producing SC islets. We believe that stage-wise characterization with previously defined markers will enable ongoing optimization to improve purity, safety, and yield. This study's proof of concept for bioreactor-based differentiation offers promise to improve yield and allow future scalability. Indeed, evaluation of large-scale suspension differentiation with approaches to improve yield and eliminate off-target growth are of interest moving forward.

3.2.6 Appendix: chapter 3 subsection 2

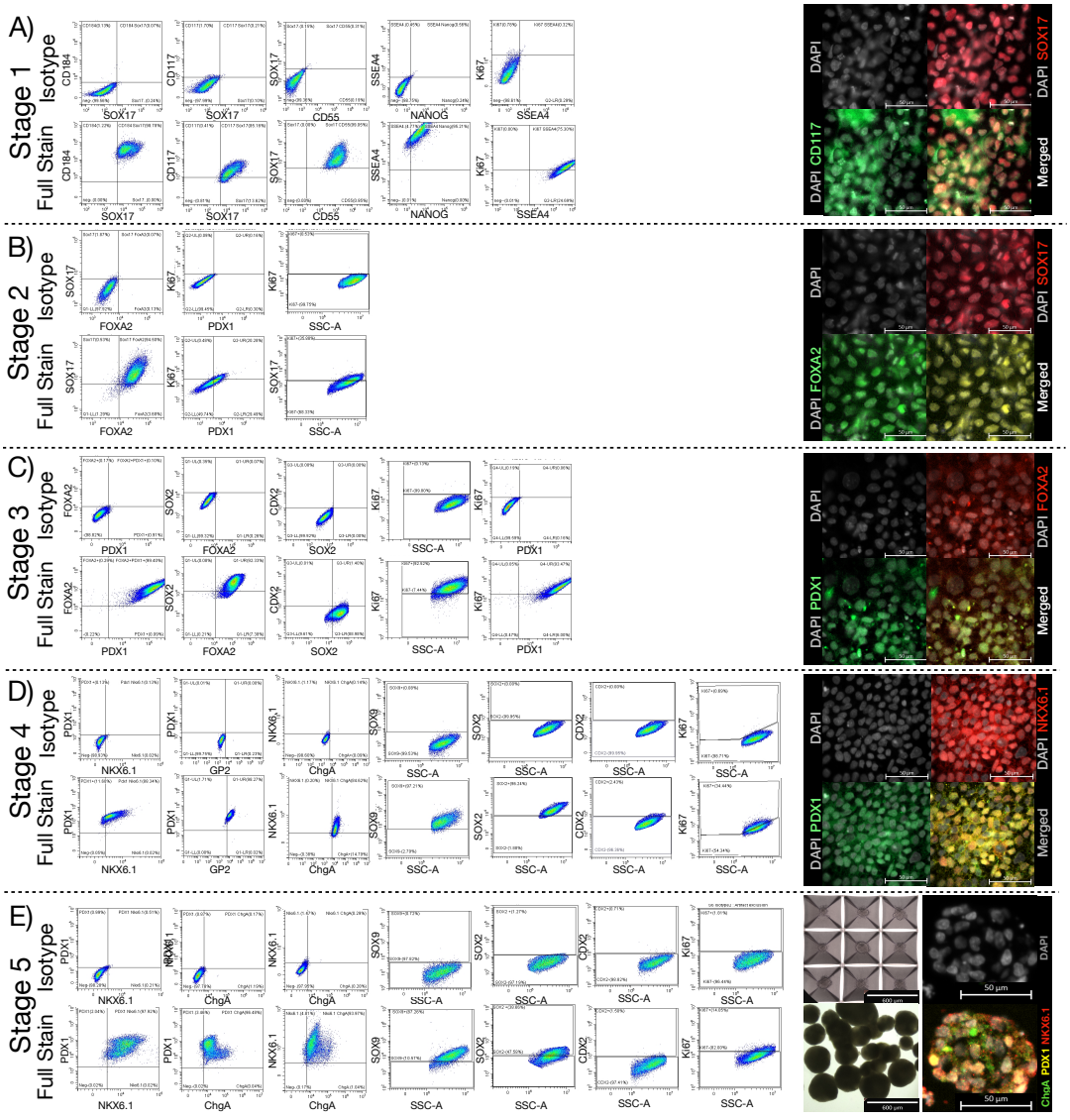

Figure S3.2.8 Flow cytometry gating strategy and immunohistochemistry of key stage-wise markers from Stages 1-5.
A) Stage 1 flow cytometry and immunohistochemistry of CD117 and SOX17. B) Stage 2 flow cytometry and immunohistochemistry of SOX17 and FOXA2. C) Stage 3 flow cytometry and immunohistochemistry of FOXA2 and PDX1. D) Stage 4 flow cytometry and immunohistochemistry of PDX1 and NKX6.1. E) Stage 5 flow cytometry and immunohistochemistry of Chromogranin A (ChgA), PDX1 and NKX6.1. Microscopy imaging

demonstrating cell clustering within aggrewell plates and the ensuing cell clusters (bottom left) following cell clustering.

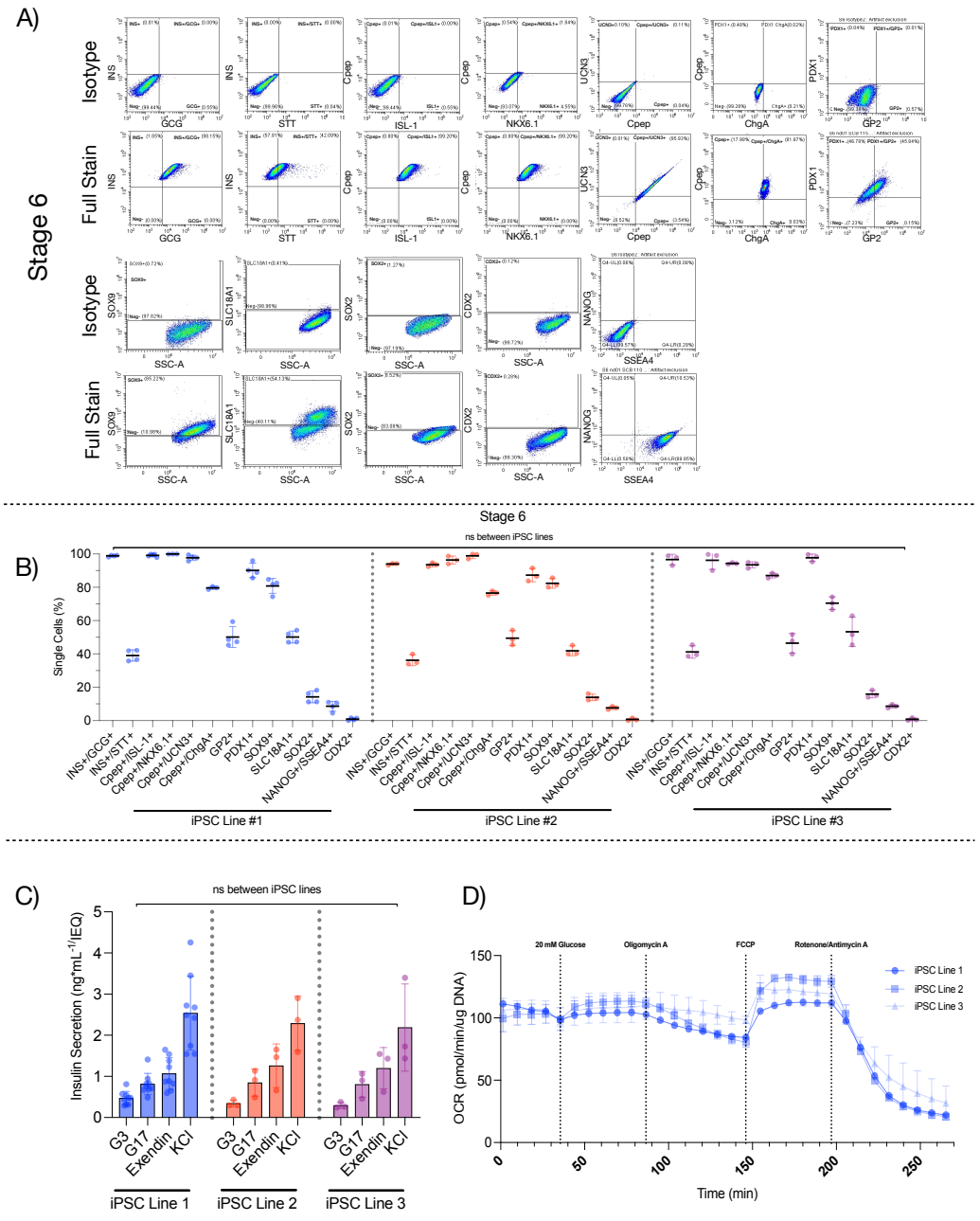

Figure S3.2.9 Stage 6 flow cytometry gating and comparison of the cell characteristics including flow cytometry, oxygen consumption ratio, and glucose stimulated insulin secretion (GSIS) from Stage 6 iPSC islets generated from three unique cell lines.
A) Flow cytometry of Stage 6 iPSC islets. B) Flow cytometry results from Stage 6 iPSC islets generated from three unique iPSC lines. Line 1 is also presented in Figure 2I. C) Oxygen consumption results from Stage 6 iPSC islets generated from three unique cell lines. Note that

results parallel those in Figure 5E. D) Glucose stimulated insulin secretion normalized to islet equivalents (IEQ) of Stage 6 iPSC islets generated from three unique iPSC lines.

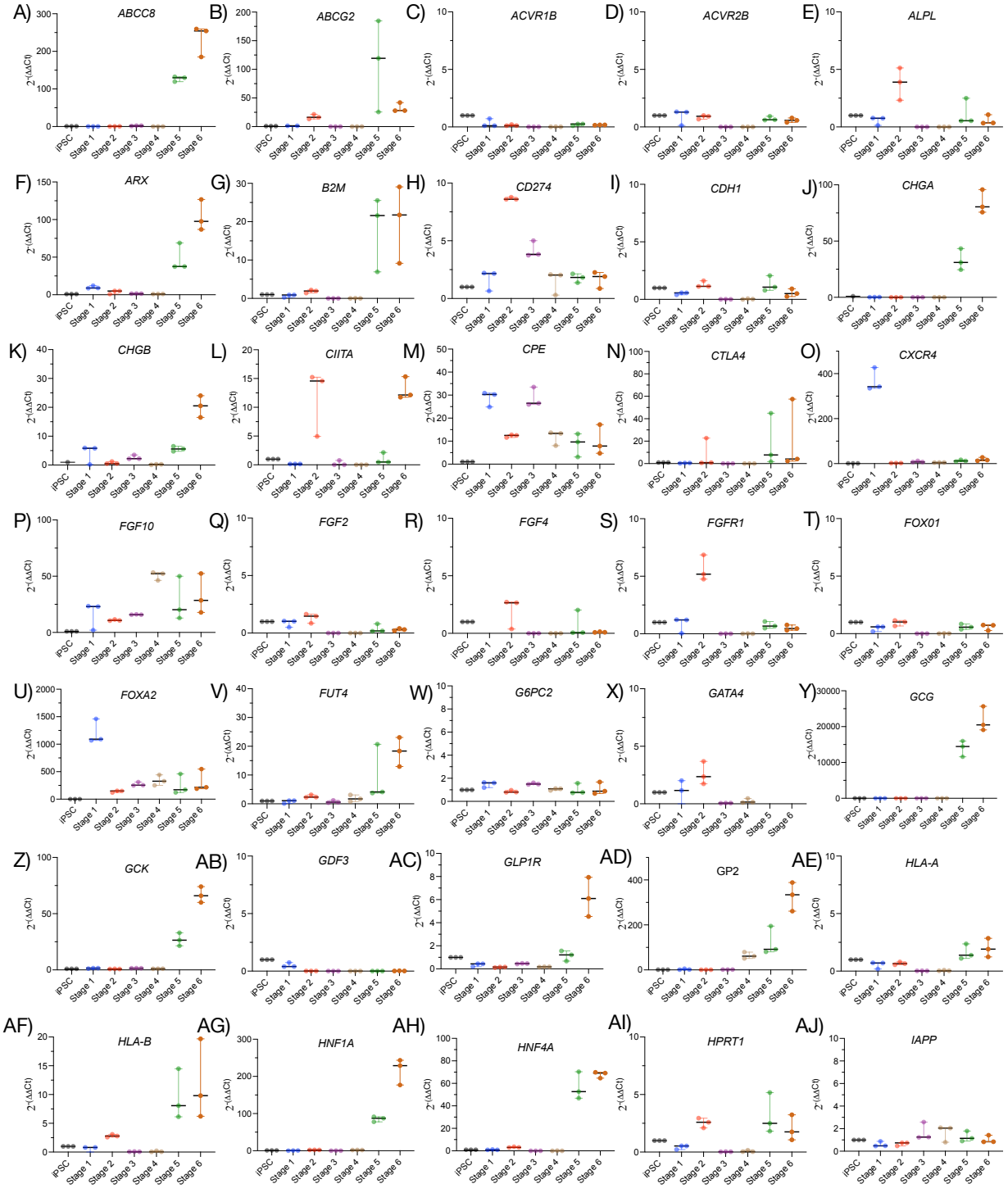

Figure S3.2.10 Stage-wise (Stages 1-6) relative expression (2- ΔΔCT) of genes compared to iPSCs (alphabetical order).

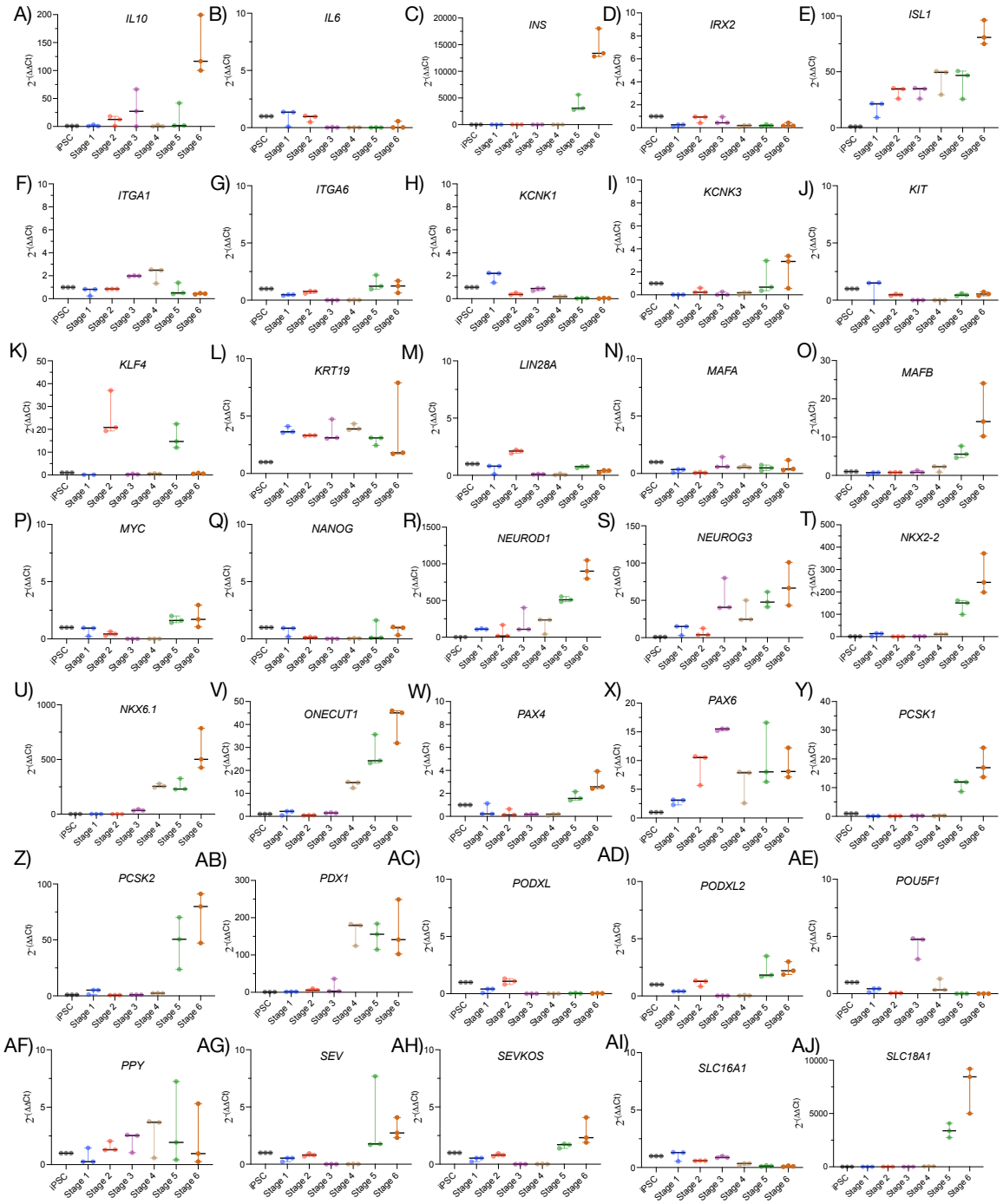

Figure S3.2.11 Stage-wise (Stages 1-6) relative expression (2- ΔΔCT) of genes compared to iPSCs (alphabetical order).

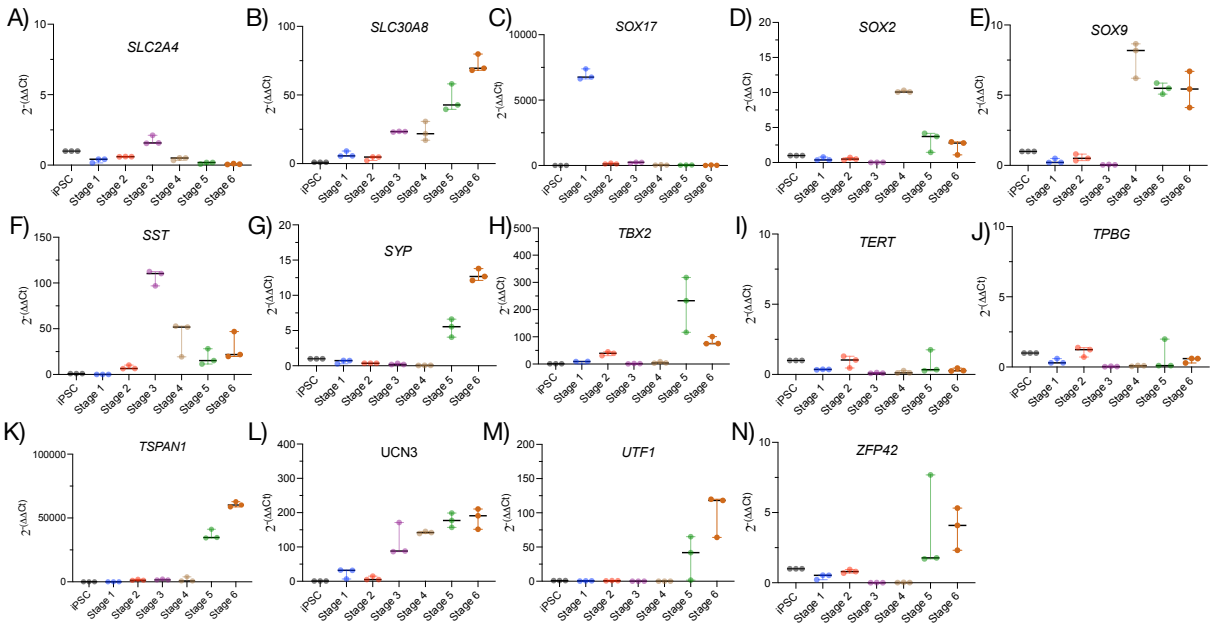

Figure S3.2.12 Stage-wise (Stages 1-6) relative expression (2- ΔΔCT) of genes compared to iPSCs (alphabetical order).

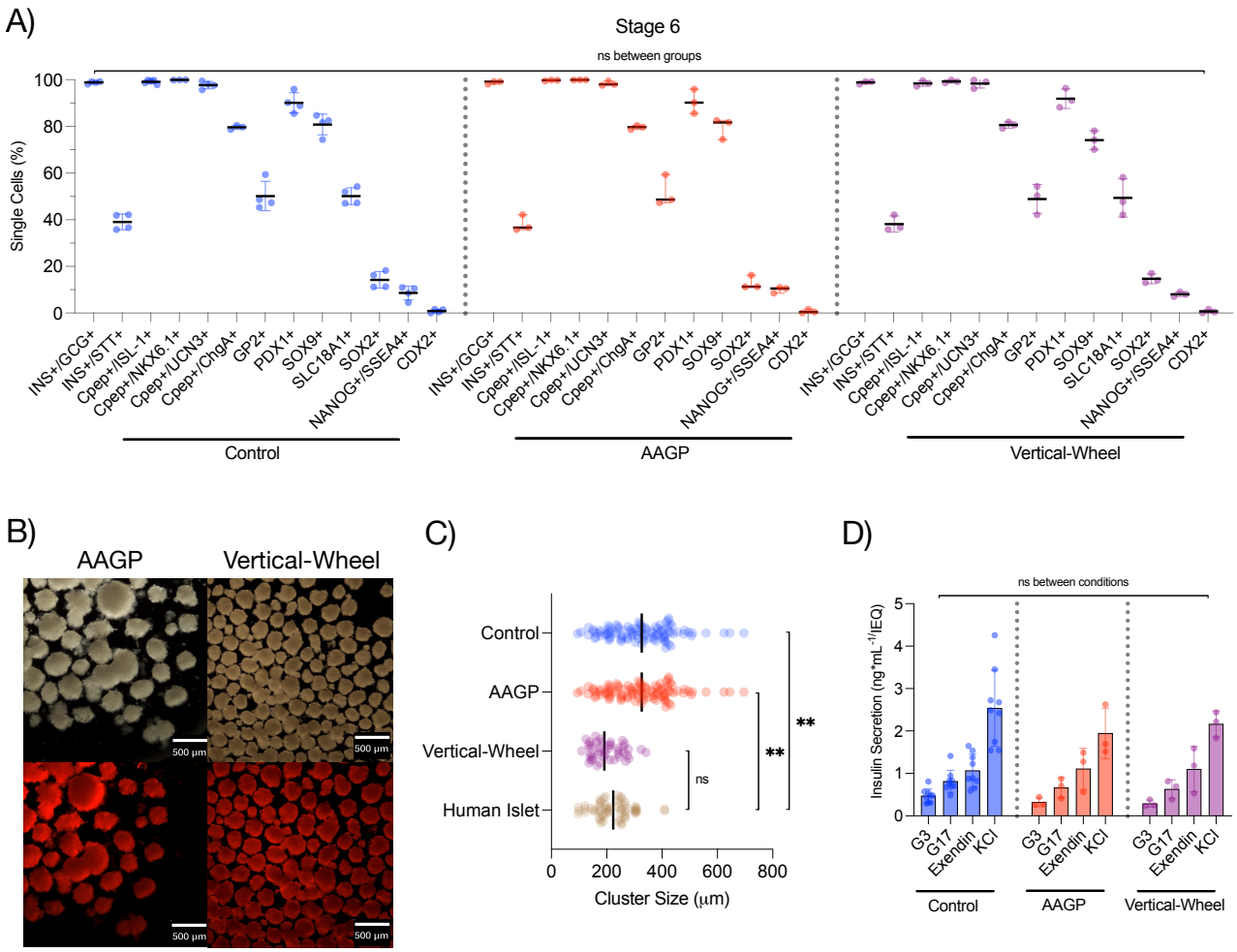

Figure S3.2.13 Flow cytometry, morphology, and functional characteristics of cells grown with the addition of anti-aging glycopeptide (AAGP) after aggregation during Stage 5 or completely in suspension culture within Vertical-Wheel® bioreactors.
A) Flow cytometry results Stage 6 iPSC islets generated using the control protocol, with addition of AAGP after aggregation during Stage 5, or in suspension culture within Vertical-Wheel® bioreactors. B) Microscopy of Stage 6 iPSC islets without (top row) and with (bottom row) dithizone staining generated with addition of AAGP after aggregation during Stage 5, or in suspension culture within Vertical-Wheel® bioreactors. C) Aggregate cluster size of Stage 6 iPSC islets generated using the control protocol, with addition of AAGP after aggregation during Stage 5, or in suspension culture within Vertical-Wheel® bioreactors and compared to human islets. D) Glucose stimulated insulin secretion normalized to islet equivalents (IEQ) of Stage 6 iPSC islets generated using the control protocol, with addition of AAGP after aggregation during Stage 5, or in suspension culture within Vertical-Wheel® bioreactors.
ns represents $p \geq 0.05$, * represents $p < 0.05$, and ** represents $p < 0.001$.

Table S3.2.2 Patient demographics for human donors of peripheral blood mononuclear cells to generate induced pluripotent stem cell lines and human islet donors

iPSC line	Age	Sex	Gender	Health status
#1	53	Female	Female	Healthy
#2	43	Female	Female	Healthy
#3	28	Male	Male	Healthy
Human Islet ID	**Age**	**Sex**	**Gender**	**Use**
R474	48	Male	N/A	-GSIS -Oxygen consumption testing
R475	51	Female	N/A	-GSIS -Oxygen consumption testing
R430	49	Male	N/A	-GSIS -Oxygen consumption testing
R473	34	Female	N/A	-GSIS -Oxygen consumption testing
R471	67	Female	N/A	-Oxygen consumption testing
R417	48	Male	N/A	-GSIS -Perifusion
R218	73	Female	N/A	-GSIS -Perifusion
R219	53	Male	N/A	-Perifusion
R224	30	Male	N/A	-Perifusion

Table S3.2.3 Differentiation media and supplements.

	Basal Media	Supplements	Duration
Stage 1	MCDB (Thermo Fisher Scientific, cat. 10372019) **DES (STEMCELL Technologies, cat. 05111)* **RPMI 1640 (Thermo Fisher Scientific, cat. 22400-089)* **MCDB 131 (Thermo Fisher Scientific, cat. 10372019)*	Activin A (7.7 nM, STEMCELL Technologies, cat. 78001.2) CHIR 99021 (43.0 nM, Thermo Fisher Scientific, cat. 2520691) Glutamax (1x, Thermo Fisher Scientific, cat. A12860-01) D-Glucose (5 mM, Sigma, cat. G7021-100G) **Human serum albumin (1%, Arkon Biotechnology, cat. AK8228-0100)* **ITX (1x, Thermo Fisher Scientific, cat. 51500-056)*	4 days Cell line dependent: 3 days is effective for some cell lines
Stage 2	RPMI **MCDB*	KGF (2.6 nM, R&D Biotechnology, cat. 251-GMP)	2 days

		Glutamax (1x) Human serum albumin (1%) ITX (1x) Retinoic acid (27.9 µM, Cedarlane, cat. 0695 Alpha-Tocopherol (4.5 µM, Sigma, cat. T3251-5G)	
Stage 3	DMEM (Sigma, cat. D0822-500ML)	KGF (1.3 nM) LDN 193189 (0.25 µM, Cedarlane, cat. 04-0074) TPPB (1 µM, Cedarlane, cat. 5343/1) Sant-1 (0.25 µM, Thermo Fisher Scientific, cat. J65294) Y-277632 (10 µM, STEMCELL Technologies, cat. 72304) Glutamax (1x) Human serum albumin (1%) ITX (1x) Retinoic acid (29.9 µM, Cedarlane, cat. 0695 Alpha-Tocopherol (4.5 µM, Sigma, cat. T3251-5G) **Nicotinamide*	2 days
Stage 4	DMEM	KGF (1.3 nM) LDN 193189 (0.25 µM) Sant-1 (0.25 µM) EGF (8.33 µM, Cedarlane, cat. 236-GMP) Glutamax (1x) Human serum albumin (1%) ITX (1x) Retinoic acid (29.9 µM, Cedarlane, cat. 0695 Alpha-Tocopherol (4.5 µM, Sigma, cat. T3251-5G) **Nicotinamide*	4 days
Stage 5	DMEM	GC-1 (1 µM, Biotechne, cat. 4554) Gamma-secratase inhibitor XXi Compound E (100 nM, Cedarlane, cat. 15579-10MG) ALK inhibitor (10 µM, abcam, cat. ab141364) Heparin Sodium (10 µg/mL, Sigma, cat. H3149-500KU) ZnS04 (10 µM, Sigma, cat. Z0251-100G) Y-277632 (10 µM) Glutamax (1x) Human serum albumin (1%) ITX (1x) Retinoic acid (27.9 µM, Cedarlane, cat. 0695	7 days

		Alpha-Tocopherol (4.5 μM, Sigma, cat. T3251-5G)	
Stage 6	RPMI 1640	Knockout Serum (10%, Life Technologies, cat. 10828-028) GC-1 (1 μM) Gamma-secratase inhibitor XXi Compound E (100 nM) ALK inhibitor (10 μM) Heparin Sodium (10 μg/mL) ZnS04 (10 μM) Y-277632 (10 μM) Glutamax (1x) Human serum albumin (0.5%) ITX (0.5x) Retinoic acid (13.95 μM, Cedarlane, cat. 0695 Alpha-Tocopherol (2.25 μM, Sigma, cat. T3251-5G)	7 days

*Starred and italicized basal media and supplements were evaluated but not included in the optimized protocol

Table S3.2.4 Antibodies and concentrations used for flow cytometry and immunohistochemistry.

Antibody	Fluorophore	Primary Antibody Supplier (catalog number)	Secondary Antibody Supplier (catalog number)	Dilution for flow cytometry	Dilution for immunohistochemistry
CD117	AF488	Invitrogen (11-1178-42)	-	1:100	1:100
SOX17	AF647	Cedarlane (IC1924A)	-	1:20	1:100
CD184	BV421	BD (562448)	-	1:100	1:100
CD55	BV786	BD (742681)	-	1:100	1:100
NANOG	PE	BD (560873)	-	1:100	1:100
SSEA4	mouse	MC-813-70	Invitrogen (A31571) or Jackson (115-115-164)	1:100	1:100
FOXA2	rabbit	Abcam (108422)	FITC/AF647	1:100	1:100

Ki67	Rabbit secondary AF405 or AF568	Abcam (ab15580)	Invitrogen (A48258) or Invitrogen (A11036)	1:50	1:50
PDX1	PE	BD (562161	-	1:100	N/A
PDX1	Mouse secondary AF647 or PE	Cedarlane (AF2419)	Invitrogen (A31571) or Jackson (115-115-164)	N/A	1:5
NKX6.1	Mouse secondary AF647 or PE	DSHB (F55A10-c)	Invitrogen (A31571) or Jackson (115-115-164)	1:20	1:5
GP2	AF405	Novus (NBP3-08243AF405)	-	1:50	1:50
CDX2	AF647	BD (560395)	-	1:100	1:100
Ki-67	PerCP-Cy 5.5	BD (561284)	-	1:50	N/A
Ki-67	Mouse secondary AF 647 or PE	Abcam (ab15580)	Invitrogen (A31571) or Jackson (115-115-164)	N/A	:50
SSEA4	Mouse secondary APC or PE	Invitrogen (MA1-021)	Invitrogen (A31571) or Jackson (115-115-164)	1:100	1:100
SOX9	Mouse secondary APC or PE	Abcam (ab76997)	Invitrogen (A31571) or Jackson (115-115-164)	1:100	1:100
SOX2	AF488	Abcam (ab195358)	-	1:100	1:100
ChgA	Rabbit secondary AF568	Novus (NB120-15160SS)	Invitrogen (A11036)	N/A	1:50

ChgA	405	Biotium (Bnc050798)	-	1:50	N/A
FOXA2	Secondary PE	Abcam (108422)	Sigma (A11036)	N/A	1:50
INS	Guinea pig secondary AF488	Gibco (12585-014)	Invitrogen (A11073)	1:500	1:500
GCG	Mouse secondary APC or PE	Sigma (G2654)	Invitrogen (A31571) or Jackson (115-115-164)	1:800	1:800
STT	Rat secondary AF647 or AF488	Cedarlane (FAB4224P)	Invitrogen (A21247) or Thermo Fisher (a21208)	1:100	1:100
ISL-1	PE	BD (Q11-465)	-	1:100	1:100
UCN3	Rabbit secondary AF405 or AF568	Cedarlane (abx100886)	Invitrogen (A48258) or Invitrogen (A11036)	1:100	1:100
SLC18A1	Rabbit secondary AF405 or AF568	Atlas Antibodies (HPA063797)	Invitrogen (A48258) or Invitrogen (A11036)	1:100	1:100
C-peptide	AF647	BD (565831)	-	1:100	-
C-peptide	Rabbit secondary AF405 or AF568	Abcam (ab14181)	Invitrogen (A48258) or Invitrogen (A11036)	1:100	1:100

*All secondaries for flow cytometry were used at a 1:500 concentration and all secondaries for immunohistochemistry were used at a 1:250 concentration.

Table S3.2.5 Thermo Fisher TaqMan Micro Array configuration.

Assay ID	Gene	Gene Name(s)	Species	Amplicon Length	Best Coverage	3' Most
Hs01053790_m1	ABCG2	ATP binding cassette subfamily G member 2 (Junior blood group)	Human	83	Yes	No

Hs01093752_m1	ABCC8	ATP binding cassette subfamily C member 8	Human	58	Yes	No
Hs00923299_m1	ACVR1B	activin A receptor type 1B	Human	74	Yes	No
Hs00609603_m1	ACVR2B	activin A receptor type 2B	Human	101	Yes	No
Hs01029144_m1	ALPL	alkaline phosphatase				
Hs00292465_m1	ARX	aristaless related homeobox	Human	96	Yes	Yes
Hs00187842_m1	B2M	beta-2-microglobulin	Human	64	Yes	No
Hs00204257_m1	CD274	CD274 molecule	Human	77	Yes	Yes
Hs01023895_m1	CDH1	cadherin 1	Human	80	Yes	No
Hs00900370_m1	CHGA	chromogranin A	Human	67	Yes	No
Hs01084631_m1	CHGB	chromogranin B	Human	112	Yes	Yes
Hs00172106_m1	CIITA	class II				
Hs00175676_m1	CPE	carboxypeptidase E	Human	106	Yes	No
Hs00175480_m1	CTLA4	cytotoxic T-lymphocyte associated protein 4	Human	93	Yes	No
Hs00607978_s1	CXCR4	C-X-C motif chemokine receptor 4	Human	153	Yes	Yes
Hs00610298_m1	FGF10	fibroblast growth factor 10	Human	70	Yes	No
Hs00266645_m1	FGF2	fibroblast growth factor 2	Human	82	Yes	No
Hs00999691_m1	FGF4	fibroblast growth factor 4	Human	130	No	No
Hs00915142_m1	FGFR1	fibroblast growth factor receptor 1	Human	62	No	No
Hs00232764_m1	FOXA2	forkhead box A2	Human	66	No	No
Hs00231106_m1	FOXO1	forkhead box O1	Human	103	Yes	Yes
Hs01106466_s1	FUT4	fucosyltransferase 4	Human	152	Yes	Yes
Hs01549772_m1	G6PC2	glucose-6-phosphatase catalytic subunit 2	Human	97	Yes	Yes
Hs99999905_m1	GAPDH	-	Human	0	No	No
Hs00171403_m1	GATA4	GATA binding protein 4	Human	68	Yes	No
Hs01031536_m1	GCG	glucagon	Human	86	Yes	No
Hs01564555_m1	GCK	glucokinase	Human	72	Yes	No
Hs00220998_m1	GDF3	growth differentiation factor 3	Human	65	Yes	Yes
Hs00157705_m1	GLP1R	glucagon like peptide 1 receptor	Human	78	Yes	No

Hs00426805_m1	GP2	glycoprotein 2	Human	75	Yes	Yes
Hs01058806_g1	HLA-A	major histocompatibility complex				
Hs00818803_g1	HLA-B	major histocompatibility complex				
Hs00167041_m1	HNF1A	HNF1 homeobox A	Human	96	Yes	No
Hs00230853_m1	HNF4A	hepatocyte nuclear factor 4 alpha	Human	49	Yes	No
Hs99999909_m1	HPRT1	hypoxanthine phosphoribosyltransferase 1	Human	100	No	Yes
Hs00169095_m1	IAPP	islet amyloid polypeptide	Human	61	Yes	Yes
Hs00961622_m1	IL10	interleukin 10	Human	74	Yes	Yes
Hs00174131_m1	IL6	interleukin 6	Human	95	Yes	Yes
Hs00355773_m1	INS	insulin	Human	126	Yes	Yes
Hs01383002_m1	IRX2	iroquois homeobox 2	Human	85	Yes	No
Hs00158126_m1	ISL1	ISL LIM homeobox 1	Human	57	Yes	Yes
Hs00235006_m1	ITGA1	integrin subunit alpha 1	Human	87	Yes	No
Hs01041011_m1	ITGA6	integrin subunit alpha 6	Human	64	Yes	No
Hs01116799_m1	KCNK1	potassium two pore domain channel subfamily K member 1	Human	140	Yes	Yes
Hs00605529_m1	KCNK3	potassium two pore domain channel subfamily K member 3	Human	134	Yes	Yes
Hs00174029_m1	KIT	KIT proto-oncogene receptor tyrosine kinase	Human	64	Yes	No
Hs00358836_m1	KLF4	Kruppel like factor 4	Human	110	Yes	Yes
Hs00761767_s1	KRT19	keratin 19	Human	116	Yes	No
Hs00702808_s1	LIN28A	lin-28 homolog A	Human	143	Yes	Yes
Hs04419852_s1	MAFA	MAF bZIP transcription factor A	Human	107	Yes	No
Hs00534343_s1	MAFB	MAF bZIP transcription factor B	Human	86	Yes	No
Hs00153408_m1	MYC	v-myc avian myelocytomatosis viral oncogene homolog	Human	107	Yes	Yes

Hs04260366_g1	NANOG	Nanog homeobox	Human	99	No	Yes
Hs01922995_s1	NEUROD1	neuronal differentiation 1	Human	110	Yes	Yes
Hs01875204_s1	NEUROG3	neurogenin 3	Human	127	Yes	Yes
Hs00159616_m1	NKX2-2	NK2 homeobox 2	Human	114	Yes	Yes
Hs00232355_m1	NKX6-1	NK6 homeobox 1	Human	93	Yes	No
Hs00413554_m1	ONECUT1	one cut homeobox 1	Human	76	Yes	Yes
Hs00173014_m1	PAX4	paired box 4	Human	115	Yes	No
Hs00240871_m1	PAX6	paired box 6	Human	76	No	No
Hs01026107_m1	PCSK1	proprotein convertase subtilisin/kexin type 1	Human	96	Yes	No
Hs00159922_m1	PCSK2	proprotein convertase subtilisin/kexin type 2	Human	76	Yes	Yes
Hs00236830_m1	PDX1	pancreatic and duodenal homeobox 1	Human	73	Yes	Yes
Hs01574644_m1	PODXL	podocalyxin like	Human	82	Yes	Yes
Hs00210532_m1	PODXL2	podocalyxin like 2	Human	73	Yes	No
Hs04260367_gH	POU5F1	POU class 5 homeobox 1	Human	77	Yes	Yes
Hs00358111_g1	PPY	pancreatic polypeptide	Human	68	Yes	No
Mr04269880_mr	SEV	Sendai	Markers & Reporters	59	No	No
Mr04421257_mr	SEV-KOS	Sendai-KLF4-KOS	Markers & Reporters	80	No	No
Hs01560299_m1	SLC16A1	solute carrier family 16 member 1	Human	95	Yes	No
Hs00915193_m1	SLC18A1	solute carrier family 18 member A1	Human	63	Yes	No
Hs00168966_m1	SLC2A4	solute carrier family 2 member 4	Human	89	Yes	No
Hs00545183_m1	SLC30A8	solute carrier family 30 member 8	Human	73	Yes	No
Hs00751752_s1	SOX17	SRY-box 17	Human	149	Yes	Yes
Hs01053049_s1	SOX2	SRY-box 2	Human	91	Yes	No
Hs00165814_m1	SOX9	SRY-box 9	Human	102	Yes	Yes
Hs00356144_m1	SST	somatostatin	Human	86	Yes	Yes
Hs00300531_m1	SYP	synaptophysin	Human	63	Yes	No
Hs00911929_m1	TBX2	T-box 2	Human	60	Yes	No

Hs00972656_m1	TERT	telomerase reverse transcriptase	Human	79	No	No
Hs00907219_m1	TPBG	trophoblast glycoprotein	Human	100	No	No
Hs00371661_m1	TSPAN1	tetraspanin 1	Human	87	Yes	No
Hs00846499_s1	UCN3	urocortin 3	Human	85	Yes	Yes
Hs00864535_s1	UTF1	undifferentiated embryonic cell transcription factor 1	Human	102	Yes	Yes
Hs01938187_s1	ZFP42	ZFP42 zinc finger protein	Human	146	Yes	Yes

Table S3.2.6 Sequences and amplicon length of primers used for RT-PCR assessment.

Gene	Forward Primer	Reverse Primer
mTOR	AGTGGACCAGTGGAAACAGG	TTCAGCGATGTCTTGTGAGG
RPTOR	actgatggagtccgaaatgc	tcatccgatccttcatcctc
MLST8	tgattgctgctgcaggttac	gttaatgggtgcgttcacct
PRAS40	agtgataatggagggctctt	acttggcgtactgctgtgtg
Deptor	caccatgtg tgtgatgagca	tgaaggtgcgctcatacttg
AMPK	TGCGTGTACGAAGGAAGAATCC	TGTGACTTCCAGGTCTTGGAGTT
mTOR	AGTGGACCAGTGGAAACAGG	TTCAGCGATGTCTTGTGAGG
RPTOR	actgatggagtccgaaatgc	tcatccgatccttcatcctc

Table S3.2.7 Fold expression of 84 genes and p-value of genes at stage 4 compared to iPSCs, stage 6 compared to stage 4, and stage 6 compared to human islets.

	Stage 4 compared to iPSCs		Stage 6 compared to Stage 4		Stage 6 compared to Human Islets	
Gene	Median Fold Expression	p-value	Median Fold Expression	p-value	Median Fold Expression	p-value
ABCC8	0.2	0.0064387	723.55	0.00001048	0.022680	0.00005418
ABCG2	0.01	0.1077761	1552.26	0.01737967	96083.142977	0.00000085
ACVR1B	0	0.0059885	8.29	0.18183399	32.618026	0.00004633
ACVR2B	0.01	0.1391238	37.61	0.27551044	8251.048000	0.00000493
ALPL	0.01	0.2091719	78.92	0.29961660	3817.543693	0.00003105
ARX	0.95	0.4117616	82.18	0.00001659	0.001229	0.00000597
B2M	0.05	0.2842063	396.39	0.04382416	599.981151	0.00009069
CD274	0.05	0.5703809	0.94	0.76014944	0.168045	0.00554795
CDH1	0.05	0.2551658	7.86	0.44033540	484.469102	0.00014243
CHGA	0.05	0.0038215	1553.00	0.00010261	0.087948	0.00022236
CHGB	0.2	0.0237983	84.68	0.00048807	0.011838	0.00002583
CIITA	0.02	0.0879112	127.84	0.03486068	13.143317	0.03546218

CPE	10.86	0.0008762	0.59	0.43811278	0.025012	0.00070383
CTLA4	0.02	0.6489281	127.84	0.27072554	429.932231	0.00973740
CXCR4	0	0.0395220	83.15	0.01082630	5.783548	0.00604377
FGF10	46.26	0.0000379	3.24	0.13779988	0.539981	0.06876522
FGF2	0.01	0.1126751	0.55	0.24385439	285.286843	0.00003024
FGF4	0	0.0063775	58.79	0.03652477	202.990252	0.00002434
FGFR1	0.03	0.3882649	127.84	0.45964315	128.006040	0.00010341
FOXA2	0.15	0.5090598	29.64	0.23953575	7.809426	0.00028328
FOXO1	0.02	0.1313078	0.47	0.23275957	191.387046	0.00019015
FUT4	1.93	0.3780159	24.50	0.01372676	104.598774	0.00004484
G6PC2	0.95	0.4117616	7.44	0.64179768	0.000004	0.00000258
GATA4	0.04	0.3616146	0.93	0.09015999	13884.367288	0.00000376
GCG	0.95	0.4117616	264.99	0.00000048	0.001537	0.00000532
GCK	0.95	0.4117616	20026.35	0.00001441	0.003501	0.00000718
GDF3	0	0.0012986	54.97	0.03844061	4939.051258	0.00002459
GLP1R	0.15	0.0013928	83.93	0.00007784	0.061226	0.00027134
GP2	60.92	0.0000209	30.19	0.00039952	0.000027	0.00000094
HLA-A	0.05	0.2195596	5.11	0.14959703	27.309092	0.00044044
HLA-B	0.21	0.8837510	22.84	0.20364161	158.386831	0.00021865
HNF1A	1.84	0.1646144	46.80	0.01782568	4.622734	0.00097203
HNF4A	0.06	0.4901506	86.48	0.00345327	0.591228	0.08502429
HPRT1	0.14	0.6568105	12.14	0.41335437	138.264178	0.00013839
IAPP	1.66	0.2531302	20.73	0.28552468	0.000145	3.67043E-06
IL10	0.02	0.0879112	0.49	0.00341982	3346.694765	0.00003092
IL6	0.02	0.0055202	3644.85	0.77069915	0.023367	0.06899274
INS	0.95	0.4117616	1.48	0.00000088	0.000054	0.00000118
IRX2	0.16	0.0142429	12243.76	0.57405362	0.016547	0.00039548
ISL1	40.2	0.0001859	1.50	0.09082697	0.029760	4.63157E-05
ITGA1	0.2	0.6250826	1.51	0.00482820	0.041779	0.00007522
ITGA6	0.04	0.2732315	0.15	0.24427153	1134.960453	0.00003328
KCNK1	0.15	0.0032820	41.36	0.02797627	0.021502	0.00108976
KCNK3	0.15	0.0798124	0.20	0.06141983	0.019642	0.00200924
KIT	0	0.0973831	15.57	0.21290997	40355.462874	0.00000186
KLF4	2.35	0.1223965	117.62	0.52206572	0.009461	0.00004866
KRT19	4.34	0.0010556	1.27	0.44638826	0.356720	0.17490372
LIN28A	0.18	0.4337722	0.47	0.77440390	1666.362799	0.00001229
MAFA	0.7	0.0883283	2.81	0.63949181	0.002538	0.00022981
MAFB	1.86	0.1946566	0.58	0.01089404	0.088263	0.00166935
MYC	0.01	0.0937403	10.65	0.05964536	338.645341	0.00007240
NANOG	0.06	0.0033110	274.85	0.07044910	175.747126	0.00012404
NEUROD1	190.5	0.0017626	3.30	0.04850251	0.010160	0.00002286
NEUROG3	46.11	0.0001611	4.47	0.11356874	267.717617	0.00004615
NKX2.2	8.46	0.1246582	1.95	0.01142426	0.004593	0.00002957
NKX6.1	243.7	0.0000034	25.27	0.00799324	2.188525	0.00799324

ONECUT1	12.33	0.0002026	2.19	0.00715320	3.133384	0.00744050
PAX4	0.15	0.0105294	2.21	0.00191226	373.540520	0.00001289
PAX6	6.39	0.0168902	20.65	0.23088532	225.066829	0.00006650
PCSK1	0.2	0.0190803	99.10	0.00504205	0.017176	0.00343932
PCSK2	2.61	0.0046533	39.86	0.00013338	0.182391	0.00331336
PDX1	182.58	0.0000089	27.65	0.38060238	11.230868	0.00048035
PODXL	0	0.0642248	30.83	0.35848189	249.198485	0.00002199
PODXL2	0.05	0.2737629	0.77	0.16112888	97.541437	0.00002762
POU5F1	0.62	0.3149670	29.68	0.003216258	14.782665	0.019437177
PPY	2.99	0.2940963	38.91	0.55781365	0.000003	0.00016018
SEV	0.02	0.0879112	0.01	0.03652477	202.990252	0.00002434
SEVKOS	0.02	0.0879112	0.18	0.04123040	142.544310	0.00005116
SLC16A1	0.29	0.0220614	127.84	0.03116010	144.974659	0.00008038
SLC18A1	40.69	0.0000450	111.84	0.00002885	1300.384553	8.82245E-06
SLC2A4	0.41	0.0641598	0.32	0.00350801	3.679129	0.01674965
SLC30A8	41.21	0.0012549	127.60	0.24136420	0.011311	1.53806E-05
SOX17	53.54	0.0001686	0.15	0.15574060	0.305591	0.093022771
SOX2	0.23	0.5557901	1.34	0.31815672	9736.557076	0.00001759
SOX9	1.25	0.4739302	10.77	0.06681842	0.008344	0.000108584
SST	42.14	0.0009105	8.94	0.31878228	0.001308	6.27853E-06
SYP	0.05	0.0002809	2.92	0.00000773	0.398636	0.009261743
TBX2	0.39	0.0490879	0.42	0.00194258	69.569681	0.00002708
TERT	0.16	0.0672913	224.67	0.89964491	6.752616	0.00107510
TPBG	0.1	0.0025543	11.41	0.01166502	109.214662	0.00010401
TSPAN1	1388.25	0.0001684	1.68	0.00201138	11.766749	0.000231386
UCN3	145.37	0.0000102	4.90	0.34338343	0.328699	0.00340379
UTF1	0.02	0.0879112	51.79	0.00386359	6878.915703	0.00000484
ZFP42	0.02	0.0879112	1.13	0.03149547	405.980504	0.00005139

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Chapter 4: Immune Considerations for Pancreatic and Stem Cell-Derived Islet Transplantation

- Chapter 4 subsection 1: The Potential of Cellular Transplantation to Harness Autoimmunity and Reverse Clinical Diabetes
- Chapter 4 subsection 2: Evaluating the Potential for ABO-Incompatible Islet Transplantation: Expression of ABH Antigens on Human Pancreata, Isolated Islets, and Embryonic Stem Cell-Derived Islets

Chapter Summary:

Chapter four focuses on the immunologic considerations for stem cell-derived islet transplantation. This includes a review (chapter 4.1) where the immunologic considerations and approaches to combat immune destruction is discussed in the context of stem cell-derived islets. Subsequently subsection 4.2 presents a preclinical study evaluating the ABH antigen expression of human pancreata, isolated islets, and embryonic stem cell-derived pancreatic progenitors before and after *in vivo* maturation. The study is of particular importance considering recent developments for the Vertex VX-880 clinical trial, which is currently limited to patients with blood type A or AB. This inclusion criteria is included due to the blood type (A) of the starting stem cell product. However, considering the results of chapter 4.2, considering ABH blood types may not be required for stem cell-derived islet transplantation.

4.1 Chapter 4 subsection 1 – The Potential of Cellular Transplantation to Harness Autoimmunity and Reverse Clinical Diabetes

CHAPTER

18

The potential of cellular transplantation to harness autoimmunity and reverse clinical diabetes

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Abstract

Diabetes is a long-standing disease with increasing prevalence that contributes to significant health care costs and patient morbidity. Over the last 100 years, since the discovery of insulin by Banting and Best, the primary treatment has remained subcutaneous insulin delivery. Although novel insulin formulations, glycemic measurement techniques, and delivery methods have been developed, complications remain common and a cure is desperately required. Understanding the pathophysiology and cellular mechanisms of autoimmunity driving diabetes is key to engineering a cure for this important disease.

Islet cell transplantation has evolved over the last 20 years as an attempt to disease cure and has now reached nearly 50% success rates due to improved understanding and management of the alloimmune and autoimmune response following implantation. However, limited cadaveric supply and ongoing immune barriers has led to the development of novel islet cell transplant via inducible pluripotent stem cells (iPSC). This novel therapy offers unlimited supply and multiple unique solutions for alloimmune control. Autologous iPSC-based islet cell transplant may resolve alloimmune concerns but requires expensive and time-consuming personalized medicine. Meanwhile, allogeneic iPSC-based islet cell transplant may enable HLA-matched transplant with less cost and time requirements but has persistent autoimmune and alloimmune barriers. Genetic modifications with CRISPR/Cas9 techniques could theoretically provide immune silenced or immune protected iPSCs for islet cell transplant but this requires further trials to strengthen the evidence. Parallel studies continue to evaluate the utility of diabetes reversal with immune reset. Clinical trials are ongoing evaluating the efficacy of resetting the immune system at the onset of diabetes to eliminate autoimmunity and prolong insulin free periods for patients. Combining these techniques with allogeneic or HLA-matched iPSC-based islet cell transplant provides a bright future for diabetes treatment and cure.

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4.1.1 **Abstract**

Diabetes is a long-standing disease with increasing prevalence that contributes to significant health care costs and patient morbidity. Over the last one-hundred years, since the discovery of insulin by Banting and Best, the primary treatment has remained subcutaneous insulin delivery. Although novel insulin formulations, glycemic measurement techniques, and delivery methods have been developed, complications remain common and a cure is desperately required. Understanding the pathophysiology and cellular mechanisms of autoimmunity driving diabetes is key to engineering a cure for this important disease.

Islet cell transplantation has evolved over the last twenty years as an attempt to disease cure and has now reached nearly 50% success rates due to improved understanding and management of the allo and auto immune response following implantation. However, limited cadaveric supply and ongoing immune barriers has led to the development of novel islet cell transplant via Induced pluripotent stem cells (iPSC). This novel therapy offers unlimited supply and multiple unique solutions for alloimmune control. Autologous iPSC-based islet cell transplant may resolve alloimmune concerns but requires expensive and time-consuming personalized medicine. Meanwhile, allogeneic iPSC-based islet cell transplant may enable HLA matched transplant with less cost and time requirements but has persistent autoimmune and alloimmune barriers. Genetic modifications with CRISPR/Cas9 techniques could theoretically provide immune silenced or immune protected iPSCs for islet cell transplant but this requires further trials to strengthen the evidence. Parallel studies continue to evaluate the utility of diabetes reversal with immune reset. Clinical trials are ongoing evaluating the efficacy of

resetting the immune system at the onset of diabetes to eliminate autoimmunity and prolong insulin free periods for patients. Combining these techniques with allogeneic or HLA-matched iPSC-based islet cell transplant provides a bright future for diabetes treatment and cure.

This chapter discusses drawbacks of historic subcutaneous insulin treatment methods, novel continuous glucose monitoring, continuous subcutaneous insulin infusion (CSII i.e., insulin pump), and closed-loop wearable insulin delivery (i.e., artificial pancreas) devices. We review our current understanding of the physiologic function and microstructure of islet cells, pathophysiology of diabetes, and current islet cell transplant methods including the autoimmune approach. We also discuss future directions for advancement including iPSC-based islet cell transplant (isogeneic versus allogeneic), genetically modified iPSC therapies, and immune reset trials.

4.1.2 **Introduction**

Descriptions of diabetes mellitus (DM) are ubiquitous across the ancient literature, with the first accurate description of the disease linked back to second century AD [1]. In 1889, its relation to pancreatic secretions was finally recognized when Oskar Minkowski and Joseph von Mering completed a canine pancreatectomy that induced fatal DM. Despite this understanding of the pancreas's importance, type 1 diabetes mellitus (T1D) remained a devastating, rapidly fatal autoimmune disease for another 30 years until 1922. It was only then that Banting, Best, and MacLeod discovered insulin. Collip subsequently improved their insulin extraction techniques using alcohol to solubilize the crystalline molecule, which allowed injectable insulin to reverse hyperglycemia and made T1D survivable – before then starvation diets were the only poor modality of treatment [1]. Approximately ten years after the discovery of insulin, it became evident that this treatment modality was far from a cure, as chronic T1D complications began to emerge. Titrating the precise dosing of insulin to account for physiologic fluctuations in supply and demand for glucose has remained a huge challenge, and inadvertent overdosing of insulin frequently leads to disruptive and occasional life-threatening neuroglycopenia. Despite 100 years of investigation since insulin discovery, and significantly improved disease understanding, insulin remains the primary treatment for DM.

Even with novel insulin formulations, the advent of continuous glucose monitoring (CGM), continuous subcutaneous insulin infusion (CSII i.e., insulin pump), and now closed-loop 'wearable' insulin delivery (i.e., artificial pancreas) devices, subcutaneous insulin delivery, and peripheral glucose monitoring remains an imprecise and burdensome treatment for many. Insulin

allows adequate metabolism of glucose to enable cellular metabolism for survival, but hardly offers physiologic glycemic control that is typically offered by dynamic hormonal release from the pancreas – insulin is released physiologically by islet β-cells in a pulsatile fashion with peaks every 15-20 minutes, and dynamic counter-regulatory control with glucagon released by α-cells provides robust homeostasis in a normal individual. In the United States, a large-scale registry with >20,000 participants demonstrated that only 21% of adults and 17% of children achieve the recommended HbA1c of <7% and 7.5%, respectively [2,3]. Even with novel technology, HbA1c is only marginally improved from 9.5% in the 1980s to 9.0% currently in 13-17-year-olds [2,3]. Life threatening hypoglycemic events still occur with an incidence of 320 episodes per 100-patient years in those living with T1D for more than 15 years [4]; a risk that increases with intensive insulin therapy [4]. Of 11,061 registry respondents, 6% reported hypoglycemic seizure or loss of consciousness within the previous three months, which is even more common for those with hypoglycemic unawareness or older age [2,5]. Hypoglycemia occurs in 31-41% of diabetic patients [6-8], often at night due to the four-fold variability of overnight insulin requirements [9]. To date, exogenous subcutaneous insulin delivery, even when provided by the most ideal closed loop systems, fails to recreate the dynamic glycemic control provided by islet cell multi-hormonal response. As practitioners and health researchers, it remains imperative that we further improve our understanding of DM management and reach for a potential cure. While technological advances continue to improve subcutaneous insulin delivery, the most likely route for long-term effective cure will involve a more effective understanding of the causes and prevention of this disease, and for those with established DM, a means to permanently reverse the diabetic state using immunogenic and cell-based therapies. To better understand diabetes and begin to develop

a cure we must understand the structure and function of the insulin producing islet cells within the pancreas, and describe their pathologic autoimmune destruction or physiologic insulin resistance that occurs in those with T1D and type 2 diabetes mellitus (T2D).

Islets of Langerhans are cell groupings that exist uniformly throughout the pancreas and represent a small fraction of the pancreatic tissue, making up only 1-4%, 2g, or 2ml of its total volume [10]. Variability is typical, but on average, islets are composed of ~60% β-cells, ~30% α-cells, <10% δ-cells, <5% γ and ε cells, which produce insulin, glucagon, somatostatin, pancreatic polypeptide, and ghrelin, respectively [10]. While much focus is directed towards insulin producing β-cells, the other islet cell components play an important, and increasingly better understood integrated role for glycemic control. Overall, euglycemia is accomplished with both autonomic nervous and hormonal systems (Figure 4.1.1). While fasting, autonomic control predominates — glucose-sensing cells in the hepatoportal vein and hypothalamus mediate a sympathetic-parasympathetic balance [11]. Hyperglycemia induced sympathetic activation leads to glucagon release from α-cells, while parasympathetic activity induces insulin release [11]. This equilibrium enables appropriate glucose availability for anabolic processes and cellular functions.

Visual introduction of food initiates the cephalic response and enables insulin release, even prior to any intravascular glycemic changes [11,12]. Activation by sight, mastication, or gastric distention initiates parasympathetic release of acetylcholine to stimulate β-cell muscarinic receptors (m3AchR), producing phospholipid-derived messengers that initiate protein kinase C (PKC) directed calcium influx and ensuing insulin release [11,12]. Once food reaches the gastrointestinal system, a biphasic pulsatile insulin release occurs lasting approximately 60 minutes in response to increased blood glucose [3,13]. Direct islet cell response to glucose occurs

with diffusion intracellularly into β-cells and ATP generation. ATP-dependent potassium channels (KATP) open to depolarize the cell and open of voltage-dependent L-type calcium channels. The calcium influx promotes SNARE protein mediated exocytosis of insulin into portal circulation (Figure 4.1.1) [3,13]. These stimuli oscillate over 3-6 minute cycles to avoid insulin receptor downregulation [3,13].

The direct effect of glucose on β-cells to produce insulin release is well known, but nearly half of the postprandial insulin release is supported by parasympathetic inputs, free fatty acids (FFA), glucagon-like peptide 1 (GLP-1), glucose-dependent insulinotropic peptide (GIP), and somatostatin (Figure 1) [3,13-16]. FFAs stimulate the GP40 G-protein-coupled receptor (GPCR), increasing intracellular 3′,5′-cyclic adenosine monophosphate (cAMP) to induce insulin release in both protein kinase A (PKA) and non-PKA-dependent pathways [14,15]. GLP-1 and GIP are also secreted in response to elevated blood glucose concentrations from pancreatic α-cells and duodenal L-cells, respectively, and function through β-cell GPCR pathways [13,14,16]. Somatostatin from δ-cells and pancreatic polypeptide from γ-cell further modify glucose homeostasis [17,18]. The mechanisms of action are poorly understood but ablation of δ-cells impairs islet cell function [17], and infusion of pancreatic polypeptide alongside insulin reduces insulin requirements [19] – further analysis of these mechanisms and cellular pathways may assist with improving glycemic control, but also highlight the complex interplay of cells required to maintain the narrow range of blood glucose. Understanding the complex cellular and autonomic interplay required for glycemic control helps appreciate the gap that exists between physiologic function and treatment with subcutaneous insulin even with the most complex single or dual hormone replacement systems.

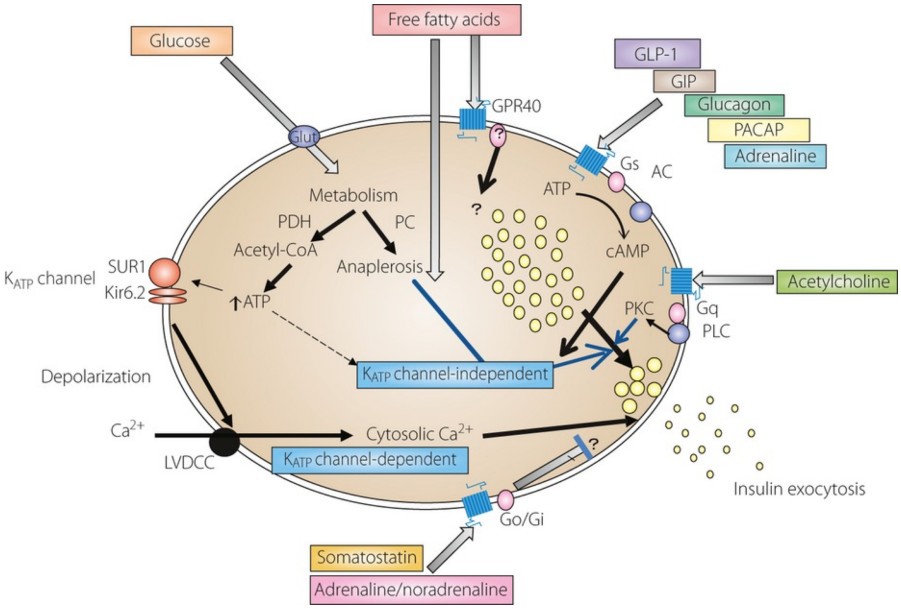

Figure 4.1.1 Mechanisms of β-cell insulin release and glycemic control.
Image reproduced with kind permission of the journal. Credit to: KOMATSU, M., TAKEI, M., ISHII, H. & SATO, Y. Glucose-stimulated insulin secretion: A newer perspective. Journal of Diabetes Investigation, 4, 516, 2013.

T1D is a unique autoimmune disease that occurs due to destruction of insulin producing pancreatic β-cells. As opposed to most other autoimmune conditions, it occurs equally in males and females [20]. Disease onset and the underlying etiology is uncertain but involves an interplay between polygenic genetic makeup and environmental cues [21]. Monozygotic twins of T1D patients have a 41.5% risk of developing the disease. Specific major histocompatibility complex (MHC) type II proteins are highly associated with T1D; DR4DQ8 homozygosity has a disease odds ratio (OR) of 9.0 [22]. DR3/DR4 and specific DQβ/DQα chains predispose patients to T1D, while <1% of T1D patients express DR2 [21]. In addition, 60 genetic loci have been associated with the disease, with the short arm of chromosome 6 playing the largest role [20,23]. However, there also appears to be an unknown environmental component, with over 350-fold variation of

incidence in populations worldwide [20,24]. Most newly diagnosed patients present with polydipsia, polyuria, weight loss, nocturia, diabetic ketoacidosis, or abdominal pain.

In patients with T1D, characteristic destruction of pancreatic β-cells occurs due to infiltrative mononuclear inflammatory cells, including macrophages, CD4+, and CD8 T cells [25]. T1D is primarily a T cell disease, with β-cell antigen presentation to CD4+ T cells by macrophages and dendritic cells [20,26,27]. It is currently unknown what triggers this antigen presentation and autoimmune activation; however, T cells activate due to failed maturation, which may be linked to poor central proinsulin 2 expression within the thymus [28,29]. Activated CD4+ cells, directed to differentiate by high concentration interleukin 2 (IL-2), subsequently trigger CD8+ direct cell mediated apoptosis, and inflammatory mediator driven insulitis within the pancreas (Figure 4.1.2) [30]. T cell inhibition with cyclosporine slows islet cell destruction demonstrating the importance of T, rather than B cells – further, agammaglobulinemic patients without B cells can still develop DM [31,32]. T cell transfer within isogeneic bone marrow transplant from a diabetic patient has also demonstrated transmission of T1D to a previously non-diabetic patient, further supporting the importance of T cells [33]. Notably, low dose IL-2 exposure leads to regulatory T cells (Treg) that inhibit this T cell maturation process, which may offer a therapeutic solution as will be further discussed later in this chapter. β-cell destruction predates clinical DM often by many years. Secondary to insulitis and β-cell destruction, patients acquire antibodies to insulin, islet cells, the cation efflux pump ZnT8, isoforms of glutamic acid decarboxylase 65 or 67 (GAD65 or GAD67), or the IA-2 secretory protein [20]. The occurrence of multiple antibodies is likely due to epitope spreading, where one primary antigen leads to cellular destruction and release, or various other reactive antigen/antibody pairs. Notably, the

occurrence of antibodies during this “pre-clinical” phase may allow for patient identification and early immunomodulation to prevent T1D progression – another approach to DM cure that we discuss below. However, up to 10% of patients with T1D do not display antibodies, and therefore, diagnosis is based on paucity of insulin rather than antibody presence [20].

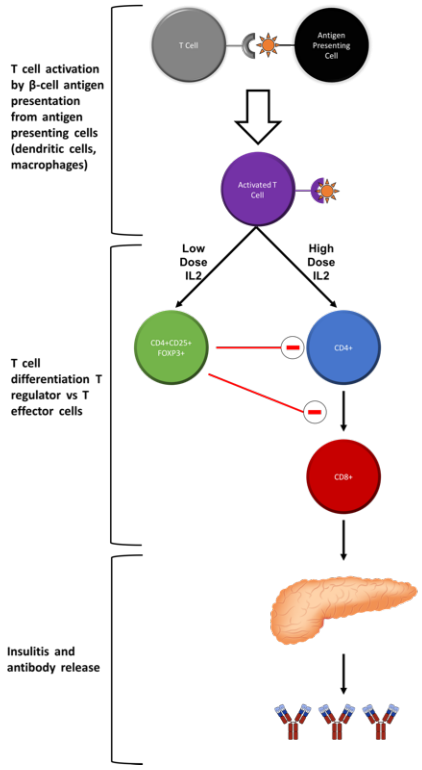

Figure 4.1.2 T cell maturation process leading to insulitis and islet cell destruction with release of antibodies.

Insulin replacement provides a gross level solution, but as previously discussed, fails to provide precision multi-hormonal, dynamic, physiologic glycemic control. Even with novel insulin preparations and delivery methods, microvascular complications including neuropathy, nephropathy, and retinopathy, and macrovascular complications such as stroke, cardiovascular disease, and peripheral vascular disease contribute to significant disease morbidity and shortened

lifespan [34]. On the low end, 6.6% of patients with DM experience stroke, while 27.8% have nephropathy (Figure 4.1.3) [34]. With over 400 million patients diagnosed with all forms of DM worldwide, and a concerning rising incidence, the health and economic burden of DM is rapidly becoming non-sustainable [35]. It is estimated that $1.3 trillion (US) are spent in managing diabetes and its complications each year by our collective global healthcare systems. Improved treatment modalities, or ideally a cure is desperately required.

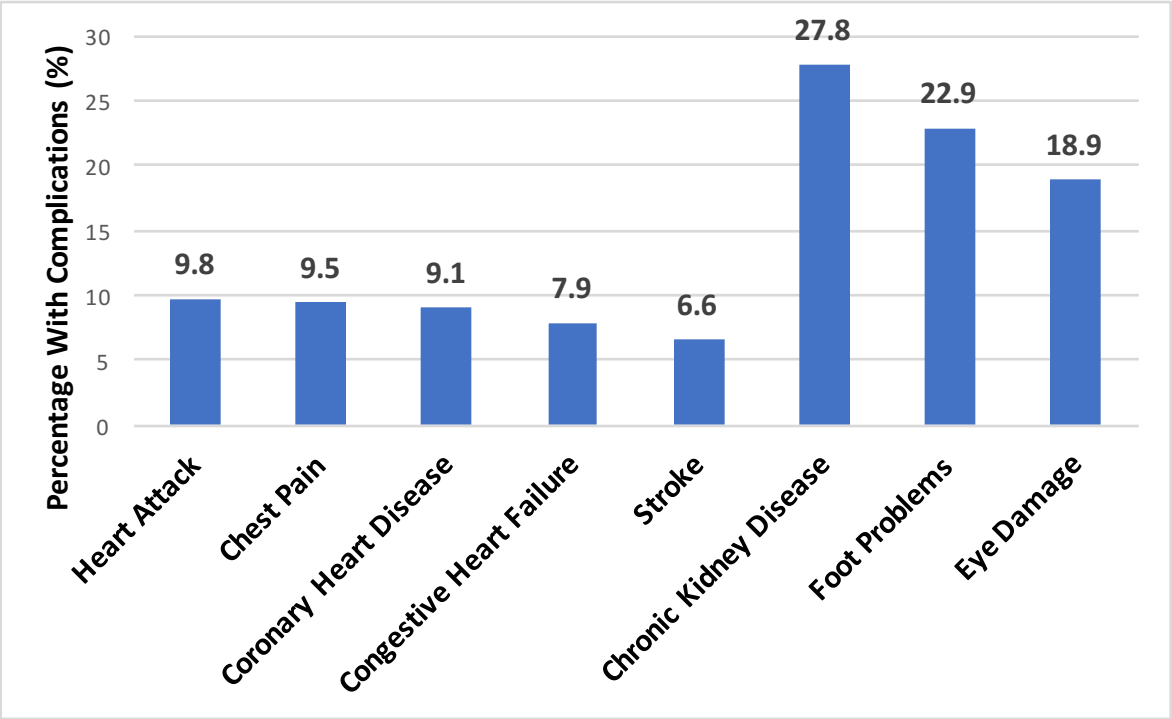

Figure 4.1.3 Prevalence of diabetes related complications amongst patients in the United Kingdom.
Image reproduced with kind permission of the journal: DESHPANDE, A. D., HARRIS-HAYES, M. & SCHOOTMAN, M. Epidemiology of diabetes and diabetes-related complications. Physical therapy, 88, 1259, 2008.

Treatment with insulin has long been the gold standard for DM. The concept of islet cell transplantation (ITx) as a potentially curative treatment for DM was established by the pathologist Paul Lacy from St. Louis, US in 1972. His techniques proved that DM could be cured in rodents by extraction and implantation of islets, and that better function was achieved when these were injected into the portal vein rather than implanted beneath the skin. Over the next 30 years, almost 250 attempts were carried out to transplant allogeneic human islets in patients, but were often fraught with difficulty due to inadequate and islet-toxic immune suppression and ineffective techniques to isolate and purify large numbers of viable islets from the human organ donor pancreas. Camillo Ricordi developed a semi-automated method for high-yield human islet isolation in 1989, and modifications of this technique remain the mainstay today. London and colleagues at the University of Leicester in the UK developed efficient and less toxic means to purify human islets using a continuous gradient centrifuge separator and Ficoll gradients. Despite these advances, fewer than 8% of patients with T1D undergoing islet transplantation were able to discontinue insulin therapy for longer than a year. James Shapiro et al. reported the results of seven patients who achieved 100% insulin independence after ITx with one year follow up [36]. This protocol was unique in that fresh islets were prepared and transplanted immediately after processing, and less toxic, steroid-free but more potent induction and maintenance immunosuppression was given to control both auto- and alloimmunity. The Edmonton Protocol advances and subsequent modifications have highlighted the potential of cell replacement therapy as a potential curative therapeutic approach, and this work has further spurred on progress in the generation of stem cell derived islet therapies for a future limitless supply. Since then, additional immunomodulatory methods for reversing or prolonging insulin

independence in newly diagnosed T1D, including regulatory T cell and immune reset therapies have also evolved. The field is now closer than ever to a tangible DM cure, yet significant work remains and is required to control this prevalent disease. This chapter discusses current approaches to ITx and barriers that persist. It also evaluates and reviews exciting novel approaches to ITx. Finally, it evaluates novel approaches to DM cure and ITx immunosuppression, including immune reset and Treg trials, which are at the forefront of DM research.

4.1.3 **Islet Cell Transplant**

Remarkably, it took just 5 years to move Paul Lacy's experiments from the benchtop to the clinic. The first islet autotransplants were carried out by John Najarian and David Sutherland in 1977. These autotransplants are unique as the patients' own islets are extracted and infused back in to the hepatic portal vein, and are thus not rejected by the immune system. The first patient remained insulin free with that approach for 6 years. The first successful islet allotransplant into a patient with T1D was reported by Scharp and Lacy in 1989 [37]. Over the next 10 years over 237 islet allotransplants were carried out in patients with T1D, but fewer than 8% remained insulin free for longer than one year. Camillo Ricordi's semi-automated method and chamber provided high yield islets (in a process that is still used routinely today). Variability in collagenase purity and activity hampered progress and made the digestion process at times inconsistent. Development of purified recombinant enzymatic blends has subsequently improved that process considerably, and made the final islet preparation much more consistent and of high quality and viability. The first consistent series of patient to achieve insulin independence state

was at the University of Pittsburgh, where Camillo Ricordi and Thomas Starzl re-implanted islets in patients with surgical induced diabetes at the time of abdominal exenteration and multi-visceral transplantation [38]. Of note, those unusual patients did not have preexisting autoimmune T1D, and were immunosuppressed with a new potent antirejection drug that became available at that time called FK506 (tacrolimus).

Our preliminary experience with the first seven patients treated with the Edmonton Protocol was striking as all seven (100%) achieved and maintained insulin independence one year after the transplantation. Keys to the success of that protocol were the combination of two potent antirejection drugs (low-dose tacrolimus with a new mTOR inhibitor sirolimus, and a non-depleting anti-IL2-receptor antibody (daclizumab) which allowed transplants to be carried out without the need for diabetogenic glucocorticoids. Prior ITx techniques required antibody induction with an anti-lymphocyte globulin combined with cyclosporine, azathioprine, and glucocorticoids, and only achieved approximately 8% insulin independence at one-year [39]. However, significant advances have been introduced over the last 20 years to combat ischemic, autoimmune, and alloimmune engraftment rejection. This series moved the needle from a previous insulin independence rate of 8% and for the first time provided proof of concept that patients with autoimmune T1D could be potentially cured with a cell-based therapy [36,40]. Long-term insulin independence still remains elusive in autoimmune T1D, but ITx protocol improvements now have five-year insulin free rates >50% — matching results from vascularized whole pancreas transplantation but with much less relative morbidity and mortality [4,41,42]. HbA1c improved from 8.2% using CSII to 6.4% with ITx demonstrating marked glycemic control benefits [43]. The Vancouver islet cell research group achieved similar results with HbA1c of 6.6%

after ITx compared to 7.5% with intensive insulin therapy [44,45]. Within-subject, paired studies comparing insulin injection, CSII, and ITx demonstrated the greatest glycemic control, least glycemic variability, and fewest hypoglycemic events with ITx; hypoglycemic reduction and glucose stability persisted even when insulin independence was not achieved [43]. A 2016 multicenter phase III clinical trial strengthened these results with 87.5% of patients having a HbA1c <7.0% one year after ITx, and 71% achieving goal HbAlc levels after two years with a median HbA1c of 5.6% [46]. Other physiologic parameters also improved following ITx, such as retinal blood flow and markers of polyneuropathy (Venturini et al., 2006, Del Carro et al., 2007). Accordingly, diabetic complications also improve following ITx compared to subcutaneous insulin delivery with lower rates of retinopathy, nephropathy, and a trend towards lower risk of neuropathy [44,45]. ITx is the first therapeutic approach that offers dynamic glycemic control that matches physiologic function of islet cells rather than gross insulin replenishment.

ITx success has occurred due to continuous innovative approaches to multi donor cell sources, islet cell isolation techniques [42], good manufacturing practice (GMP) protocols [42,47], and novel targeted immunosuppression and inflammatory mediation therapies. Islet isolation, culture, preparation, and transplant is now well described and standardized by the clinical islet transplantation consortium allowing >50% utilization of donated organs [42,48]. The capability and understanding of islet cell culture has further enabled islet cell-source optimization and selection prior to transplantation to further improve clinical outcomes [49-52]. However, improvement of immunosuppression and anti-inflammatory protocols for induction and management of transplanted islets is likely the greatest contributor to insulin independence over the last 20 years, especially the use of a potent anti-TNF-α chimeric antibody etanercept. Agents to resist immune

reaction, inflammation, apoptosis, and the instant blood-mediated inflammatory reaction (IBMIR) have advanced significantly, while gaps still exist, and more understandings and inventive approaches are required to enable widespread use of ITx.

4.1.4 **Current Immunoreactivity Approach**

Immune acceptance following ITx is difficult to achieve, as it involves controlling both complex ischemic, autoimmune, and alloimmune reactions that limit islet engraftment and survival (Figure 4.1.4). Islet cell engraftment first faces ischemic inflammatory damage prior to neovascularization during the 10 to 24 days leading to apoptosis, thrombosis, ischemia, inflammation, and IBMIR [42,53-56]. This is coupled with recurrent autoimmune T cell directed insulitis as shown in Figure 4.1.1, and alloimmune acute and chronic rejection. Current immunosuppressant techniques focus on limiting the recipient's immune system to cope with the multitude of insults and improve long-term islet cell engraftment.

Figure 4.1.4 Barriers to islet engraftment following islet cell transplant.
Image reproduced with kind permission of the journal: SHAPIRO, A. M. J. State of the Art of Clinical Islet Transplantation and Novel Protocols of Immunosuppression. Current Diabetes Reports, 11, 345, 2011.

To prevent recurrent autoimmune graft destruction, interleukin-2 (IL-2) receptor blockade combined with monoclonal antibodies inhibit IL2 driven inflammatory cytokine release and T-cell activation [36,57]. IL2 plays a major role for effector T cell differentiation of antigen receptor activated CD4+ T cells [58-63]. High dose IL-2 supports CD4+ differentiation into destructive T helper (Th)1 and Th2 cells, and inhibits protective Th17 and T follicular helper (Tfh) cells through various cytokine pathways [58,60,64]. More recently, using the anti-CD52 monoclonal antibody alemtuzumab (30mg given s.c. or i.v.), or alternatively rabbit antithymocyte globulin (up to 6mg/kg IV given over 3 days) as opposed to less potent

daclizumab or basiliximab alongside etanercept have demonstrated improved graft survival with inhibition of IL-2 mediated T cell activation [42,46,65].

Other approaches have also been introduced to inhibit thrombosis, ischemia, inflammation, and IBMIR. These present the greatest barrier to engraftment over the first 10 to 24 days prior to islet cell neo-vascularization [55]. Thrombosis and hypoxia initiate a significant reaction with larger islet cells demonstrating significant core hypoxia on microscopy that leads to a series of events producing inflammation and apoptosis [56,66]. Reactive oxygen species are produced and result in islet cells releasing inflammatory TNF-α, interleukin-1β, and interferon gamma, alongside procoagulant monocyte chemoattractant protein-1, tissue factor (TF), and macrophage inflammatory protein 1α. Approaches to manage these inflammatory reactions have included dosing patients' manganese superoxide dismutase to decrease reactive oxygen species, which has demonstrated enhanced *in vitro* islet cell viability with augments *in vivo* murine marginal islet mass engraftment [67,68]. Downstream pathway intervention with interleukin-1 antagonist anakinra and TNF-α inhibitor etanercept also demonstrates improved insulin independence in patients with ITx [41,42,65,69-71]. Additionally, the improved IBMIR understanding with pro-coagulant mediators has led to post-ITx heparin infusion to limit TF related IBMIR, while insulin infusion allows islet rest and reduced inflammation to improve engraftment [72,73]. Other agents, including liraglutide or pan-caspase inhibitors, are also being investigated to inhibit the inflammatory cascade that stems from ischemia and may further improve ITx engraftment success [4,41,74-77]. Despite these advances, the combined effect of autoimmune and alloimmune reactivity against allogeneic islet cells remains a fierce competitor for engraftment. Additional

innovations are required and continue to be investigated, as immunosuppression requirements is a significant barrier, amongst others, to widespread ITx.

4.1.5 **Barriers to Islet Cell Transplant**

Although it represents a potential cure for longstanding DM, ITx faces numerous ongoing barriers. Access to ITx is not available to most patients due to poor procedure funding, donor shortage, and immunosuppression requirements. In 2012, only eight regions funded ITx under non-research, clinical streams, including Canada, Australia, the United Kingdom, France, Switzerland, Norway, Sweden, and other parts of Europe [4,78]. Even when funded, cadaveric islet cell transplant is the only well-established source of islet cells; each procedure often requires 2-4 pancreata to isolate >5,000 islet equivalents (IEQ) per kg and ideally >7000 IEQ/kg for optimal likelihood of insulin independence [42]. In brief, the donor pool supply does not remotely meet recipient demand. Even when recipients can access ITx programs, they are faced with strict criteria for consideration due to immunosuppression requirements and its associated complications. For islet alone transplant (i.e., without kidney), patients must have recurrent severe hypoglycemic episodes with hypoglycemic unawareness, glycemic lability not managed with intensive insulin, pumps, and/or continuous glucose monitoring therapies [4]. They should also have had T1D for >5 years, be over the age of 18, have normal renal function, and have BMI (<30 kg/m2) and/or weight <90 kg, and/or daily insulin requirement <1.0 U/kg.

The long-term increased risks of potentially life-threatening opportunistic infection or various forms of malignancy including skin cancers and post-transplant lymphoproliferative disorder (PTLD) make the risks of ITx higher than the background risks from T1D alone in

patients with very stable glycemic control. Thus if immunosuppression could be reduced or eliminated this would dramatically shift the risk:benefit ratio for future patients considering cell replacement therapy. Risk of malignancy, primarily squamous and basal cell carcinoma of the skin, is approximately 2% and combined ITx and immunosuppression related mortality is 0.19% [42,79]. Direct toxicity of immunosuppressant medications, namely calcineurin-inhibitors, are also important as they are diabetogenic and nephrotoxic [41,80,81]. DM patients would ideally receive ITx as early in their lives at disease onset prior to diabetic complications; unfortunately, earlier ITx leads to longer immunosuppression exposure and risks. The acute peri-operative, early infectious and toxic immunosuppression effects must also be considered for 50% of ITx recipients who fail to achieve insulin independence. Although many ITx benefits persist regardless of insulin independence, dynamic risk benefit analysis must be completed. With improved ITx results and marked recent improvement in wearable, closed loop insulin (and glucagon) pump and integrated continuous glucose monitoring systems, future prospective randomized control trials would greatly assist this undertaking. Meanwhile, significant advances continue to occur with novel islet cell sources and immunosuppression approaches, which may eventually render this analysis mute.

4.1.6 **Stem Cells for Islet Cell Transplant**

Recent and ongoing advances in stem cell therapy may resolve many of the remaining barriers that currently face ITx. If a potentially limitless islet supply could be generated from stem cells, it would eliminate issues of supply and demand currently posed by the organ donor shortage, and could enable more widespread access to ITx. Remarkably, stem cells may also

offer a potential solution to immunosuppression requirements via autologous transplantation of HLA-identical cell products, genetic engineering or HLA silencing – all of which could open new doors for this curative approach. Enthusiasm for these techniques was initially limited due to the ethical and access barriers associated with human embryonic stem cells (ESC) [82]. However, discovery of the reprogramming factors by Yamanaka et al. and Thomson et al. to induce stem cells from various tissues has revitalized the concept [83]. Creating functional islet cells from ESCs and Induced pluripotent stem cells (iPSC) is currently a reality. Multiple groups have demonstrated a seven-step stem cell differentiation protocol to generate mature β-cells that are glucose-responsive, insulin-positive, and co-express PDX1, NKX6.1, MAFA, C-peptide, and prohormone processing enzymes *in vivo* (**Figure 5**) [84-88]. These techniques have been optimized in both 2D and 3D media to produce islet cells capable of consistently reversing diabetes in murine models following ITx [85,87,88]. It appears likely that generating a large volume of islet cells is within grasp . Additional excitement also arises from the potential of stem cell-based ITx offering various novel approaches to eliminating immunosuppression requirements.

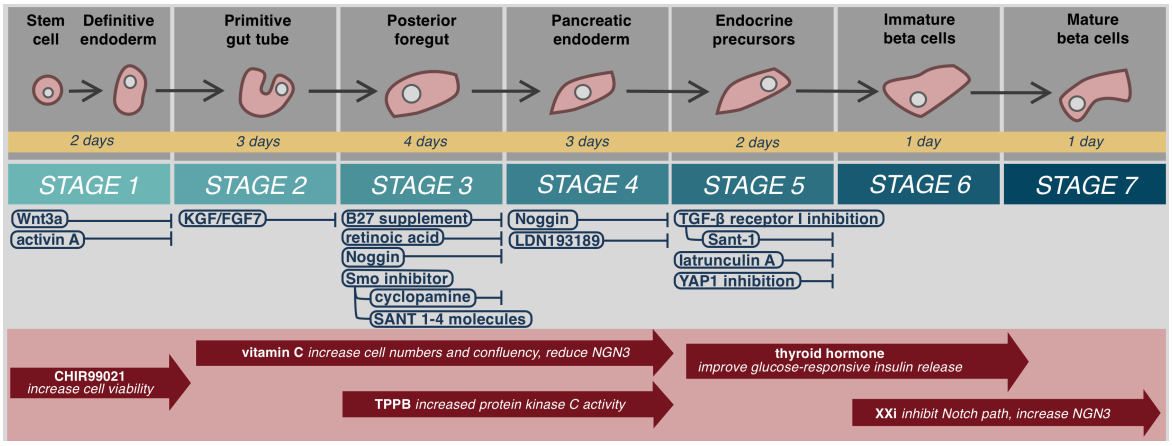

Figure 4.1.5 Seven Stage Process for Islet Cell Maturation from Stem Cells.

The most obvious pathway to eliminating immunosuppression requirements with iPSC-based ITx is through autologous transplantation. This would involve isolating a DM patient's cells, reverting them into iPSCs as described by Yamanaka et al. and Thomson et al., and subsequently developing personalized islet cells for autotransplantation [83]. Islet cell autotransplantation in patients with chronic pancreatitis demonstrated an example of this technique and was highly successful. While there have been concerns that maturation alters cellular immunogenicity and may confer allogeneity [89], further investigation demonstrates that immunoreactivity is only conferred following retrovirus-derived iPSCs due to leakage of transgenes and activation of neighboring genes, whereas plasmid-derived iPSCs demonstrate negligible immune reactions [90-92].Therefore, autologous iPSC ITx may potentially eliminate alloimmunity but this is entirely unproven at present and remains of concern. Of course, techniques would be required to also control autoimmunity, and this may prove to be a much harder challenge. The combination of iPSC ITx with lower dose immunosuppressant or ideally novel immunosuppressant approaches presents a realistic option for a T1D and especially for non-autoimmune T2D curative therapy. The final barrier to utilizing this approach then becomes economic feasibility; isogeneic iPSC generation requires personalized medicine, which requires a highly complex and expensive procedure that would potentially limit target delivery to only a few patients. The regulatory challenges with the preparation of a patient-by-patient personalized product may prove to be insurmountable, especially compared to the mass-production of a unified and validated GMP-grade 'off-the-shelf' allogeneic product – and it will be interesting to see how these divergent processes will eventually emerge in practice. Each patient's unique iPSC islets would require screening for off target growth and other genetic variabilities [92]. Variability

may also exist between different iPSC lines, mostly due to genetic background differences, with an inconsistent ability to differentiate into functional cells of a given lineage [93,94]. While this offers an easy solution to allogenecity, other barriers may limit its widespread patient use.

The alternative to iPSC-based cell lines involves allogeneic islet cell generation. Allogeneic sources allow for mass generation of islet-like cells from a single, optimized iPSC source. Large pools of HLA-specific iPSC-generated islet cell lines could be generated for transplant HLA matching. This may offer a homogenous transplantation source with optimal glycemic control, less off target growth, and easily accessible HLA matched islets for ITx. However, despite HLA matching, patients are likely to require immunosuppression with HLA matched ITx as minor antigens can still induce aggressive and irreversible destructive rejection [95]. The most significant barrier to allogeneic transplant is therefore immunoreactivity and post-ITx immunosuppression requirements.

To resolve allogenic iPSC immunoreactivity barriers, genetic editing may offer a realistic option. Uniquely, this would enable immune acceptance while leaving the recipients' immune system intact and capable of conducting regulatory and infectious roles. CRISPR/Cas9 techniques enable genetic alteration and have grown exponentially in recent years. Historically, it has been used to create and study genetic disease states such as Rett syndrome [96], HIV [97], and Parkinson's disease [98]. More novel approaches have used the genetic modification capabilities of CRISPR/Cas9 to modify iPSCs and reverse disease states *in vitro* by increasing expression of protective factors [99-101]. For example, increased interleukin-10 (IL-10) expression in animal models transplanted with tissues for liver, lung, and corneal transplant show less immune activation and improved graft survival without immunosuppression [102-104]. While this technique

limits immunosuppression requirements, another novel approach may eliminate immunosuppression needs entirely – CRISPR directed deletion of HLA class-I molecule from transplantable stem cells may enable a creation of single population of widely accepted iPSCs for all transplants regardless of their genetic makeup. HLA-silenced iPSC lines have been generated by targeted disruption of both alleles of the Beta-2 microglobulin gene, and produce non-reactive iPSC cells in lymphocyte reaction assays with retained ability to differentiate into multiple cell lineages [105-107]. HLA-silenced iPSC islet cells have yet to be generated but offer significant possibilities for the future. Of course, long-term stability and clinical efficacy have yet to be demonstrated, and we still need to be certain that a heavily gene-edited islet cell product can still function with similar potency and manufacture insulin in a physiologic fashion.

The ability to genetically modify cell lines may enable economic allogenic iPSC ITx but also solves other concerns about this therapy. Genetic modifications can enable drug induced apoptosis (i.e., kill switches) to solve concerns regarding off-target growth. Much concern has been discussed regarding the potential for iPSC cells to develop off-target growth of teratomas. However, researchers have effectively genetically inserted a drug suppressed essential cell-division gene [108], or a drug-inducible caspase-9 effector of cellular apoptosis into T cells [109]. Reproducing similar drug inducible “kill switches” increases the safety profile of iPSC ITx, as cells can be eliminated if off-target growth occurs. These ‘kill-switches’ may be especially important as a safety step as these cell products are transplanted within the more physiologic (and less easily retrievable) intraportal hepatic site. Implantation of cells beneath the skin is currently hampered by hypoxia and low cell survival. If insulin independence and function comparable or superior to that achievable with current allogeneic islet cell transplantation is to be

realized, then moving these therapies to a site such as the portal vein that permits better functional engraftment and survival will be the key to progress.

Similarly, CRISPR aided insertion of protective factors within iPSCs may enable them to combat autoimmunity – even HLA silenced allogenic iPSC lines will be exposed to autoimmunity. Exogenous IL-10 supplementation [110], and more recent gene transfer and increased islet cell IL-10 expression have demonstrated delayed recurrences of DM after isogeneic transplantation [111-113]. This action occurs through induction of Treg cell populations [112,113]. Additional studies have demonstrated alloantigen-specific immunosuppressive capacity of Treg treatment with transplantation [114], and clear GMP protocols now exist to generate protective Tregs specific for recipient alloantigens under GMP conditions [115]. Genetic expression of IL-10 has not been conducted within iPSC generated islet cells but may offer additional allogeneic immune protection and isogeneic autoimmune protection for patients with T1D to allow for immunosuppression-free ITx.

Many of these solutions to immunoreactivity have been proven but remained to be tested in ITx. Combining allogeneic protection with HLA-silenced iPSCs, autoimmune protection with IL-10 expression for Treg upregulation, and enabling drug induced apoptosis to enable safe intraportal transplantation may enable iPSC ITx. This would allow for a single source of allogeneic, but HLA-silenced and autoimmune protected islet cells, with controlled “kill switches” to enable intraportal transplant.

4.1.7 **Islet Cell Regeneration and Immune Reset**

Another large volume, easily accessible source of stem cells not yet widely considered are a patient's own stored bone marrow-derived hematopoietic and mesenchymal stem cells (BMSC). *In vitro* differentiation of insulin producing islets from BMSC is well described [116-119]. Differentiation protocols initially required prolonged culture time [116], or genetic manipulation [117-119]. Hisanaga *et al.* (2008) developed a technique like iPSC differentiation with insulin producing cells five to seven days after culture that were capable of reversing streptozotocin induced murine DM [120]. iPSC discovery has largely supplanted BMSC interest, since acquisition of bone marrow and culture to generate multipotent BMSC is much more difficult than iPSC generation and differentiation. However, improved understanding of the regenerative role played by mobilized BMSC may provide an alternative novel approach to curing newly diagnosed T1D. We currently have an ongoing clinical trial in Edmonton that began in 2019 and is exploring the potential of the drug plerixafor to mobilize CD34+ stem cells into the peripheral blood. This trial, approved for adults and adolescent children with new onset T1D, also uses a single dose of T-depletional therapy, dual anti-inflammatory medications and a long-acting GLP-1 analogue to promote regeneration and repair. Using this technique, BMSC are mobilized from a patient's own bone marrow, travel to the inflamed pancreas, and allow pancreatic progenitor cells to recover and regenerate insulin producing islet cells. Similar to isogeneic iPSC ITx, these regenerated islets still require immune protection from recurrent autoimmunity.

In vivo BMSC directed islet cell regeneration in murine models receiving isogeneic bone marrow transplant has been demonstrated [121]. BMSC transplant following experimentally

induced DM in streptozotocin-treated mice [122,123], streptozotocin-treated rats [124], E2f1/E2f2 mutant mice [125], and non-insulin-dependent KKAy mice [126] have shown DM reversal. While insulin production and DM improvement occurred, only 1.7 - 3% of functional islets were linked to donor BMSC differentiation [127]. The mechanism for regeneration of functional islets initially remained unclear. A beautifully designed study by Hasegawa et al. (2007) helped to clarify these results – they demonstrated that BMSC localize to pancreatic ducts and islets to initiate islet regeneration from pre-existing pancreatic progenitor cells [127]. Endothelial nitric oxide synthase (eNOS) acts through matrix metalloproteinase-9 to mobilize BMSC for tissue regeneration [128]. Without eNOS, BMSCs could not localize to pancreatic tissues or restore islet function regeneration [127]. Unfortunately, early BMSC mobilization prior to complete islet cell destruction is needed. Clinically, this means that BMSC mobilization is required soon after T1D diagnosis. DM reversal did not occur if mobilization transpired >30 days after streptozotocin induced islet injury [127].

Mechanistically, AHSCT results in extended duration CD4+ T cell depletion, with little effect on CD8+ T cells, leading to inverted CD4+/CD8+ ratio [129,130]. This is in keeping with our understanding of insulitis, as CD8+ directed apoptosis that is CD4+ activation-dependent [30]. Previously activated CD8+ T cells, or CD4+ cells to activate them, are needed to produce insulitis. Patients with increased activated CD8+ T cells before AHSCT stem cell transplant were therefore more likely to reacquire insulin dependence early [129]. Additionally, patients with increased CD4+ memory cells had early AHSCT failure and insulin dependence [129].

Although this approach is unlikely to cure longstanding T1D, it may enable T1D reversal if applied early after disease onset. This theory has been termed “immune reset” and involves

eliminating the autoreactive immune system, and replacing it with a more tolerant system through intravenous non-myeloablative autologous hematopoietic stem cell transplantation (AHSCT). Voltarelli *et al.* (2007) was the first to apply this technique clinically. They utilized cyclophosphamide to suppress the immune system and granulocyte colony-stimulating factor (GCSF) to mobilize patient's own CD34+ cells (hematopoietic BMSC) for collection via leukopharesis. Patients were then treated with five days of intensive immunosuppression with cyclophosphamide and rabbit antithymocyte globulin for immune ablation and subsequently injected with the previously collected BMSC [131]. Following autologous intravenous injection, they achieved 87% medication independence and 96% insulin-independence in 23 patients [131,132]; twelve of these patients were insulin-free for a mean 31 months and even when insulin independence was not achieved, C-peptide levels increased and insulin requirements decreased [132]. Several other international studies with small protocol changes have shown similar results following AHSCT [130,133-135]. Long-term follow up in these studies has shown HbA1c levels at four years of 7.1% from 10.9% at transplant [134], and significantly decreased microvascular complications regardless of insulin independence [136]. Unfortunately, current immune reset techniques do not offer long-term insulin independence, primarily due to recurrence of autoimmunity.

4.1.8 **Novel Approaches to Immunosuppression**

Despite significant advances, auto- and alloimmunity continue to pose significant barriers to cellular transplantation therapies in diabetes, whether that be through immune reset or via ITx. As discussed, the risks associated with immunosuppression remain a significant barrier to

widespread ITx. Autoimmune, alloimmune, and inflammatory pathways must be countered to enable ideal islet engraftment and long-term insulin independence. Immune reset trials have attempted to target autoimmunity with early autoimmune reversal and islet cell regeneration prior to complete autoimmune directed islet cell loss. While currently being investigated as a single approach for DM cure, combining autoimmune protection from immune reset with ITx may also present an important future direction. Alternatively, other novel approaches to modify recipient allo- and autoimmune response following ITx are also being investigated. As our understanding of the immune reaction producing auto- and alloimmunity improves, we become closer to countering them to eradicate DM.

Previous immune reset trials have demonstrated proof of concept for reversing autoimmunity but effects were transient. Based on the results of preliminary AHSCT studies, a first-in-human pilot clinical trial (ClinicalTrials.gov Identifier: NCT03182426) is currently evaluating a novel approach to immune reset. The hope of this trial is to achieve similar or improved long-term results with a better tolerated approach to autoimmune suppression. Immune ablation in this trial is being conducted with anti-CD25 antibody alemtuzumab, IL-1 antagonist anakinra, and TNF-α inhibitor etanercept. These agents have much greater tolerability than cyclophosphamide and rabbit antithymocyte globulin as demonstrated by current ITx practices. Plerixafor, rather than GCSF, will mobilize hematopoietic CD34+ cells (BMSC) into peripheral circulation and patients will not undergo apheresis to isolate them. As opposed to previous trials, this approach will continue to manage autoimmunity with ongoing anakinra and etanercept treatment, in addition to a second “immune reset” following a year of treatment. The long-acting GLP-1 analogue (liraglutide) will also be used to provide trophic and metabolic support to

regenerated islets. If well-tolerated and efficacious, patients could potentially undergo multiple rounds of immune reset to achieve long-term insulin independence. Alternatively, immune reset may provide a novel approach to immunoprotection following islet cell transplant . Both allogeneic and isogeneic iPSC ITx face significant autoimmune reactivity that likely limit their long-term graft survival. Future endeavors to combine iPSC ITx and immune reset may enable immunosuppression-free or immunosuppression-limited transplant. This would significantly shift the risk-benefit analysis in favor of ITx benefits and potentially increase funding access to all patients with DM.

A promising alternative approach to control of T1D autoimmunity is currently being explored through autologous expansion of a potent T cell suppressing cell population called Tregs. Further review of patients with prolonged insulin independence following AHSCT demonstrated significantly increased Treg populations [129]. Tregs are relatively newly discovered CD4+ T cell subtype that are CD25+ and control immune response to self-antigens [137]. Various Treg subpopulations have now been discovered, but most evidence focuses on natural Treg cells, which are CD4+CD25+FOXP3+. Loss of CD25+, inhibition with monoclonal antibodies or absence of its transcription factor FOXP3, leads to uncontrolled organ failure through autoimmune processes [137-140]. If harnessed for islet cells, these cells provide a powerful tool for immune tolerance. Understanding of these cells is still growing, but we do know that they are activated in the thymus through a CTLA-4 T cell receptor (TCR), and upregulate an ever-growing number of immune suppressive molecules [141-143]. Their activation is antigen specific, but immunoprotection actions are antigen non-specific with the capability of suppressing polyclonal T effector cells regardless of those cells antigen specificity, a unique process termed

bystander suppression [141,144]. Once activated, they also create a regulatory milieu with activation of other Treg cells with distinct antigen specificities, termed infectious tolerance. The combination of infectious tolerance and bystander suppression creates a tolerant state that is active against various antigen-specific T effector cells and not reliant on a single Treg antigen population. Following single antigen Treg activation post-ITx, immunoprotection without immunosuppression was achieved, even when the original Treg population was eliminated [141,145,146]. The exact mechanism of Treg immunoprotection is not well understood but a long list of protective cytokines has been described [141], as has inhibition of CD8+ T cell activation [147-149] and direct CD8+ T cell apoptosis [150,151]. Some of the most convincing evidence suggests that Tregs induce long-lasting anergy in CD4+ cells; these CD4+ cells then produce IL-10 and inhibit other CD4+ cells from proliferating [152-154]. Coupled with IL-10 directed CD4+ inhibition, Tregs also appear to block CTLA-4 TCRs on antigen presenting cells to inhibit CD4+ and CD8+ activation [155-157]. This explains how blocking CD8+ activation occurs without Treg-CD8+ contact [158,159], and provides a mechanism for infectious tolerance and bystander suppression.

Like AHSCT, when Treg cells are expanded and injected in 13-week-old NOD mice they can establish autoimmune protection and reverse expected DM [146]. Novel studies have demonstrated that low dose IL-2 is necessary for intra-thymic Treg production [139,160,161] and peripheral expansion [139]. While high dose IL-2 expands T effector cells, low doses can effectively increase *in vivo* Treg concentrations [59,162-164]. Low dose IL-2 has thus been used to expand *in vivo* Treg populations, eliminating the need for *ex vivo* Treg expansion. Similarly, low dose IL-2 reverses T1D in young NOD mice [165,166].

Phase one human clinical trials have demonstrated strongly promising results with expanded autologous Treg transplantation proving safe; unfortunately, inadequate study sizes have been achieved to comment on C-peptide production or glycemic control [144]. Low dose IL-2 injection has also demonstrated increased Treg populations during phase one human clinical trials, but preliminary data suggested that IL-2 could accelerate immune destruction in new onset T1D [167,168]. No data currently demonstrates T1D reversal if provided early after T1D diagnosis. However, AHSCT and Treg therapy likely act through similar mechanisms by achieving improved Treg to T effector ratios and autoimmune downregulation. If true, the large AHSCT clinical trials will provide important data to determine T1D reversal if provided to patients soon after diagnosis. Further, this study will enable discussion regarding prolonged autoimmune islet cell protection that may be utilized in combination with ITx. The primary use of Treg and AHSCT therapy initially focused on reversing early T1D but combination therapy with iPSC ITx may also provide significant therapeutic benefits. Allogeneic and isogeneic iPSC ITx has persistent autoimmune activation that limits engraftment and graft longevity. Providing autoimmune protection to islets following transplantation with Tregs may enable elimination or markedly decreased immunosuppression requirements [169-171].

4.1.9 **Conclusion**

DM is has become a pervasive and most costly chronic disease both in terms of patient morbidity and healthcare burden. As the prevalence and complications continue to grow, standard subcutaneous insulin replacement will not suffice. Technology has attempted, and succeeded to improve subcutaneous insulin delivery techniques with continuous glucose

monitoring, continuous insulin delivery, and closed-loop wearable devices that attempt to respond dynamically to blood glucose levels. Although they represent an improvement from prior treatments, these technologies fall short of curing diabetes, and likely also fall short of even effectively treating the disease. Even with the most complex devices, patient and disease factors lead to inadequate glycemic control and a significant risk of diabetic complications. An improved treatment or ideally a cure is desperately needed.

Like most other diseases, a potential cure is best achieved by better understanding the disease pathophysiology to reverse or inhibit physiologic pathways that led to the clinical presentation at first. In terms of T1D, this has involved discovering insulitis and islet cell destruction. With current practice of allogeneic ITx from organ donors, immunosuppression and preparation improvements have led to 50% insulin independence with ongoing improvements that will further improve efficacy. Meanwhile, discovery of ESC-derived or iPSC ITx will certainly meet islet cell supply that will match demand. Determining whether allogeneic or isogeneic sources are suitable in terms of immunosuppression requirements and economic efficiency will require a period of intense parallel research. Taking iPSC islet generation further with genetic modification to create HLA-silenced islets, possibly with drug induced “kill switches” will advance the field remarkably, but may also make initial clinical approval more complex.

Alongside ITx, ongoing improved understanding of the allo and auto immunity cellular pathways are likely capable of supporting ITx, but may also provide novel curative techniques that reverses newly diagnosed T1D. Immune reset has demonstrated promising data to transiently reverse newly diagnosed T1D, and ongoing clinical trials may prove that T1D is

indeed reversible. Combined with ITx, a cure for longstanding T1D is likely not far from a reality. Similarly, advances in Treg therapy may be able to reverse newly diagnosed T1D or be combined with ITx to cure longstanding DM.

Could similar therapies be used to treat T2D? – This is a question we have not evaluated within this chapter but is clearly well within the realm of possibility – indeed, the absence of autoimmunity may well substantially enhance the early success in that patient population. We do not yet know for certain whether augmented islet mass cell transplantation will be able to overcome peripheral insulin resistance alone, but there are no several T2D medications that can enhance insulin sensitivity and alter insulin clearance. Considering the obesity epidemic, future investigations will be much needed to answer this question definitively.

Regardless of which technique advances furthest, discussion around DM has shifted from improving treatment therapies, towards discussion about a realistic cure with longstanding insulin independence. Surely, it seems that reversing newly diagnosed T1D is likely in the near future. Combining multiple approaches alongside ITx is also likely to enable cure of longstanding T1D. The discussion about curing or treating T2D with similar therapies has also begun. With improved pathophysiologic and immunologic understanding and rapidly developing novel approaches to combat those processes, the future of diabetes treatment is exciting. A cure may be within reach and we may witness a remarkable medical shift for treatment, something that cannot realistically be said for many diseases we face.

4.1.10 References

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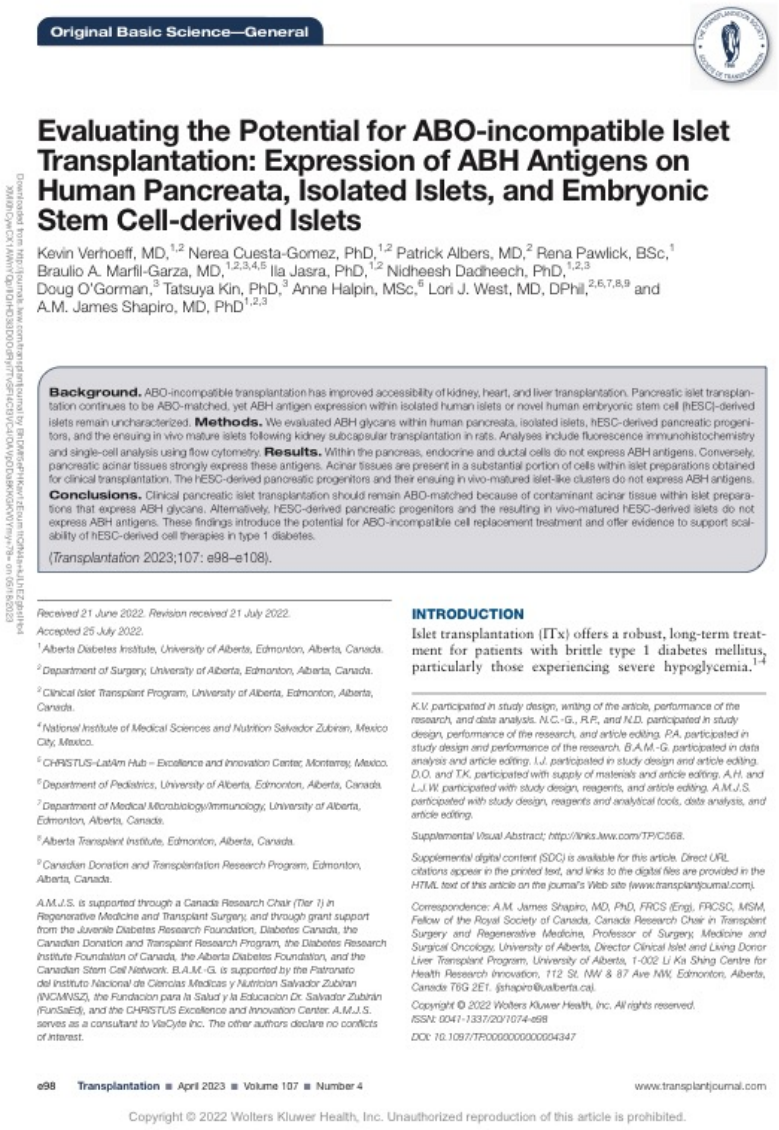
4.2 Chapter 4 subsection 2 – Evaluating the Potential for ABO-Incompatible Islet Transplantation: Expression of ABH Antigens on Human Pancreata, Isolated Islets, and Embryonic Stem Cell-Derived Islets



Original Basic Science—General

Evaluating the Potential for ABO-incompatible Islet Transplantation: Expression of ABH Antigens on Human Pancreata, Isolated Islets, and Embryonic Stem Cell-derived Islets

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Background. ABO-incompatible transplantation has improved accessibility of kidney, heart, and liver transplantation. Pancreatic islet transplantation continues to be ABO-matched, yet ABH antigen expression within isolated human islets or novel human embryonic stem cell (hESC)-derived islets remain uncharacterized. **Methods.** We evaluated ABH glycans within human pancreata, isolated islets, hESC-derived pancreatic progenitors, and the ensuing in vivo mature islets following kidney subcapsular transplantation in rats. Analyses include fluorescence immunohistochemistry and single-cell analysis using flow cytometry. **Results.** Within the pancreas, endocrine and ductal cells do not express ABH antigens. Conversely, pancreatic acinar tissues strongly express these antigens. Acinar tissues are present in a substantial portion of cells within islet preparations obtained for clinical transplantation. The hESC-derived pancreatic progenitors and their ensuing in vivo-matured islet-like clusters do not express ABH antigens. **Conclusions.** Clinical pancreatic islet transplantation should remain ABO-matched because of contaminant acinar tissue within islet preparations that express ABH glycans. Alternatively, hESC-derived pancreatic progenitors and the resulting in vivo-matured hESC-derived islets do not express ABH antigens. These findings introduce the potential for ABO-incompatible cell replacement treatment and offer evidence to support scalability of hESC-derived cell therapies in type 1 diabetes.

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INTRODUCTION

Islet transplantation (ITx) offers a robust, long-term treatment for patients with brittle type 1 diabetes mellitus, particularly those experiencing severe hypoglycemia.[1-4]

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4.2.1 **Abstract**

4.2.1.1 Background:

ABO-incompatible transplantation has improved accessibility of kidney, heart, and liver transplantation. Pancreatic islet transplantation continues to be ABO-matched, yet ABH antigen expression within isolated human islets, or novel human embryonic stem cell (ESC)-derived islets remains uncharacterized.

4.2.1.2 Methods:

We evaluated ABH glycans within human pancreata, iolated islets, ESC-derived pancreatic progenitors, and the ensuing *in vivo* mature islets following kidney subcapsular transplantation in rats. Analyses include fluorescence immunohistochemistry and single-cell analysis using flow cytometry.

4.2.1.3 Results:

Within the pancreas, endocrine and ductal cells do not express ABH antigens. Conversely, pancreatic acinar tissues strongly express these antigens. Acinar tissues are present in a substantial portion of cells within islet preparations obtained for clinical transplantation. ESC-derived pancreatic progenitors and their ensuing *in* vivo-matured islet-like clusters do not express ABH antigens.

4.2.1.4 Conclusions:

Clinical pancreatic islet transplantation should remain ABO-matched due to contaminant acinar tissue within islet preparations that express ABH glycans. Alternatively, ESC-derived pancreatic progenitors and the resulting *in vivo*-matured ESC-derived islets do not express ABH antigens. These findings introduce the potential for ABO-incompatible cell replacement treatment and offers evidence to support scalability of ESC-derived cell therapies in type 1 diabetes.

4.2.2 **Introduction**

Islet transplantation (ITx) offers a robust, long-term treatment for patients with brittle type 1 diabetes mellitus, particularly those experiencing severe hypoglycemia[1-4]. Optimal clinical outcomes are independently and strongly associated with β-cell mass and islet engraftment as measured by C-peptide levels[3]. To limit acute immune-mediated islet destruction following transplant, recipients are evaluated for allosensitization to human leukocyte antigens (HLAs), and transplanted with ABO-compatible (ABOc) islet preparations[5]. Despite the substantial efforts required to reduce acute immune responses, including ABO-matching, the presence of ABO glycan structures has only been evaluated within pancreatic tissues[6,7], suggesting that islets don't express ABH structures. ABH antigen expression remains uncharacterized within human islet preparations following isolation for transplantation. Furthermore, as stem cell-derived cell replacement therapies move forward in early human clinical trials[8,9], evaluation of their ABH antigen expression and potential for ABO-incompatible (ABOi) transplant remains undescribed.

For kidney, heart, and liver transplantation, ABOi transplantation has achieved clinical outcomes nearly equivalent to those observed following ABOc transplant[10-17]. Application of ABOi organ transplant occurred in response to donor supply limitations, which has substantially increased the capacity to transplant these organs[10,12,13,18]. Achieving these outcomes in other fields has required modified induction immunosuppression to limit acute rejection[16,19-21]. However, overcoming acute rejection following ABOi ITx by relying on immunosuppression alone is unlikely to be clinically feasible since islets have comparatively greater susceptibility to acute rejection due to their increased exposure to the immune system as a cell suspension[5].

Furthermore, islet transplants, while purified, typically still have substantial contamination by pancreatic exocrine tissue components, and even purified islet preparations remain only 30-50% pure. ITx has therefore remained ABOc. To consider ABOi ITx, characterization of ABH antigen expression on human islet preparations and human embryonic stem cell (ESC)-derived islets is critical. If human islets or ESC-derived islets do not express ABH structures, the transplant matching process may change dramatically by potentially increasing donor pancreas utilization and thus expanding ITx access. Specific to ESC-derived islets, if cell products do not express ABH glycans, the potential for a single, expandable and scalable cell product for all patients may be achievable.

Herein, we characterize ABH antigen expression in human pancreata, donor pancreatic islets, ESC-derived pancreatic endocrine progenitor cells (PECs) prior to transplant, and ESC-derived *in vivo*-matured islet-like clusters following transplantation into immunocompromised rats.

4.2.3 **Materials and Methods**

All procedures have been reviewed and approved by the Health Research Ethics Board (Pro00001620, Pro00084032) and all animal protocols were approved by the Institutional Animal Care and Use Committee (AUP00004036). Patients included in this study as islet and ESC donors provided written consent for experimental use of tissues. All experiments reported were completed in biological triplicates with evaluation of three patients with ABO-A blood type, three with ABO-B, and three with ABO-O in all experiments. Where ESCs are used, only technical triplicates were completed because only one cell line is utilized (Cyt49), which is blood

type O. Thus, three separate ESC-derived PEC preparations were evaluated to reproduce biological triplicates. Because the ABO blood type of all samples was known, samples were evaluated for only their expected ABH-antigen.

4.2.3.1 Collection and Preparation of Human Pancreata, Isolated Islets, ESC-Derived Pancreatic Progenitors, and In vivo-matured ESC-Derived Islet-like Clusters

Human pancreas tissue was collected from a biopsy of deceased donor pancreata and fixed in 10% formalin. The remainder of the pancreas was used for islet isolation, as previously described by the Alberta Diabetes Institute IsletCore at the University of Alberta[22]. Once isolated, islets were separated into two microcentrifuge tubes, one for immunohistochemistry containing 1000 islet equivalents, and the remainder of cells used for flow cytometry.

ESC-derived PECs were collected following ESC differentiation using protocols previously described by Schulz et al. (2012)[23]. These cells were provided without cost by ViaCyte Inc.. Stage 4 ESC-derived PEC clusters were either prepared for immunohistochemistry as described below, or transplanted into the renal subcapsular space. Transplants occurred in Rowett Nude (RNU) immunodeficient rats, aged between 16-18 weeks (Charles River Laboratories) following previously described protocols[24,25]. Each rat was transplanted with $5x10^6$ (i.e. 40μL packed cell volume) PEC-01 cells. Cells were allowed to mature *in vivo* for 24-weeks, followed by renal graft recovery and fixation using 10% formalin.

4.2.3.2 Immunohistochemistry

4.2.3.2.1 Immunohistochemistry Preparation of Islets, Pancreatic Progenitor Clusters, and Pancreatic Biopsies

Fixed pancreas biopsies and renal grafts were paraffinized and 5 µm sections were placed on glass slides. Isolated islets and PEC clusters used for immunohistochemistry were collected and washed with PBS followed by fixation in 2 mL of 4% paraformaldehyde (PFA) in a microcentrifuge tube for 20 minutes at room temperature (RT). Cells were allowed to settle by gravity and placed within 1% low melting agarose (Invitrogen cat. 16520-050). The subsequent paraffinized samples were sectioned to 8 µm and placed on glass slides.

4.2.3.2.2 Immunohistochemistry Staining

For immunohistochemistry preparation slides were incubated for 40 minutes at 60°C to melt the paraffin and allow cell adherence to the glass. Slides were deparaffinized and rehydrated followed by heat-induced epitope retrieval by submerging cells for 20 minutes in 98°C citrate buffer (0.0126 M citric acid, Sigma cat. C-0759; 0.0874 M sodium citrate, Sigma cat. S-4641; pH 6.0).

Samples were blocked with 5% normal donkey serum (Sigma cat. S30-M) in FoxP3 permeabilization buffer (Biolegend cat. 421402) and incubated for 1 hour at RT. Blocking buffer was then removed and primary antibodies diluted in blocking buffer were incubated for 2 hours at RT in a humid dark chamber (antibodies and dilutions as per **Appendix** Table S4.2.2). As above, the ABO blood type of all samples was known, therefore, only the matching A, B, or H

antibody was applied for each sample. Slides were washed with PBS-T (1x PBS with 0.1% Tween, Sigma cat. P1379) for 2-minutes, three times. Secondary antibodies were then diluted as per **Appendix** Table S4.2.2 and incubated for 40 minutes at RT in a humid dark chamber. Following the final wash, DAPI (Fisher cat. D1306) was applied for 4 minutes at RT in the dark. Slides were washed once in PBS and mounted with fluoromount-G (Thermo Fisher cat. 00-4958-02), which was allowed to dry in the dark at RT overnight. Slides were visualized using the Zeiss Observer Z1 inverted fluorescence motorized microscope.

4.2.3.3 Flow Cytometry

Human islets were dissociated, filtered, counted, and fixed prior to analysis. For dissociation, human islets were transferred to a 50 mL conical and allowed to settle by gravity. Media was removed and human islets were washed with PBS and then incubated with 10 mL of StemPro accutase (Fisher Scientific cat. A11105-01) supplemented with Y-27632 dihydrochloride ROCK inhibitor (Rocki, 2 µL/mL) for 10 minutes at 37°C. Clusters were further disrupted mechanically, leaving only single cells. The conical tube was then centrifuged at 450xg for 2 minutes, followed by removal of the supernatant, and resuspension of the pellet with 10 mL of PBS supplemented with Rocki (2 µL/mL). The single cell solution was filtered through a 40 µm cell strainer and cells were counted and assessed for viability (Trypan Blue, Fisher Scientific cat. 15250061) using the Thermo Scientific Invitrogen Countess II AMQAX1000 Cell Counter. Cells were fixed with 4% paraformaldehyde (PFA) for 20 minutes at RT and cells were distributed into microcentrifuge tubes with 2 million live cells each. Cells were centrifuged at

700xg for 2 minutes and the supernatant was removed; cells were stored in PBS until staining was performed.

For staining, cells were permeabilized and stained using the BD Cytofix/Cytoperm Fixation/Permeabilization Solution Kit (BD Biosciences cat. 554714) as per manufactures instructions. Primary antibodies were incubated for 1 hour and secondary antibodies for 30 minutes according to the dilutions in **Appendix** Table S4.2.2. ABH antigen antibodies were again matched to the sample being evaluated. Cells were resuspended in fluorescence – activated cell sorting (FACS) buffer (2% FCS, 2 mM EDTA in dPBS) and kept on ice until needed for flow cytometry. Isotype controls were used to gate positive staining and data were acquired using the CytoFLEX S flow cytometer and analysed using the CytExpert software (Beckman Coulter).

4.2.3.4 Statistical Analysis

Continuous data is presented as medians and interquartile ranges (IQR). Normality testing was performed with the D'Agostino-Pearson normality test to determine the need for non-parametric testing, which was utilized for all subsequent analyses. Between group comparisons of continuous data were carried out using the non-parametric Mann–Whitney U test or Kruskal–Wallis tests with Dunn's Multiple Comparison Test. All statistical analysis was performed using STATA 17 (StataCorp, College Station, TX, USA) with the alpha was set to 0.05.

4.2.4 **Results**

4.2.4.1 Human pancreata: Human pancreatic acinar tissues strongly express ABH antigens, whereas ABH antigens are absent in endocrine and ductal cells

ABH antigen expression was evaluated on the previously demonstrated major cell populations within the pancreas including the endocrine (Chromogranin A expressing i.e. $ChgA^+$), acinar ($HPX1^+$), and ductal (cytokeratin 19^+ i.e. $CK19^+$) cells[26]. Analysis of the acinar and endocrine tissues through immunochemistry suggested that $ChgA^+$ cells do not express ABH antigens, while $HPX1^+$ cells showed ABH antigen expression (Figure 4.2.1A). Evaluation of the $CK19^+$ cell populations demonstrated that ductal cells lacked ABH antigen expression (Figure 4.2.1B).

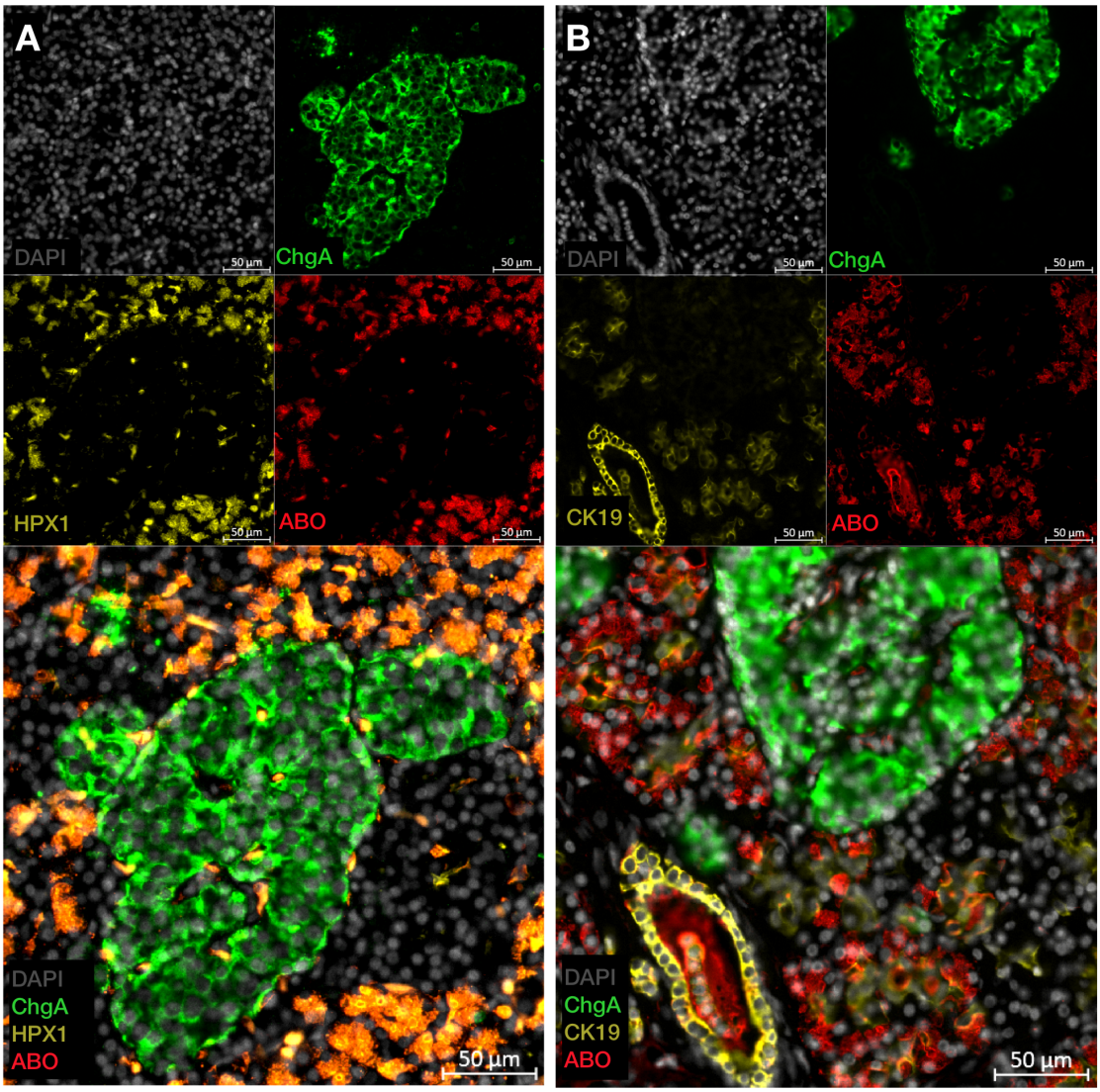

Figure 4.2.1 Fluorescence immunohistochemistry evaluation of the major cell populations within the pancreas and the associated ABH antigen expression within those cell populations.
A) Evaluation of the cells expressing chromogranin A (ChgA), pancreatic acinar (HPX1), and human ABH antigen markers with the ensuing merged image. B) Evaluation of the cells expressing CK19 ductal marker (CK19), pancreatic acinar (HPX1), and human blood type (i.e. ABO) antigen markers with the ensuing merged image. Figure is a representative image from patient with ABO-A blood type, with representative images of ABO-B and ABO-O presented in supplementary material. n = 3 per ABO blood type completed with representative images displayed.

Interrogation of endocrine subpopulations including C-peptide expressing ($Cpep^+$) β-cells, glucagon expressing ($Gluc^+$) α-cells, and somatostatin expressing (Stt^+) δ-cells, showed a lack of ABH antigen expression (Figure 4.2.1A-C). Overall, *in situ* pancreas immunohistochemistry suggested that acinar cells express ABH antigens, while endocrine and ductal cells do not. All blood groups were evaluated independently (n = 3 *per* blood group) and no immunohistochemistry differences were noted within pancreas biopsies regardless of the patient's ABO blood group (i.e. ABO-A, B, or O, **Appendix** Figure S4.2.8).

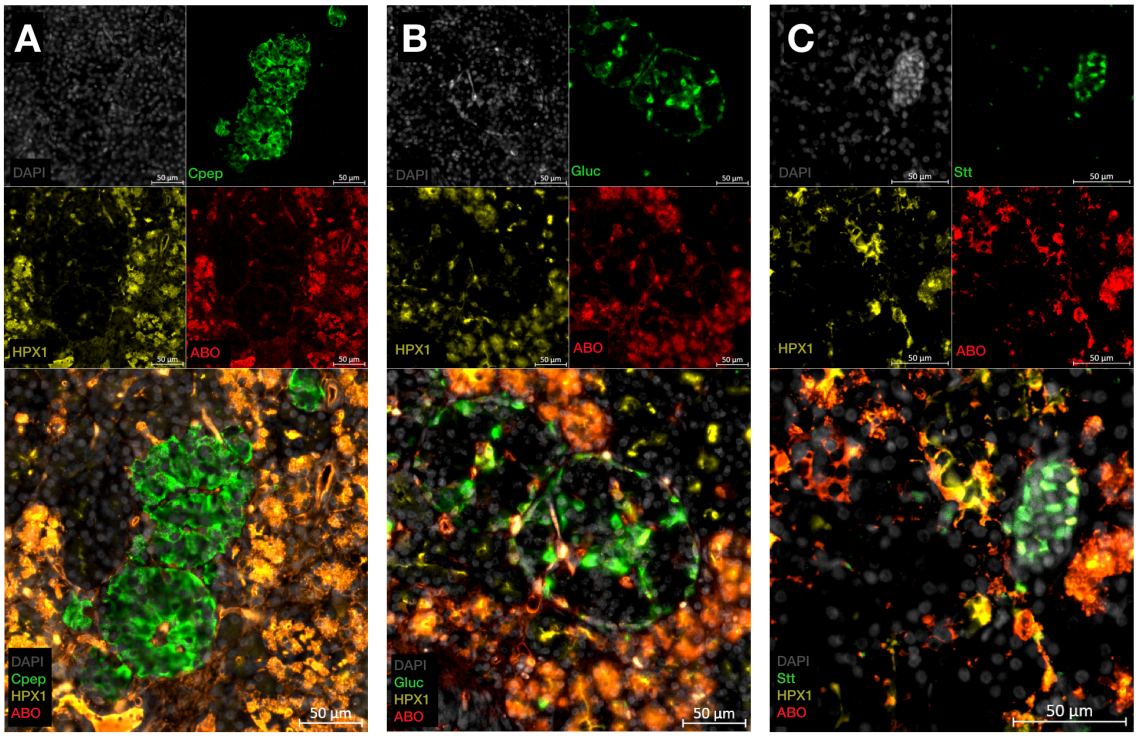

Figure 4.2.2 Fluorescence immunohistochemistry evaluation of the endocrine cell subpopulations within the pancreas and the associated ABH antigen expression within those cell populations.
A) Evaluation of the cells expressing C-peptide (Cpep), pancreatic acinar (HPX1), and human ABH antigen markers with the ensuing merged image. B) Evaluation of the cells expressing Glucagon (Gluc), pancreatic acinar (HPX1), and human ABH antigen markers with the ensuing merged image. C) Evaluation of the cells expressing Somatostatin (Stt), pancreatic acinar

(HPX1), and human blood type (i.e. ABO) antigen markers with the ensuing merged image. Figure is a representative image from patient with ABO-A blood type, with representative images of ABO-B and ABO-O presented in supplementary material. n = 3 per ABO blood type completed with representative images displayed.

4.2.4.2 Isolated human islets: Flow cytometry confirms ABH antigen expression within acinar cells but not endocrine or ductal cells

Flow cytometric analysis demonstrated that isolated islet preparations were composed primarily of $ChgA^+$ cells (45.3%, IQR 15.8), with fewer $CK19^+$ cells (21.5%, IQR 3.6, p = 0.0093 vs $ChgA^+$ cells) and $HPX1^+$ cells (21.7%, IQR 12.2, p = 0.0093 vs $ChgA^+$ cells, Table 4.2.1). Notably, islet preparation purity was variable leading to relatively wide IQRs (Figure 4.2.3). Flow cytometry was completed on n = 9, representing 3 samples for each A, B, and O blood type with data grouped due to a lack of differences between groups on immunohistochemistry. Flow cytometry gating strategies are described in **Appendix** Figure S4.2.9.

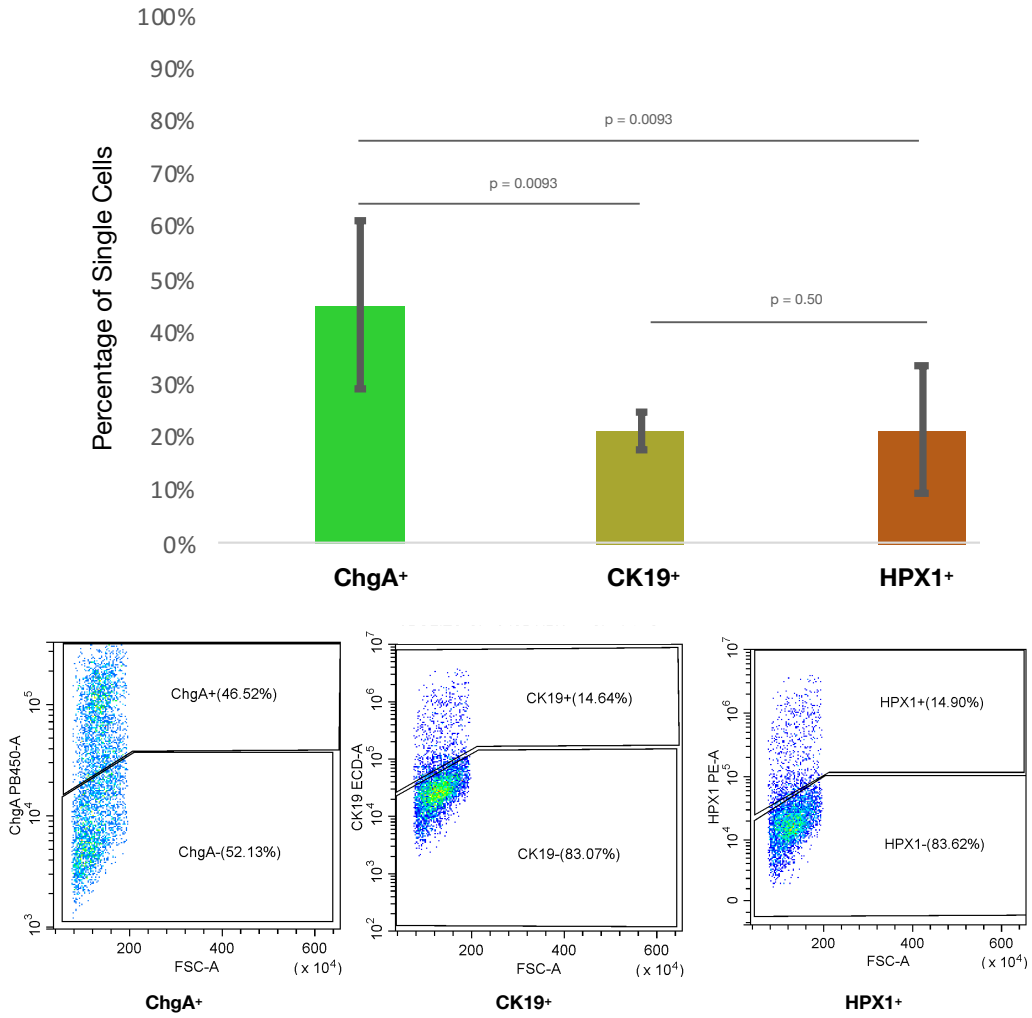

Figure 4.2.3 Flow cytometry results of single cells obtained from human islet preparations demonstrating the percent (%) of cells being endocrine (ChgA+), ductal (CK19+), and acinar (HPX1+).
Data represents n = 9 with three samples from each ABO blood group.

Assessment of ABH antigen expression on these cell populations demonstrated that few ChgA$^+$ (3.7%, IQR 0.5), or CK19$^+$ (5.8%, IQR 0.9) cells expressed ABH antigens, while significantly more HPX1$^+$ cells expressed ABH antigens (64.5%, IQR 1.1, $p < 0.05$ compared to

both ChgA$^+$ and CK19$^+$ cells, Figure 4.2.4). Gating strategies are demonstrated in **Appendix** Figure S4.2.10.

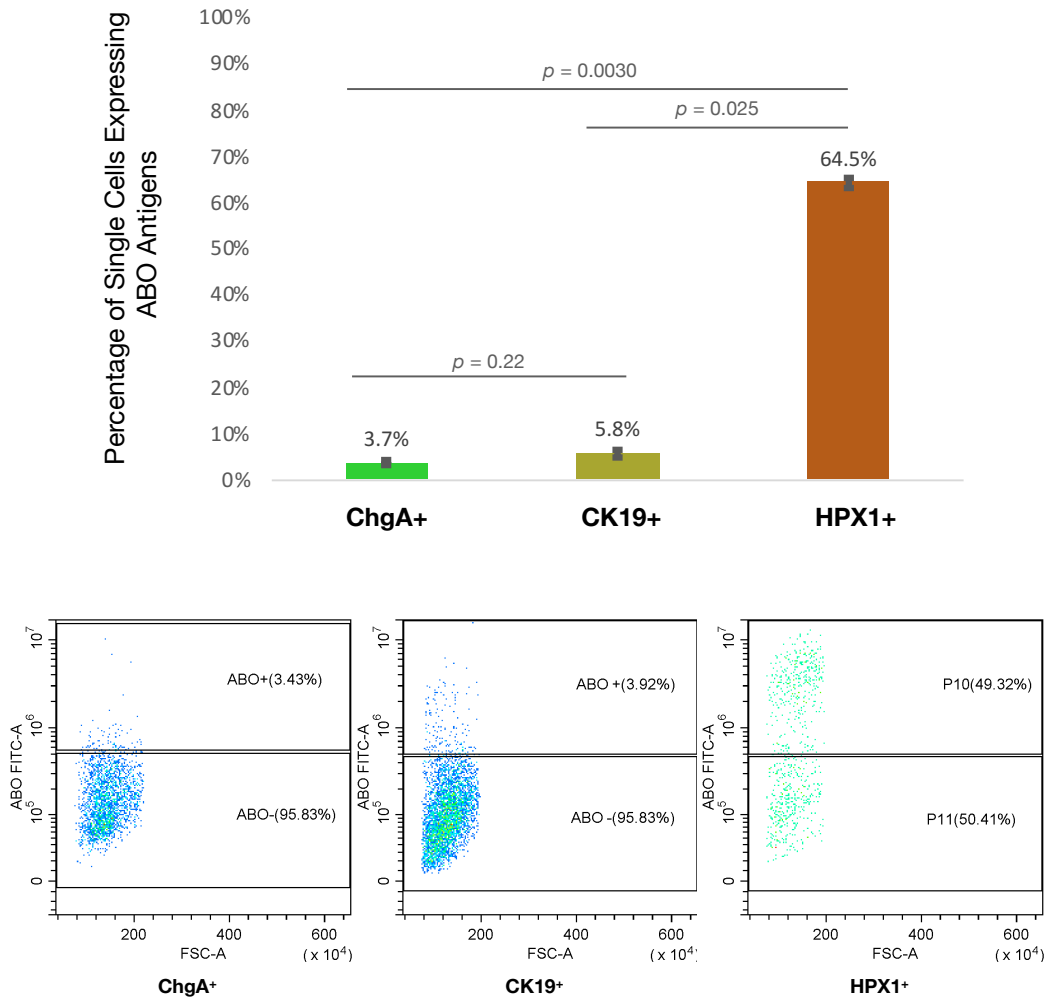

Figure 4.2.4 Flow cytometry results of single cells from human islet preparations demonstrating the percent (%) of endocrine (ChgA$^+$), ductal (CK19$^+$), and acinar (HPX1$^+$) cells that express human ABH antigens.
Data represents n = 9 with three samples from each ABO blood group.

Because the β-cells represent the most clinically relevant cell population for ITx, we also performed subgroup evaluation of Cpep$^+$ cells. This demonstrated that 6.3% (IQR 1.5) Cpep$^+$

cells expressed ABH antigens (Table 4.2.1). Other endocrine subpopulations were inadequately represented to accurately evaluate their ABH antigen expression.

Table 4.2.1 Results of flow cytometry evaluating single cells from islet cell preparations.

Major Cell Populations		
	Percent of Single Cells within Islet Preparation n = 9 Median (IQR)	p-value
$ChgA^+$	45.3 (15.8)	0.025
$CK19^+$	21.5 (3.6)	
$HPX1^+$	21.7 (12.2)	
ABH Antigen Expression		
	Percent of Single Cells Expressing ABH Antigens n = 9 Median (IQR)	
$Chga^+$	3.7 (0.5)	0.0183
$CK19^+$	5.8 (0.9)	
$HPX1^+$	64.5 (1.1)	
$Cpep^+$	6.3% (1.5)	

Demonstrates the major cell populations within islet cell preparations and the percent of ABH antigen expression within those cell populations. Expression of ABH antigen expression on C-peptide cells is also presented as a cell population of special interest to islet transplantation.

4.2.4.3 Human islets: Acinar cell ABH antigen expression is further confirmed within human islet preparations using immunohistochemistry

To further evaluate islet preparations, and confirm that the small number of cells found to be positive for ABH antigens with flow cytometry represented autofluorescence, we performed additional immunohistochemistry of isolated islet preparations (n = 3 *per* blood group). In these samples, we again evaluated $ChgA^+$ and $HPX1^+$ cells, demonstrating that only acinar cells expressed ABH antigens (Figure 4.2.5A). $CK19^+$ cells also failed to express ABH antigens (Figure 4.2.5B).

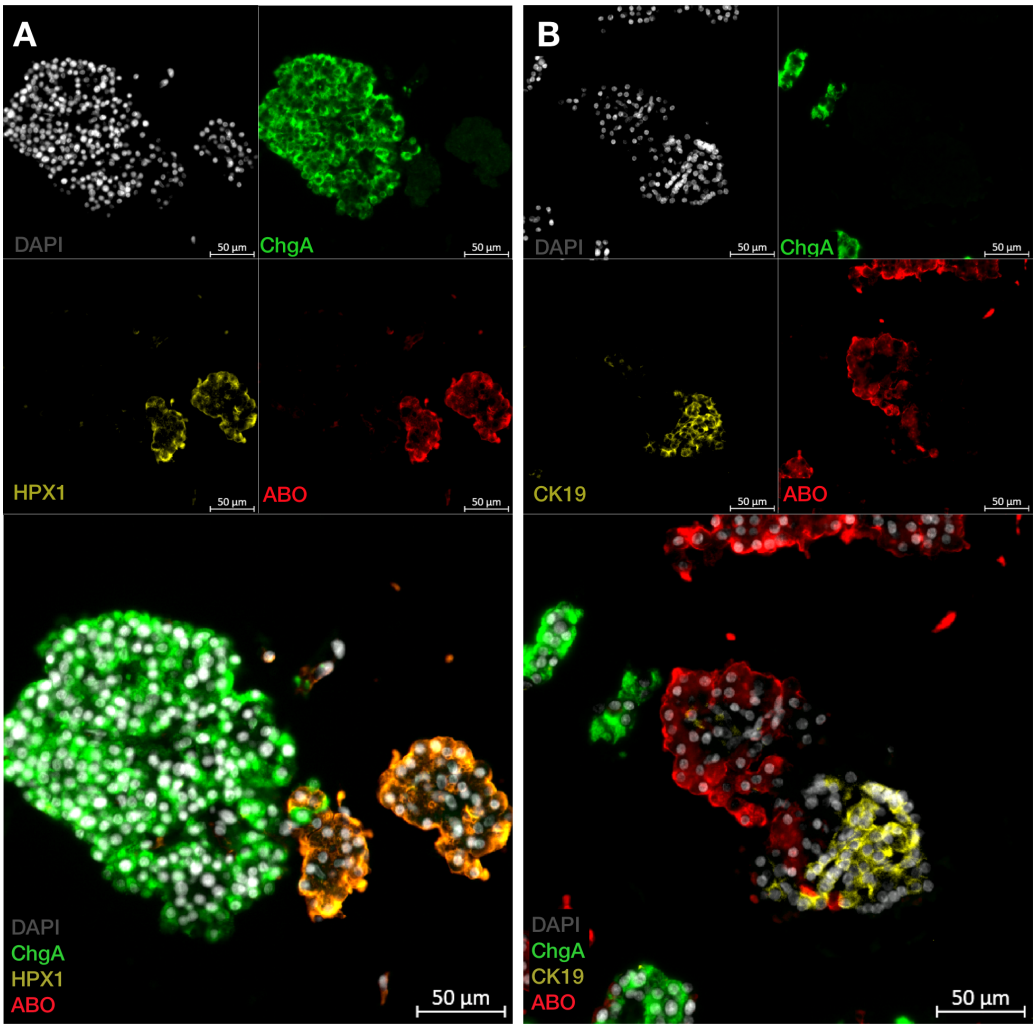

Figure 4.2.5 Fluorescence immunohistochemistry evaluation of the major cell populations within human islet preparations and the associated ABH antigen expression within those cell populations.
A) Evaluation of the cells expressing chromogranin A (ChgA), pancreatic acinar (HPX1), and human ABH antigen markers with the ensuing merged image. B) Evaluation of the cells expressing CK19 ductal marker (CK19), pancreatic acinar (HPX1), and human ABH antigen markers with the ensuing merged image. Figure is a representative image from patient with ABO-A blood type, with representative images of ABO-B and ABO-O presented in supplementary material. n = 3 per ABO blood type completed with representative images displayed.

Cpep$^+$, and Gluc$^+$ cells also demonstrated a lack of ABH expression (Figure 4.2.6A-B). Too few Stt$^+$ cells were present in islet preparations to accurately determine ABH antigen expression. A substantial proportion of HPX1$^+$ clusters remained and expressed ABH antigens within all islet preparations. No immunohistochemistry differences were noted in islet preparations regardless of ABO blood group (n = 3 *per* blood group, i.e. A, B, or O, **Appendix** Figure S4.2.11).

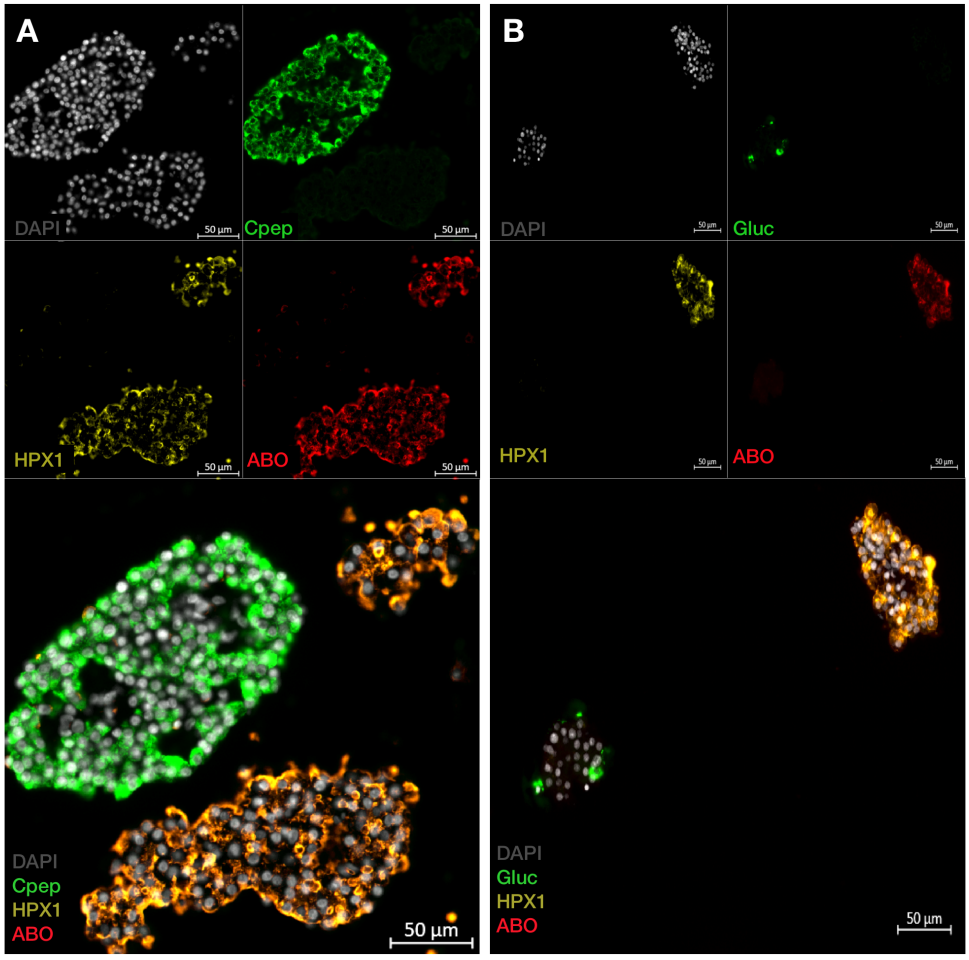

Figure 4.2.6 Fluorescence immunohistochemistry evaluation of the endocrine cell subpopulations including the A) C-peptide (Cpep+) and B) glucagon (Gluc+) expressing cells within human islet preparations.

Figure is a representative image from patient with ABO-A blood type, with representative images of ABO-B and ABO-O presented in the Appendix. n = 3 per ABO blood type completed with representative images displayed.

4.2.4.4 Pancreatic endoderm cells: PEC-01 pancreatic endocrine progenitors and post-transplant PEC-01-derived islets do not express ABH antigens

Finally, we evaluated PEC-01 cells prior to transplant and following *in vivo* maturation into islets after renal subcapsular transplantation. Three PEC samples, each differentiated using previously published protocols from one cell line (Cyt49), were evaluated. Evaluation of the immature PEC-01 clusters demonstrated expression of pancreatic and duodenal homeobox 1 ($PDX1^+$) and $ChgA^+$ but without any ABH antigen expression (Figure 4.2.7A). Quantification of PEC-01 cells using flow cytometry demonstrated that 48.5% (IQR 2.4%) expressed ChgA, while 32.3% (IQR 2.6) were NKX6.1+. Assessment of these subpopulations demonstrated a negligible number of ABH antigen expressing ChgA+ cells (1.7%, IQR 0.4) or NKX6.1+ cells (0.7%, IQR 0.5). Analysis of *in vivo* islet-like clusters following 24-weeks of maturation within the renal subcapsular space demonstrated that $ChgA^+$ cells within grafts showed no ABH antigen expression and a complete lack of human pancreatic acinar ($HPX1^+$) cells (Figure 4.2.7B). Similarly, the evaluation of the $Cpep^+$, $Gluc^+$, and Stt^+ cells demonstrated no ABH antigen expression throughout the graft (**Appendix** Figure S4.2.12A-C). While there appeared to be $CK19^+$ ductal cells that had matured from transplanted PECs, these cells also lacked ABH antigen expression (**Appendix** Figure S4.2.12D). On the contrary, when isolated human islets were transplanted into the renal subcapsular space, ChgA+ cells did not express ABH antigens,

while remnant acinar (HPX1+) tissues continued to express ABH antigens (n = 1, **Appendix Figure S4.2.13**).

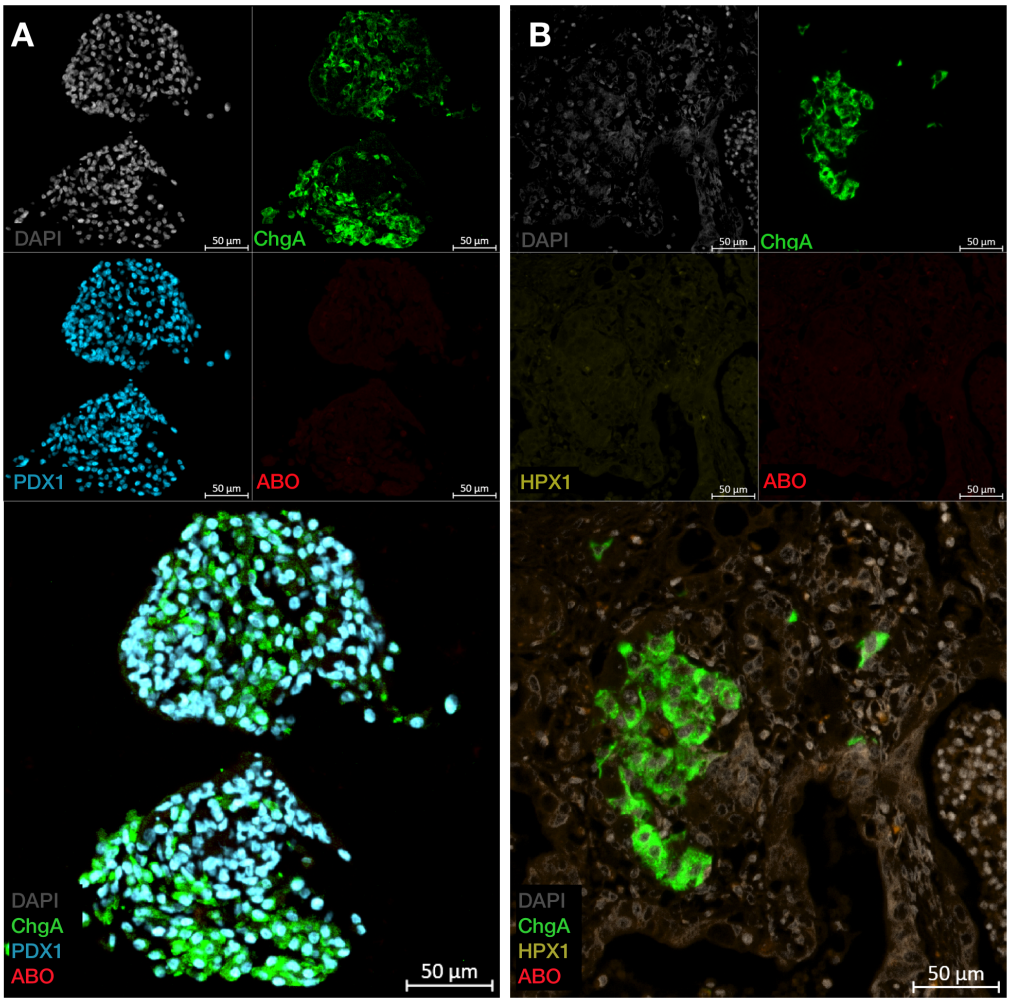

Figure 4.2.7 Fluorescence immunohistochemistry evaluation of ABH antigen expression within human embryonic stem cell-derived pancreatic endocrine progenitor cells and mature embryonic stem cell-derived islet-like cells within the renal subcapsular space of Rowett Nude (RNU) immunodeficient rats.
A) Human embryonic stem cell-derived pancreatic endocrine progenitor cells evaluated for endocrine cells (ChgA+), pancreatic and duodenal homeobox 1 (PDX1), and ABH antigen expression. B) Evaluation of the cells expressing chromogranin A (ChgA), pancreatic acinar (HPX1), and human ABH antigen markers within the renal subcapsular graft. n = 3 pancreatic endocrine progenitor cell preparations evaluated.

4.2.5 **Discussion**

This is the first study to evaluate ABH antigen expression within isolated human clinical-grade islets, ESC-derived PEC, and engrafted ESC-derived islet-like clusters. We demonstrate that within the pancreas, endocrine cell populations and ductal cells do not express ABH antigens. However, pancreatic acinar tissues strongly express ABH antigens and represent an important proportion of cells within an isolated human islet preparation, likely limiting the potential for safe ABOi ITx. Conversely, ESC-derived PEC and their ensuing engrafted, matured *in vivo* islets do not appear to express ABH antigens, suggesting that ABO compatibility may not be required for ESC-derived ITx.

These findings suggest that ABOi ITx using isolated human pancreatic islets should not be applied without further interventions or purification techniques. Acutely, ABOi ITx could result in islet destruction, acute graft loss, inflammation-mediated portal venous embolism, or vasculopathy, which could place patients at risk of life-threatening complications. The instant blood-mediated inflammatory reaction (IBMIR) following ITx provides a low-grade example of what may occur if current islet preparations were transplanted across blood groups[27]. During IBMIR, inflammatory cell infiltration and coagulation cascade activation occur, with substantially increased levels of thrombin-antithrombin III complex, inflammatory interleukins, and inflammatory cells leading to islet destruction[27]. We suspect the ABOi inflammatory reaction would be even greater than IBMIR and produce substantial islet loss with negative effect on islet engraftment and vascularization[28]. An example of this is also seen following ABO-incompatible allogeneic stem cell transplantation, where graft loss occurs unless plasma

exchange to eliminate the recipients ABO antibodies occurs[29]. Even if a small number of islets were not destroyed and successfully engrafted, we know that engrafted islet volume is independently associated with favorable clinical outcomes[3]. Overall, we hypothesize that based on current ITx experience and evidence from non-islet ABOi cell transplant that substantial acute graft loss and early graft failure would occur with ABOi human pancreatic ITx and may limit any clinical utility.

Despite these findings, further islet purification to eliminate ABH antigen presenting acinar tissues could be considered. Dorrell et al. (2008) have previously shown the capacity to dissociate islets into single cell suspensions and apply fluorescence antigen cell sorting (FACS) to achieve cell preparations without $HPX1^+$ cells[26]. Similarly, others have used FACS to isolate cells expressing unique antigens to generate pure β-cell populations[30]. Magnetic microbead immunoprecipitation represents another alternative technique capable of isolating highly pure islet cell populations based on cell surface markers[31,32]. However, purifying islet preparations using FACS[33] or magnetic microbeads[32] causes substantial cell death, dramatically reducing islet yield. Again, this cell loss may completely abrogate any benefits of ABOi ITx, and potentially lead to clinically inadequate islet cell preparations. Alternatively, a recent study demonstrated >97% ABH antigen elimination following enzymatic treatment of human lungs, introducing a potential intervention that could be applied to eradicate ABH antigen expression within human islet preparations[34]. Finally, it remains possible that culturing islet preparations, or even whole pancreatic tissues, with ABOi serum could lead to immune-mediated acinar tissue lysis leaving highly pure islet cells for transplant. Evaluation of approaches to further purify or eliminate ABH antigen expression within islet preparations may be of interest, however, with

current promise of stem cell-derived transplant and their lack of ABH antigen expression, a solution may already exist.

In this study, we show that stage 4 differentiated ESC-derived PECs and their ensuing engrafted islet-like cells do not express ABH antigens. This suggests that ABO-incompatible ITx using this cell source may be achievable. As current allogeneic ITx is only matched for ABO group, a single ESC cell source for transplant may be possible. This may improve the scalability of ESC-derived islet cell therapies. Investigation of the *in vivo* immune response to ABOi PEC transplant potentially using humanized mouse models or subcutaneous devices to enable safe in-human evaluation would be beneficial. The expression of other HLA antigens on PECs also remains unclear and recipients with sensitization to specific HLA markers may still require matching for HLA class I- and class II-reactive antibodies[35]. Finally, it should be mentioned that ViaCyte's PEC-01 cells tested in the current study are derived from a universal blood group O donor, and thus the argument about ABO-matching in this context could be mute.

Findings should be contextualized within the study's limitations. This includes the single-center nature of our study and its potential impact on islet isolation protocols and islet quality While islet isolations in this study were conducted by experts in the field with thousands of previous human islet isolations, our findings may not be generalizable to islets obtained using alternative techniques. Moreover, while we have studied the major pancreatic cell populations in this study, we did not address the capillary intra-islet microvasculature that presumably also expresses ABH antigenic targets, and could lead to rapid islet demise in an ABOi transplant. We did attempt to stain and quantify this small population but were unable to reliably identify these. Furthermore, while we demonstrate ABH antigen expression within pancreatic acinar tissue, the

ABH-subtype (I-IV) was not evaluated, and studies characterizing the ABH subtype expressed by pancreatic tissues would be of interest to better characterize the expected immune reaction following ABOi ITx[36,37]. Characterization of ABH-subtype expression within islets may facilitate ABOi transplant, as seen for ABOi renal transplantation[36,37]. While we demonstrate a lack of ABH antigen expression within PEC and ESC-derived islets, the *in vivo* immune response remains unclear and requires investigation. Additionally, while stage 4 ESC-derived PECs and *in vivo*-matured islet-like clusters do not appear to express ABH antigens, these findings should not be extrapolated to earlier stages and investigation of ABH antigen expression clusters from earlier differentiation stages prior to their transplant would be beneficial if transplantation of less mature cells is to be considered. Similarly, evidence suggests that induced pluripotent stem cells express ABH antigens[38], and extrapolation of these results to islets derived from other stem cell sources should be cautioned. Evaluation of ABH antigens on cells differentiated from additional cell lines (such as UES8 or induced pluripotent stem cell lines) would be beneficial.

This is the first study evaluating ABH antigen expression within isolated human islets, ESC-derived PEC, and post-transplant ESC-derived islets. Results suggest that human pancreatic ITx should remain ABOc due to contaminant acinar tissue expressing ABH antigens within islet isolates. Conversely, ESC-derived PEC and *in vivo* ESC-derived islets do not express ABH antigens, offering the potential for ABOi β-cell replacement treatments and supporting scalability of these cell therapies. Future evaluation of the *in vivo* immune response and other immunogenic antigens on these cells remains of interest for future examination.

4.2.6 **Appendix: chapter 4 subsection 2**

Table S4.2.2 Antibodies and concentrations used for flow cytometry and immunohistochemistry.

Antibody	Fluorophore	Primary Antibody Supplier (catalog number)	Secondary Antibody Supplier (catalog number)	Dilution for flow cytometry	Dilution for immunohistochemistry
Anti-A*	FITC	BD (550807)	N/A	1:200	1:100
Anti-B*	FITC	Fisher (**MA1-7672)**	N/A	1:200	1:100
Anti-O(H)*	FITC	Fisher (53-9810-82)	N/A	1:200	1:100
HPX1	PERCP	Novus (NBP1-18951PCP)	N/A	N/A	1:100
HPX1	PE	Novus (NBP1-18951PE)	N/A	1:100	N/A
Chromogranin A	AF647	Sigma (SAB5500082)	eBioscience (51-7177)	1:50	1:100
C-peptide	AF647	BD (565831)	N/A	1:100	1:100
Glucagon	AF647	Sigma (G2654)	Invitrogen (A31571)	N/A	1:100
Somatostatin	AF647	R&D (MAB2358)	Invitrogen (A21247)	N/A	1:100
CK19	Secondary AF 594	Abcam (ab52625)	Fisher (A11012)	1:100	1:100
PDX1	PE	BD Pharmingen (562161)	N/A	1:100	1:100
NKX6.1	AF647	BD Pharmingen (563338)	N/A	1:100	1:100

All secondary antibodies for immunohistochemistry were diluted to 1:250 and all secondary antibodies for flow cytometry were diluted to 1:500. *Antibodies represent anti-human blood group A, B, and O (H) antigens.

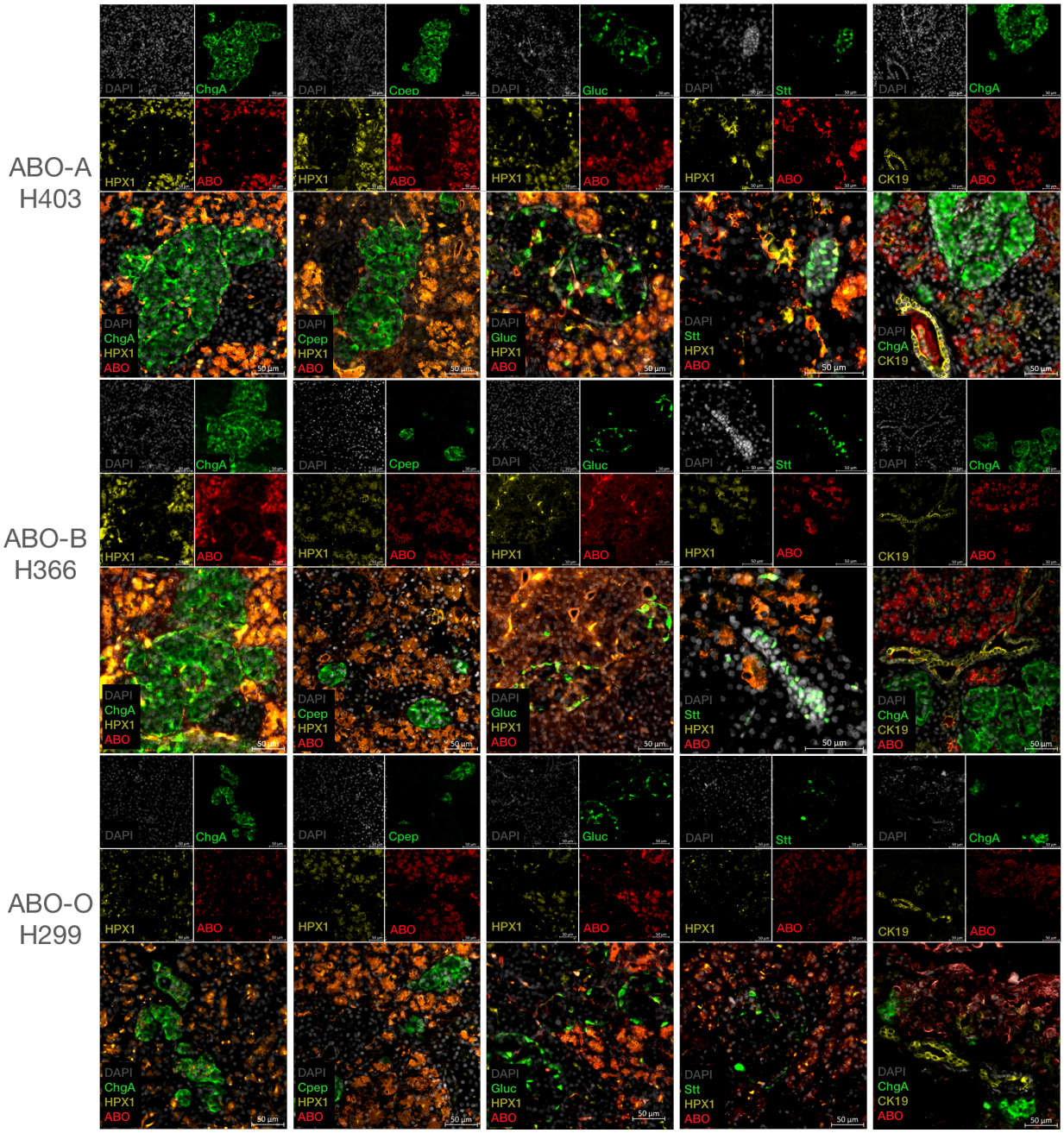

Figure S4.2.8 Fluorescence immunohistochemistry evaluation of the endocrine (including subpopulations), acinar, and ductal cells within pancreas tissue samples from patients with blood type A, B, and O and the associated ABH antigen expression within those cell populations.

n = 9 with three samples from each ABO blood group.

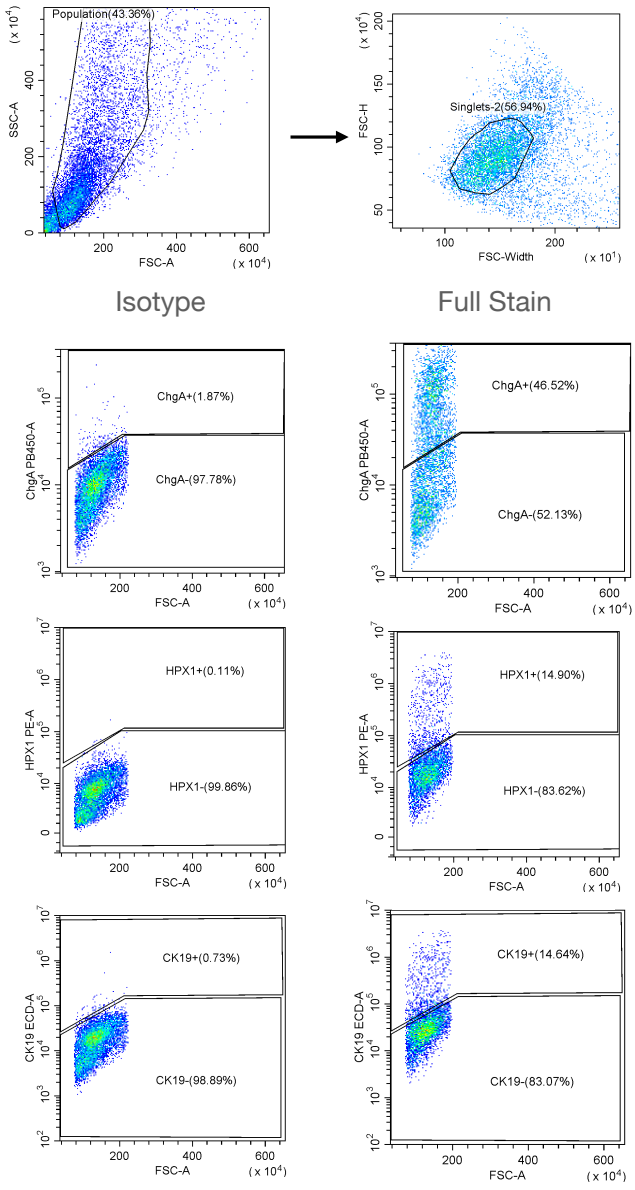

Figure S4.2.9 Gating strategy to evaluate single cells from isolated islet preparations. As shown, we selected the singlet cell population and used isotype controls to delineate the negative population. Fully stained cells were then interrogated and demonstrate the percent of singlets expressing being endocrine cells ($ChgA^+$), ductal cells ($CK19^+$), and acinar cells ($HPX1^+$).

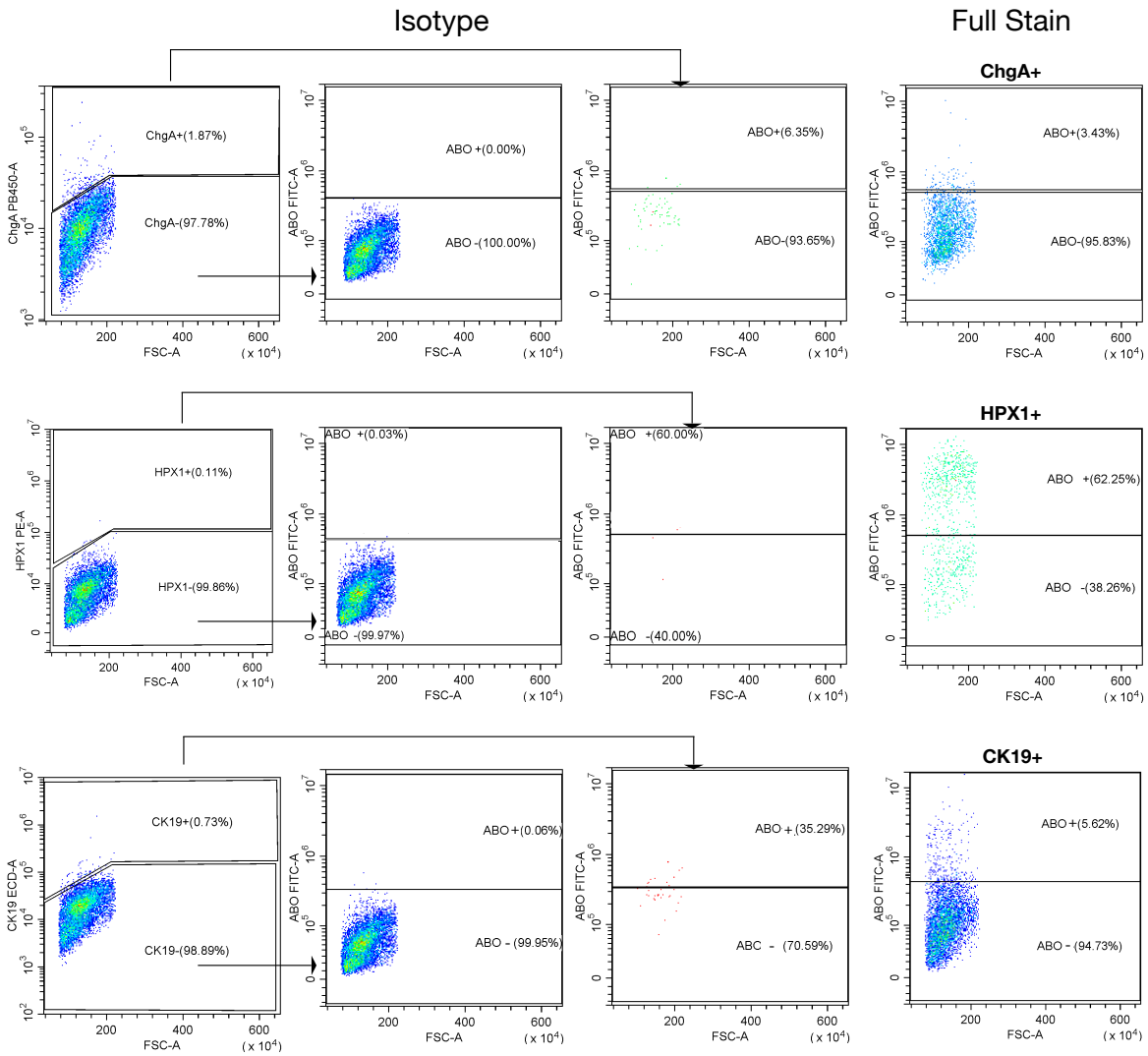

Figure S4.2.10 Gating strategy to evaluate human ABH antigen expression on single cells determined to be endocrine (ChgA$^+$), acinar (HPX1$^+$), or ductal (CK19$^+$) cells.
Within the isotypes we characterized the negative ChgA, HPX1, and CK19 cells and described the ABH negative gating. Those gates were applied to fully stained cells (far right) where ABH expression was demonstrated in each single cell population.

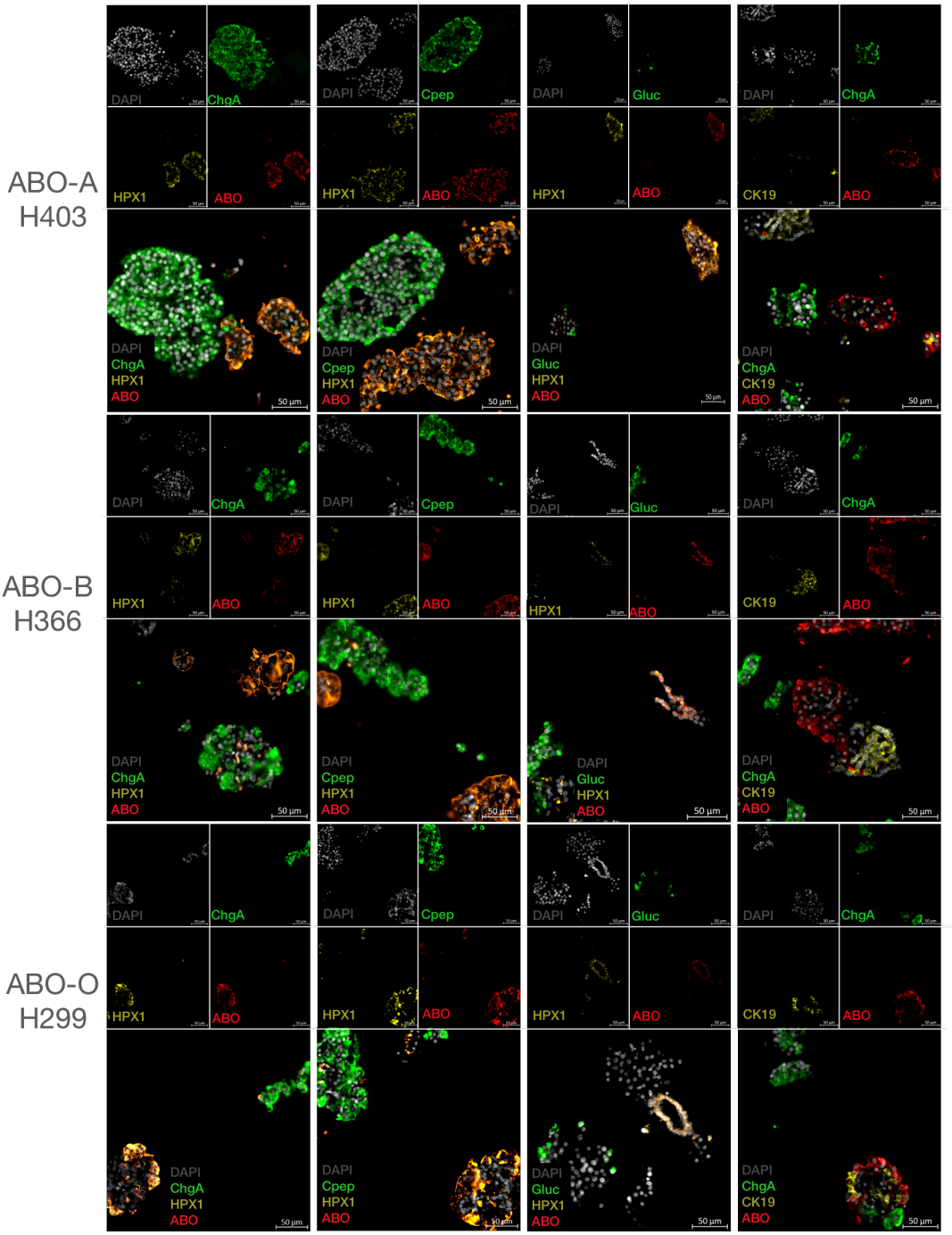

Figure S4.2.11 Fluorescence immunohistochemistry evaluation of the endocrine (including subpopulations), acinar, and ductal cells within the isolated islet preparations from patients with blood type A, B, and O and the associated ABH antigen expression within those cell populations.

n = 9 with three samples from each ABO blood group.

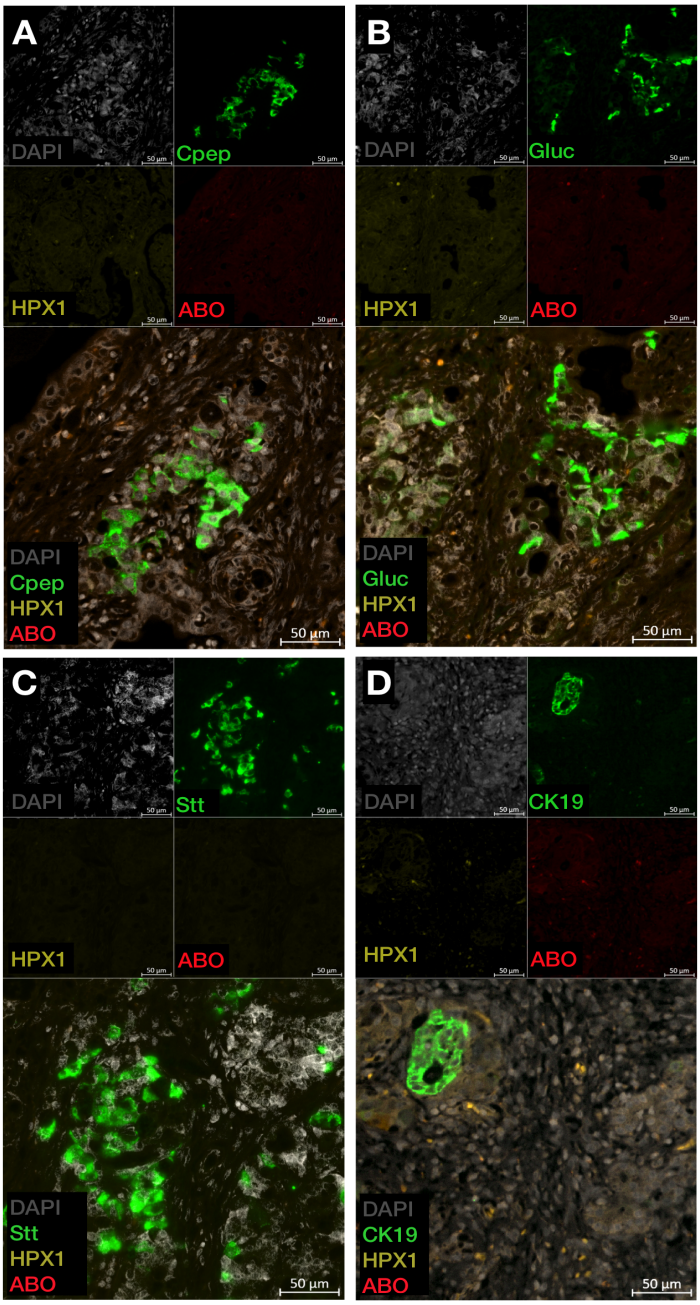

Figure S4.2.12 Fluorescence immunohistochemistry evaluation of ABH antigen expression within human endocrine cell subpopulation, human pancreatic acinar (HPX1+), and human ductal (CK19+) following transplantation of embryonic stem cell-derived pancreatic endocrine progenitors in the renal subcapsular space of immunodeficient rats. ABH antigen and HPX1 expression within A) C-peptide ($Cpep^+$), B) Glucagon ($Gluc^+$), C) somatostatin (Stt^+), and D) ductal ($CK19^+$) cells. n = 3 transplanted pancreatic endocrine progenitor cell preparations evaluated with representative images shown.

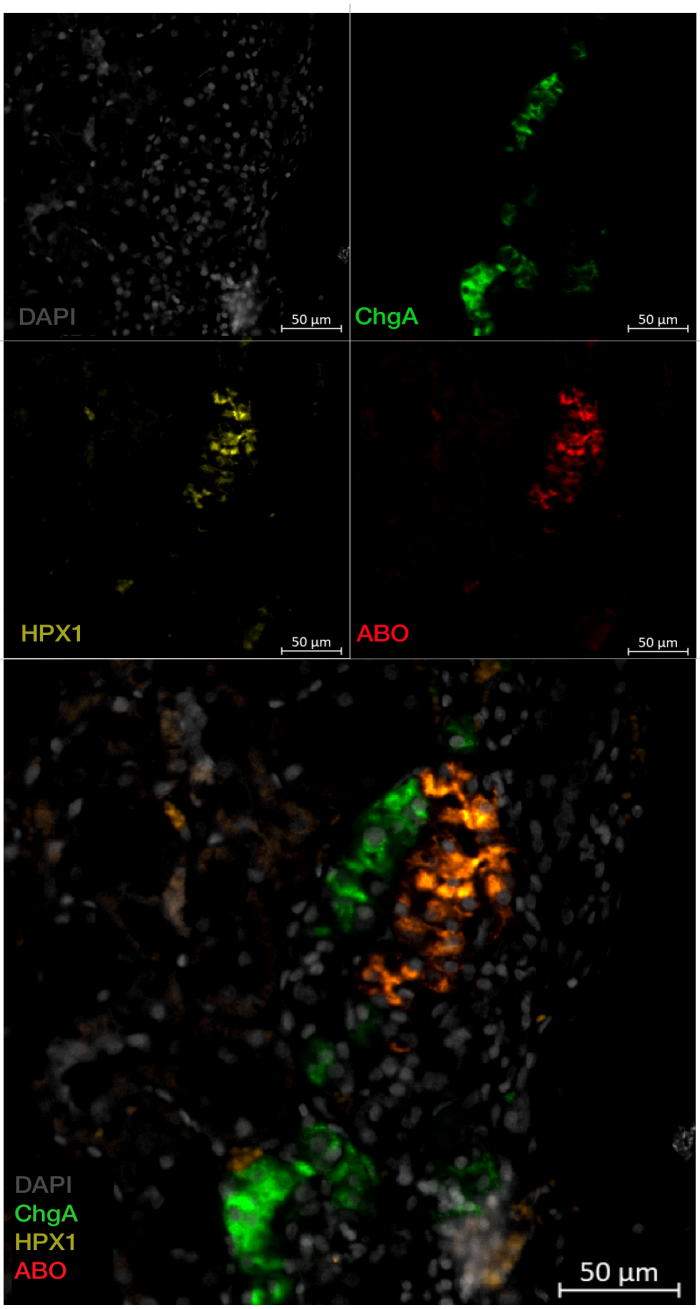

Figure S4.2.13 Fluorescence immunohistochemistry evaluation of ABH antigen expression within human endocrine cell subpopulation, human pancreatic acinar (HPX1+), and human ductal (CK19+) following transplantation of human islets into the renal subcapsular space of immunodeficient rats.

ABH antigen and HPX1 expression within A) C-peptide ($Cpep^+$), B) Glucagon ($Gluc^+$), C) somatostatin (Stt^+), and D) ductal ($CK19^+$) cells. n = 1 transplanted human islet preparations evaluated as a positive control for Figure 4.2.7.

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Chapter 5: Strategies for Implementation, Evaluation, and Further Optimization of Stem Cell-Derived Islet Transplantation

- Chapter 5 subsection 1: Current Status, Barriers, and Future Directions for Humanized Mouse Models to Evaluate Stem Cell Based Islet Cell Transplant
- Chapter 5 subsection 2: Outcomes Following Extrahepatic and Intraportal Pancreatic Islet Transplantation: A Comparative Cohort Study
- Chapter 5 subsection 3: C-peptide Targets and Patient-Centered Outcomes of Relevance to Cellular Transplantation for Diabetes
- Chapter 5 subsection 4: Implementation of Stem Cell-Derived Islet Transplantation in Patients with Type 2 Diabetes: Can Novel Diabetes Disease Subtypes Guide Clinical Implementation?

Chapter Summary:

Chapter 5 focuses on approaches to implement and evaluate stem cell-derived islet transplantation, grouping together two reviews and two clinical studies. First, chapter 5.1 evaluates the potential and limitations of humanized mouse models to evaluate immune considerations for stem cell-derived islet transplantation. Considering the limitations of humanized models and limited high quality alternative animal models chapter 5.2 evaluates the potential of using extrahepatic sites for evaluation of stem cell-derived islets by evaluating in human results from omental, gastric submucosa, and subcutaneous transplant. Subsequently, chapter 5.3 provides a second clinical study evaluating patients receiving intraportal islet transplant to define optimal cut-offs for C-peptide, stimulated C-peptide, and BETA2 scores associated with patient important outcomes. This data is provided to enable evaluation of stem cell-derived islet clinical trials and as a benchmark for such therapies. Finally the chapter concludes with a review of diabetes subtypes to evaluate potential targeted populations with type 2 diabetes that may benefit from stem cell-derived islet transplantation. Overall, the chapter is intended to provide data and insight to allow clinical translation of stem cell therapies.

5.1 Chapter 5 subsection 1 – Current Status, Barriers, and Future Directions for Humanized Mouse Models to Evaluate Stem Cell Based Islet Cell Transplant

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Current Status, Barriers, and Future Directions for Humanized Mouse Models to Evaluate Stem Cell–Based Islet Cell Transplant

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Abstract

Islet cell transplant (ITx) continues to improve, with recently published long-term outcomes suggesting nearly 80% graft survival, leading to improvements in glycemic control, reductions in insulin doses, and near-complete abrogation of severe hypoglycemia. Unfortunately, access to ITx remains limited by immunosuppression requirements and donor supply. Discovery of stem cell–derived functional islet-like clusters with the capacity to reverse diabetes offers a renewable, potentially immunosuppression-free solution for future widespread ITx. Evaluation and optimization of these therapies is ongoing, but may one day provide a realistic cure for type 1 diabetes. However, stem cell–based ITx has unique immunologic questions that remain unanswered. Here, we briefly synthesize current approaches for stem cell–derived ITx, review humanized mice models, and elaborate on the potential of humanized mice models for bridging the gap between current small rodent models and human clinical trials for allogeneic and autologous inducible pluripotent stem cell (iPSC)–based ITx while highlighting limitations and future directions.

Keywords

Diabetes · Humanized mouse model · Immunosuppression · Inducible pluripotent stem cells · Islet cell transplant · Transplant

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5.1.1 **Abstract**

Islet cell transplant (ITx) continues to improve, with recently published long-term outcomes suggesting nearly 80% graft survival leading to improvements in glycemic control, reductions in insulin doses, and near complete abrogation of severe hypoglycemia. Unfortunately, access to ITx remains limited by immunosuppression requirements and donor supply. Discovery of stem cell derived functional islet-like clusters with the capacity to reverse diabetes offers a renewable, potentially immunosuppression free solution for future widespread ITx. Evaluation and optimization of these therapies is ongoing, but may one day provide a realistic cure for type 1 diabetes. However, stem cell-based ITx has unique immunologic questions that remain unanswered. Here, we briefly synthesize current approaches for stem cell-derived ITx, review humanized mice models, and elaborate on the potential of humanized mice models for bridging the gap between current small rodent models and human clinical trials for allogeneic and autologous iPSC ITx while highlighting limitations and future directions.

5.1.2 **Introduction**

Twenty two years ago islet cell transplant (ITx) provided proof-of-concept for a cell-based cure of type 1 diabetes (T1D) when 100% insulin independence was achieved one year post-ITx in a small number of subjects using glucocorticoid-free immunosuppression [1]. Although long-term insulin independence only occurred in some patients, ITx has since proven to be a highly efficacious treatment for T1D patients with severe and recurrent hypoglycemia or severe glycemic lability [2]. With ongoing technical, immune, and engraftment techniques, substantial advances continue to be recognized [3-6]; new evidence has demonstrated comparable twenty-year patient survival between ITx and other T1D patient cohorts and 10-year graft survival rates of nearly 80% with sustained improvements in glycemic control, reductions in insulin doses and near complete abrogation of severe hypoglycemia [4,7-9]. The primary barriers to a more widespread use of ITx are lifelong immunosuppression requirements and limited donor supply. Embryonic stem cells (ESCs) and Induced pluripotent stem cells (iPSCs) present an exciting development and a path to simultaneously overcoming both barriers [3,4,10,11]. With iPSC ITx, the reprogramming transcription factors (Oct3/4, Sox2, Klf4 and c-Myc) are overexpressed in diverse mature cells (e.g., peripheral blood cells) to reverse them into a pluripotent state [12,13]. Using ESCs and iPSCs functional islet cell clusters for transplant can be generated following a well-defined protocol [3,14,15]. However, as stem cell-based ITx advance towards clinical trials, the question remains whether evaluation in rodents is sufficient or indeed confounding to demonstrate translational efficacy and safety.

While mice offer economical and ethical models to study ITx, their immune systems are dissimilar to those of humans, which limits their utility to evaluate immunogenic responses related to transplantation [16-18]. Historically, chimpanzees have helped bridge the gap between small rodent animal models and clinical trials in humans, however this has become less feasible due to ethical and funding limitations [19,20]. These limitations have led to an expanding interest in chimeric and humanized mouse models that could better replicate the human immune system.

In this review, humanized mice models refer to immunodeficient mice engrafted with portions of a human immune system. Ideally, these models would display an entire, innate and acquired, human immune system. Current efforts have focused on generating mouse strains capable of representing important aspects of human immune responses, more than the whole immune system. While it has been postulated that humanized mice may provide a way of evaluating stem cell-based ITx [21], it remains unclear whether they can be utilized for evaluation of autologous iPSC ITx. Here, we briefly synthesize current approaches for stem cell-based ITx, review humanized mice models, and elaborate on the potential of humanized mice models for bridging the gap between current small rodent models and human clinical trials for allogeneic and autologous stem cell-based ITx while highlighting limitations and future directions.

5.1.3 **Stem cell-based Islet Cell Transplantation**

Currently, clinical ITx demonstrates proof-of-concept for a cell-based treatment for T1D [2,5-8,22,23]. As a way of addressing limitations in organ donor supply, ongoing work with ESCs and iPSCs have shown capacity to differentiate into functional islet-like clusters capable of reversing diabetes (Figure 5.1.1) [3,13-15,24,25]. Their promise offers a path to personalized cell-based therapies

that could ultimately offer a true cure for T1D [3,10,12]. Herein, we discuss ESCs and iPSCs as they relate to humanized mouse models, for a recent review on stem cell-based cellular therapies in diabetes see Verhoeff et al. (2021) [3,4]. As it relates to humanized mouse models, two approaches for stem cell-based ITx have been suggested: allogeneic and autologous, each with unique immune-related considerations that require further evaluation.

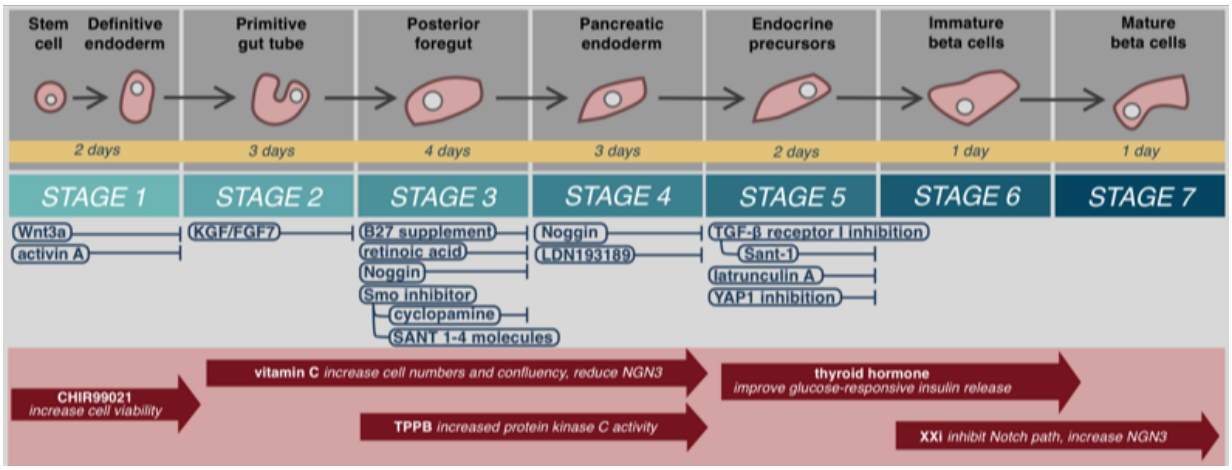

Figure 5.1.1 Embryological differentiation and maturation of islet cells.
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Allogeneic stem cell-based ITx involves generating human leukocyte antigen (HLA)-matched or immune silenced ESC or iPSC banks that can be used as a source of islet differentiation for all patients. It is highly likely that matching for major HLA antigens alone would be doomed to failure and mismatched minor antigens still generate potent, destructive immune response. Therefore generalized immunosuppression or alternatively some local or CRISPR-Cas9 genetic edit-based approaches would be needed to sustain allograft cell survival. Allogeneic approaches do however offer a technique that would simplify up-scaling stem cell-based ITx to provide cells

for >8 million T1D patients [26]. However, modified allogeneic islets represent a technically difficult approach, and currently only offer reduction and not complete elimination of immunosuppression. Several approaches to generating immune tolerated allogeneic stem cell-based islets are being considered. Genetically-modified stem cells may enable expression of immunotolerant molecules such as IL-10 or PD-L1 [27,28]. Alternatively, generating ESCs or iPSCs without HLA class 1 molecule expression may further reduce graft rejection and immunosuppression requirements. Han et al. (2019) and others have recently generated iPSCs without HLA class I molecules and expressing the immunomodulatory factors PD-L1, HLA-G, and CD47, which resulted in with blunted T-cell reactivity, minimal NK cell-mediated death and macrophage phagocytosis [29-31]. Similarly, Viacyte's PEC-QT multitiered approach also takes advantage of these concepts and combines a genetically-modified clonal ESC line expressing PD-L1 and lacking HLA class I molecules (i.e., β microglobulin), with their PEC-Direct subcutaneous maroencapsulation device, and is expected to enter clinical trials soon [3,4,11,32]. However, islet cell maturation and their physiologic capacity to maintain normoglycemia after genetic modification remains unproven. Similarly, it remains uncertain whether allograft rejection will still occur despite genetic manipulation. Ongoing studies are certainly required prior to clinical implementation of allogeneic stem cell-based ITx.

Alternatively, individual iPSCs could be generated for each patient to create personalized islet-like cells for autologous ITx, which may eliminate the need for immunosuppression altogether. However, autologous islet cell clusters may still be subjected to recurrent autoimmune graft destruction [3,4]. While autologous iPSC islets would not face allogenecity, similar to what occurs following autologous ITx after total pancreatectomy, the effect of recurrent autoimmunity

represents remains a key issue with uncertain effects. However, if recurrent autoimmunity does prove to be a barrier, combining "immune reset" approaches with autologous ITx, may provide an effective solution to control recurrent autoimmunity [33-37]. Additionally, autologous iPSC ITx is more difficult for scale-up since it requires generation of unique iPSC lines for each patient, amplification, maturation and subsequent safety screening prior to ITx to identify and prevent genetic mutations or off-target effects. Automation, artificial intelligence (i.e. machine learning), process automation, large-scale bioreactors, standardized protocols, and increased efficiency of processes will be required to enable cost-efficient autologous iPSC ITx [3]. Despite substantial promise, there remains unanswered questions regarding recurrent autoimmunity and uncertainty regarding feasibility of up-scaling with research to evaluate these questions ongoing.

5.1.4 **Immune Responses**

Differences between allogeneic and autologous responses for stem cell-based ITx are key when evaluating the utility of humanized mice models for preclinical evaluation. Alloimmune responses occur through direct recognition of donor major histocompatibility complex (MHC) molecules and indirect recognition of graft-derived peptides through recipient MHC molecules. In humans, the key MHC molecule is the human leukocyte antigen (HLA). Recognition of MHC molecules by recipient antigen-presenting cells (APCs) and co-stimulattory signals leads to activation and amplification of recipient effector T-cells with ensuing direct cytotoxic graft destruction [38]. Alloimmunity begins immediately after islet infusion and can lead to massive islet cell destruction that could prevent any substantial benefit following ITx. In contrast, autoimmune responses, such as in T1D, involves destruction of pancreatic β-cells by infiltrative mononuclear

inflammatory cells, including macrophages, CD4+, and CD8+ T cells [39]; high concentrations of interleukin 2 (IL2) lead to activated CD4+ cells, that stimulate CD8+ direct-cell mediated apoptosis, and inflammation-driven insulitis within the pancreas [40]. While macrophages and dendritic cells act as antigen presenting cells (APCs) that initiate the cytotoxic T-cell response [41-43], it is T cells that are central in the pathogenesis of T1D. In support of the key role of T-cells, inhibiting T-cells with cyclosporine slows T1D onset, and agammaglobulinemic patients without B-cells but with T-cells can still develop T1D [44,45]. Furthermore, transferring T-cells from a patient with T1D to a non-diabetic patient has shown to induce T1D in the recipient [46]. Secondary to insulitis and β-cell destruction by T-cells, specific antigens are exposed and patients acquire antibodies to insulin, islet cells, the cation efflux pump ZnT8, isoforms of glutamic acid decarboxylase 65 or 67 (GAD65 or GAD67), or the IA-2 secretory protein [42]. Autoimmunity occurs over months, with patients eventually becoming symptomatic once islet mass is reduced significantly. Recurrent autoimmunity in patients with T1D is largely understudied and remains difficult to evaluate, as patients receiving ITx are currently immunosuppressed. Concerning autologous iPSC ITx, theoretically, these patients continue to express islet specific antibodies, and may mount autoimmune responses to autologous iPSC islets, but this remains an unanswered question.

5.1.5 **Differences in Human and Murine Immune Systems**

While human and murine immune systems are highly conserved and unique with regards to only approximately 300 genes [47], acknowledging these differences provides context to current findings obtained from immunodeficient mouse. Additionally, understanding these differences

highlights the role of humanized mice to enable ITx evaluation under the effects of a human-immune system. Differences between mouse and human immune systems is only discussed briefly here, and specifically as they pertain to ITx. Reviews by Mestas and Hughes (2004), and Haley (2003) provide complete evaluation of differences between human and mouse immune systems [16,48].

The first major difference between humans and mice is the composition of circulating white blood cells (WBCs). Mice have a much higher proportion of lymphocytes (75-90% in mice vs. 30-50% in humans) and lower neutrophils (10-25% in mice vs 50-75% in humans) (Table 5.1.1). For ITx, a lower proportion of neutrophils may explain successful subcutaneous ITx in mice due to less neutrophil-directed foreign body response [49-52], while device and device-free techniques have shown less promising results in humans [53]. The proportion of lymphocytes and neutrophils that exist in humanized mouse models varies, and should be considered when evaluating outcomes; higher or lower lymphocyte populations may inadequately represent allograft responses or recurrent autoimmunity in these models.

Important differences for innate immunity are also present when comparing humans to mice (Table 5.1.1). Neutrophils, one of the primary cells responsible for early graft death and the instant blood mediated inflammatory reaction, express defensins as one of the key effector molecules in humans but not in mice [54]. Similarly, activation pathways for NK cells and macrophages is different between humans and mice [16,55,56]. The clinical significance of this for ITx evaluation remains uncertain; however, both NK cells and macrophages play key roles in islet cell allo- and auto- immunity [41,57-59], with macrophages also contributing to islet

angiogenesis and survival [60]. These cells also play an important role as antigen presenting cells (APCs), with species specific antigen identification and presentation capabilities [59,61].

Acquired immunity also has notable differences between species (Table 5.1.1). Immunoglobulin (Ig) subtype activation pathways are different between mice and humans; as an example, interleukin 13 induces IgE class switching in humans but has no effect for mice [16]. In evaluation of ITx this may lead to different Ig being activated with allogeneic or autoimmune reaction, although this has never been evaluated. Potentially most significant is that the differentiation of T-cells occurs via stimulation by different cytokines in each species. For example, in humans interferon-alpha produces Th1 T-cells, while it does not have an effect in mice [62]. Additionally, differentiated T-cells release species specific inflammatory molecules [16]. Again, Th1 and Th2 cells make IL-10 in humans, while only Th1 cells release IL-10 in mice [63]. While the effect of these differences have not been studied specifically for T1D or ITx, we know their outcomes are crucial. The balance of Th1 and Th2 cells is directly related to development of T1D and modification of cytokine expression, including IL-10, has clearly been shown to affect autoimmunity and allograft survival in islet cell transplant [42,64-66].

Table 5.1.1 Differences in Human and Murine Immune Systems

Immune System Difference	Mouse	Human	Effect for Islet Cell Transplantation
Proportion of neutrophils and lymphocytes in peripheral blood	10-25% neutrophils 75-90% lymphocytes	50-75% neutrophils 30-50% lymphocytes	Improved outcomes in subcutaneous and implantable devices for mice (less foreign body response)
Neutrophils with leukocyte defensins	Not present, defensins are expressed within small intestine	Present	Potentially reduced effect of IBMIR and early graft apoptosis
Macrophage and NK cell activation pathways	Different cytokines and messenger peptides lead to activation of macrophages and NK cells in mice and humans		Unclear clinical significance but both are crucial for allo and auto immunity including antigen presentation
Immunoglobulin class switching pathways	Interleukins and inflammatory markers produce variable immunoglobulin classes		Unclear clinical significance but may produce variable allo- or auto-immune reaction.
T-cell differentiation and function	Interferon-alpha produces Th1 T-cells Th1 and Th2 cells make IL-10	Interferon-alpha has no effect Only Th2 cells make IL-10	Inflammatory markers and cell types found to cause insulinitis or allograft rejection may be variable between groups

These differences highlight the need for evaluating interventions and novel immune therapies within a true human immune environment. The importance of these humanized mouse models has become clear in research related to human immunodeficiency virus vaccines [67,68], targeted oncologic immunotherapies [69], and human immunity [70], amongst others. However, the use of humanized models in the field of islet transplantation remains in its infancy and continues to face significant barriers. Understanding the history, current status and major limitations is paramount to move this area of study forward.

5.1.6 **Current Humanized Mouse Models**

It has been nearly 20-years since the discovery that immunodeficient mice would accept, engraft, and display specific aspects of the human immune system [71-73]. Since then, substantial

work has focus on optimizing both the recipient mice and the methods for immune system engraftment. In this section we review historical and current immunocompromised mice that accept immune system engraftment with evaluation of efficacy between types. We also review techniques used for human immune system engraftment into these recipient mice, with discussion of the benefits and drawbacks of these approaches specific to stem cell-based ITx evaluation. Finally, we discuss current evidence evaluating humanized models for ITx evaluation and consider the feasibility of these models for autologous and allogeneic stem cell-based ITx evaluation.

5.1.6.1 Immune Deficient Mice: Suitable Homes for a Human Immune System?

Early generation of humanized mouse models began with engraftment of NOD-scid mice. These models are homozygous for the severe combined immunodeficiency (*scid*) mutation and do not display any functional lymphoid tissue, which precluded development of both T and B cells. Given their highly immunocompromised state [74], these mice can accept engraftment of diverse tissues and cell types, including those from a human immune system. However, these models have numerous limitations that preclude their use to evaluate the complexity of a fully functional human immune system. Firstly, NOD-*scid* mice "leak" native T and B cells, which means that these cells slowly reappear and destroy human tissues and cells [21,75,76]. These mice also reintegrate Emv30 into their genome leading to thymic lymphoma and early death [76,77]. Finally, NOD-*scid* mice continue to exhibit NK cell function, leading to high rates of hematopoietic stem cell (HSC) and stem cell death, which severely limits efficient immune system engraftment [76,78,79]. Ongoing investigations have led to development of improved mouse strains to act as recipients for immune system engraftment.

To eliminate the initial barriers for humanization using of NOD-*scid* mice, the addition of *Rag1* and *Rag2* knockout mutations eliminated adaptive immune system "leak" [75,80,81]. Subsequent manipulation of NOD-*scid*-*Rag* knockout mice with targeted disruption of the IL-2 gamma-chain markedly reduced occurrence of lymphoma [80,82]. Combinations of the NOD-*scid*, *Rag* knockout, and IL-2-gamma disrupted mice have now generated the NOD-*scid* gamma strain (NSG), the NOD-*Rag1* gamma (NRG) and BALB/c-*Rag2* gamma (BRG) strains, which now represent the primary recipient strains for humanization [73,80,83]. Evaluation of immune system engraftment into NSG, NRG, and BRG immunodeficient mouse strains has demonstrated that NSG mice, and a similar NOG model, accept engraftment better than others [73,75,84-86]. As outlined by Ito et al. (2012), immune system engraftment success appears to occur with greatest success in NSG and NOG mice, followed by NRG>BRG>NOD/SCID [75].

5.1.6.2 Approaches to Engraftment

Optimization of immune system engraftment techniques have also occurred since the original description of HSC injection into immunocompromised mice (Figure 5.1.2). Importantly, each technique to generate humanize mouse models creates a unique immune system with relevant benefits and drawbacks, which can be exploited to evaluate allogeneic and autologous stem cell-based ITx (Table 5.1.2).

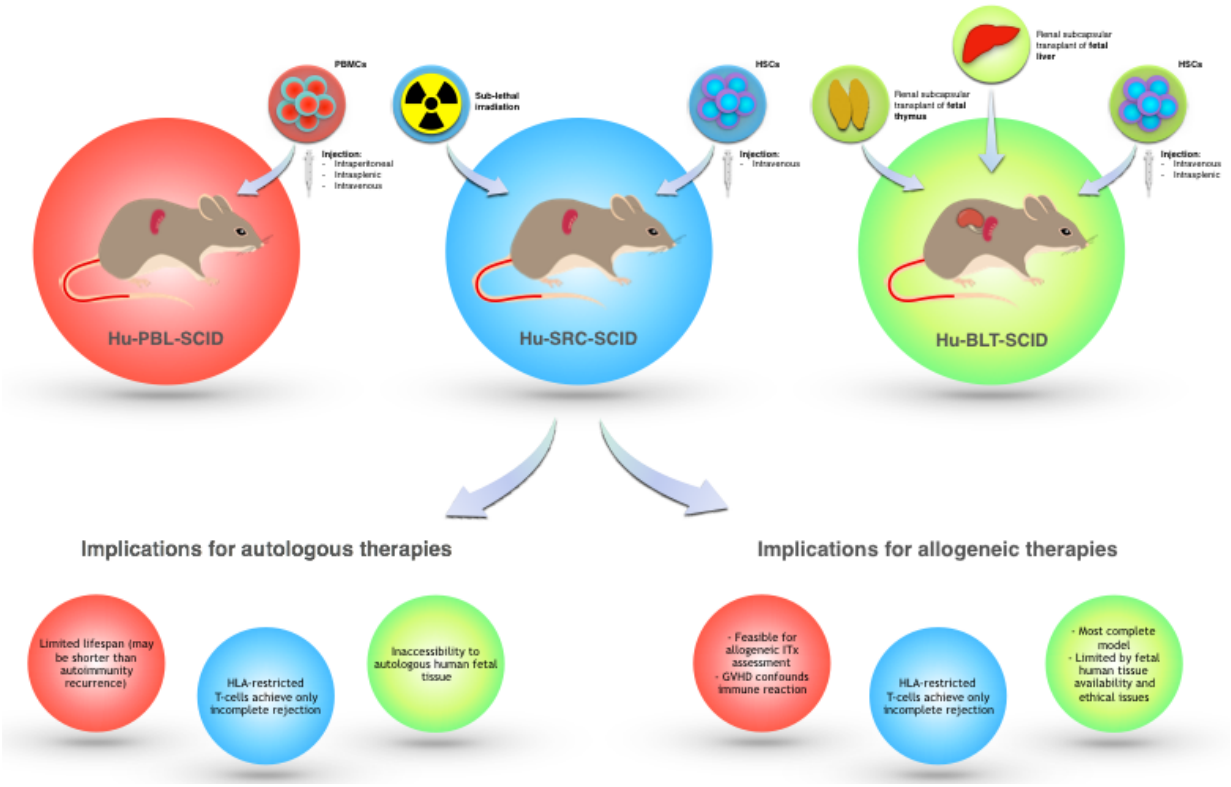

Figure 5.1.2 Techniques used to engraft the human immune system into immunocompromised mice and key considerations for their use to evaluate allogeneic and autologous iPSC ITx

Table 5.1.2 Benefits and limitations to various human immune system engraftment techniques to generate humanized mouse models.

Engraftment Technique	Benefits	Limitations
Hu-PBL-SCID	100% engraftment success Engraftment of activated T-cells	Limited lifespan due to GVHD Lack of humoral immunity HLA-restricted immune responses and APC presentation
Hu-SRC-SCID	Enables engraftment of multiple immune cell lineages (T-cells, B-cells, NK-cells)	HLA-restricted immune responses and APC presentation Lack of class switching
BLT	Only model with mucosal human immune system Most complete immune system	Limited lifespan due to GVHD Requires fetal tissue Technically challenging

iPSC: Induced Pluripotent Stem Cells; GVHD: Graft Versus Host Disease; HLA: Human Leukocyte Antigen, APC: Antigen Presenting Cell; ITx: Islet Cell Transplant.

Human peripheral blood lymphocyte SCID (Hu-PBL-scid) mice, first described by Mosier et al. (1988), are generated through intravenous, intraperitoneal or intrasplenic injection of peripheral blood lymphocytes (PBLs) [87]. This technique offers nearly 100% engraftment success, is technically simple, and enables engraftment of already activated T-cells [83,87]. However, hu-PBL-scid mice have a shortened lifespan due to graft versus host disease (GVHD), and live approximately one month before meeting end points requiring euthanasia [78,83,85,88,89]. Naturally, GVHD confounds assessment of immune responses in these mice [88]. Additionally, although Hu-PBL-SCID models enable engraftment of pre-activated T-cells, antigen specific T-cell activation and humoral immunity within the engrafted mouse does occur [19,90]; this happens because the mice display antigens on H2 molecules whereas engrafted APCs are human-HLA restricted [78,85,88,91].

The second approach is the human SCID-repopulating cell scid mice (hu-SRC-SCID), and involves engraftment of CD34+ HSCs injected into recipients via an intravenous or intrafemoral route into mice pre-treated with sublethal irradiation. This approach benefits from technical feasibility, and development of a broader human immune system displaying all lineages of hematopoietic cells, including T cells, B cells, NK cells, myeloid cells, and precursors for red blood cells, megakaryocytes and granulocytes [19,85]. The most significant limitation with hu-SRC-SCID mice is that, although they produce adequate T-cell populations, those T-cells fail to recognize, migrate, and reject allogeneic human antigens [92,93]. This is likely due to an absence of human thymic cells and lack of T-cell education to human MHC molecules [73,85,94,95]. Others have also postulated that lack of human-specific cytokines to direct these

engrafted human T-cells limits their migration, or that lack of peripheral lymph tissues in the recipient mouse strains limits their maturation [16,85,96].

To resolve HLA-restriction and partially developed acquired immunity, SCID-Hu and BLT models were developed. These involve implantation of fetal liver and thymus fragments under the renal capsule in adult mice to enable HLA expression, immunoglobulin class switching, and T-cell activation [19,85,96]. The BLT model adds intravenous injection of HSCs from the same fetal liver. The BLT model displays the most complete human immune system and is also the only model to have a mucosal immune system. These models have been useful for evaluation of immune response to vaccination and HIV, but are severely limited due to their need for embryonic tissues, technical complexity, and reproducibility [85].

5.1.7 **Humanized Mouse Models to Study Stem Cell-Based Islet Cell Transplantation**

5.1.7.1 Current Evidence

Initial studies evaluating the utility of humanized mouse models utilized NOD-SCID and NRG mice with engraftment of human peripheral blood mononuclear cells (PBMC) [91]. After xenotransplantation of transgenic mouse islets expressing human HLA-A2.1 allograft rejection occurred, but only in 70% of mice [91]. In this study, high PBMC doses were required and engraftment was highly variable, likely due to the use of NOD-SCID and NRG mice strains. Since then, several studies evaluating the utility of humanized mouse models have been developed and tested, each with their own benefits and limitations (Table 5.1.3). King et al. (2008) improved upon this study substantially by first testing engraftment in NOD-SCID versus

NSG mice, showing better success with NSG strains [83]. They also evaluated intraperitoneal, intravenous, and intrasplenic PBMC engraftment and demonstrated optimal results with intravenous injection [83]. Using those techniques, they achieved 100% human immune system engraftment and after injection of human islets, all mice with humanized immune systems demonstrated islet graft rejection in twenty-one days with microscopic evidence of allogeneic response [83]. Unfortunately, mice in this study died due to GVHD after approximately one month after immune system engraftment. However, utilizing this model, Nadig et al. (2010) transplanted skin allografts onto humanized mice and demonstrated rejection; subsequent co-transplantation with regulatory T-cells (Tregs) prevented graft rejection [97]. This technique was evaluated with ITx, showing human islet allograft rejection after hu-PBL-SCID generation and prolonged islet allograft survival (45-days) when *ex vivo*-expanded Tregs were co-transplanted with PBLs [98]. These studies demonstrate successful use of humanized mouse models to evaluate islet allograft rejection, and similar techniques have been postulated to be helpful to evaluate allogenic stem cell-based ITx [21]. However, no study has demonstrated evaluation of recurrent autoimmunity in autologous iPSC ITx. This is partly due to restricted lifespan of current humanized models. While allogeneic responses occur soon after transplant, recurrent autoimmunity is delayed and occurs later, requiring models to evaluate it to have longer lifespans [99,100].

Alternatively, using Hu-SRC-SCID models has been attempted but remains highly limited due to the immature immune system they possess and lack human specific MHC activation and T-cell education [93,101]. Because of this, most Hu-SRC-SCID models have failed to completely reject allografts [92] (Table 5.1.3). The most promising Hu-SRC-SCID model has been

developed by Brehm et al. (2010), where NRG mice, crossed with mice heterozygous for *Ins2*^{Akita}, showed spontaneous hyperglycemia similar to T1D in immunodeficient mice capable of being engrafted with human immune system cells [102]; however, when they received human ITx, only 60% of islets were rejected, likely owing to poor T-cell function in Hu-SRC-SCID models [102]. In these models, lack of T-cell education directed towards human MHC molecules within the thymus means that although human T-cells develop, they do not mount a robust peripheral immune response directed at human tissues [78,85,94,96]. Additionally, others have postulated that poor peripheral lymphatic development in immunosuppressed recipient mouse strains [103], and the poor interaction of murine cytokines with both human immune cells and cytokine receptors may further limit allograft responses [16,85,96]. To resolve these barriers, several genetically-modified Hu-SRC-SCID models have been developed to express or interact with human cytokines [85,104-109], or express important human MHC molecules [96,110-112]. Overall, similar to Hu-PBL-SCID models, Hu-SRC-SCID models may be of use to evaluate alloimmunity but are unlikely to display adequate immunogenicity to fully comprehend recurrent autoimmunity after autologous iPSC ITx.

Finally, evaluation of BLT models in the context of ITx remains highly limited. A single study has demonstrated islet xenograft rejection in a BLT model, but no studies evaluating human allografts have been conducted [113]. A recent BLT model has demonstrated capacity to induce immune mediated T1D in humanized mice without concerns for GVHD [114]. In their model, transplantation of HLA-DQ8+ human fetal thymus and CD34+ cells into HLA-DQ8 transgenic mice developed hyperglycemia and diabetes [114]. While testing allogeneic stem cell-based ITx in this model could be attempted, technical and ethical concerns regarding BLT

models, as well as restrictions on the source of fetal liver or thymus fragments from patients with T1D profoundly limit their use for the study of immune responses following autologous iPSC ITx.

Table 5.1.3 Studies evaluating humanized mouse models in islet cell transplant, their benefits, and drawbacks.

STUDY	KEY FINDINGS	LIMITATIONS	COULD THIS BE USED FOR IPSC EVALUATION?	
			Allogeneic	Autologous
HU-PBL-SCID MODELS				
BANUELOS ET AL. (2004)	- First study showing islet allograft rejection	- Variable engraftment - High PBMC doses required	No, inconsistent immune engraftment	No, inconsistent immune engraftment
KING ET AL. (2008)	- Demonstrated the NSG mice has improved immune system engraftment - Proved IV PBMC injection was superior to intrasplenic or intraperitoneal - 100% allograft rejection in 21 days	- Early mouse death after approximately 1 month due to GVHD	Possibly, although examination beyond 1-month would be limited	Probably not, GVHD occurs soon after transplant and could confound assessment of autoimmunity
WU ET AL. (2013)	- Demonstrated human islet allograft rejection in Hu-PBL-SCID model Improved allograft survival with Treg co-transplantation	- Study only evaluated mice for 45 days	Yes, effective model for early allograft rejection but not studied beyond 45 days, likely due to GVHD	No, GVHD is likely a limitation
BREHM ET AL. (2019)	- Developed a Hu-PBL-SCID model with MHC class 1 and 2 knockout that rejects islet allografts	- Chronic GVHD still occurs but is significantly decreased	Yes, however, chronic GVHD may confound later results	Possibly, however the lifespan is currently limited to 4-months and chronic GVHD may confound results

HU-SRC-SCID MODELS				
JACOBSON ET AL. (2010)	- Generated Hu-SRC-SCID model with 100% immune engraftment	- Failed to reject islet allografts	No, allografts were not rejected, likely due to inadequate human specific MHC activation and T-cell education	No, allografts were not rejected, likely due to inadequate human specific MHC activation and T-cell education
BREHM ET AL. (2010)	- Developed mice that became diabetic spontaneously and accepted immune engraftment	- Only achieved ~60% islet allograft rejection	No, allografts were not rejected, likely due to inadequate human specific MHC activation and T-cell education	No, allografts were not rejected, likely due to inadequate human specific MHC activation and T-cell education
BLT MODELS				
TONOMURA ET AL. (2008)	- Developed a BLT model that rejected porcine islets (xenorejection)	- Unclear pathophysiology of xenograft rejection and whether it would apply to human allografts	Unclear, no evidence of allograft rejection. The study only showed xenograft rejection	No, acquiring fetal tissues for autologous iPSC donor/recipients is not possible. GVHD is also a limitation.
TAN ET AL. (2017)	- Developed a BLT model with spontaneous development of T1D in DQ8+ transgenic mice	- No evaluation of islet cell transplant to reverse spontaneous diabetes	Possible, but has yet to be tested and would be limited by ethical concerns and fetal tissue availability	No, acquiring fetal tissues for autologous iPSC donor/recipients is not possible. GVHD is also a limitation.

iPSC: Induced Pluripotent Stem Cells; GVHD: Graft Versus Host Disease; MHC: Major Histocompatibility Complex.

5.1.7.2 Future Directions

An ideal humanized mouse model for preclinical evaluation of autologous iPSC ITx currently does not exist. While generating individualized Hu-PBC-SCID mice for each iPSC

donor is theoretically possible, the utility of those models remains highly limited by their shortened lifespan, while Hu-SRC-SCID models remain limited by their incomplete immune response (Figure 5.1.3). When evaluating recurrent autoimmunity, humanized models with up to a year (or more) lifespan will be required. Current research is attempting to produce Hu-PBL-SCID models with MHC knockout mice that do not express murine MHC class I or II molecules, which limits GVHD and prolongs their lifespans [96,115-117]. Yaguchi et al. (2018) generated a NOG mouse strain deficient in MHC class I and II molecules and have demonstrated life spans over 4-months [115]. Their model also showed human antigen-specific immune activation including primary T-cell responses and B-cell activation. Other similar models without MHC class I or II molecules have also demonstrated human-islet allograft rejection [116]. However, it should be noted that while diminished, chronic GVHD still occurred with T-cell infiltration throughout the model's organs. It remains unclear how long these new models can live, and what effect GVHD plays, especially when evaluating alloimmune or autoimmune reactions. Alternatively, transgenic Hu-SRC-SCID models expressing human MHC molecules may enable improved T-cell immune response and enable primary immune responses within mouse models [96,110-112]. However, in transgenic mice expressing human MHC molecules, HSC engraftment is variable and may only occur for fetal sourced cells [111]. Future work is needed to demonstrate humanization in transgenic Hu-SRC-SCID models and subsequent evaluation of their utility for evaluation of immune responses following ITx.

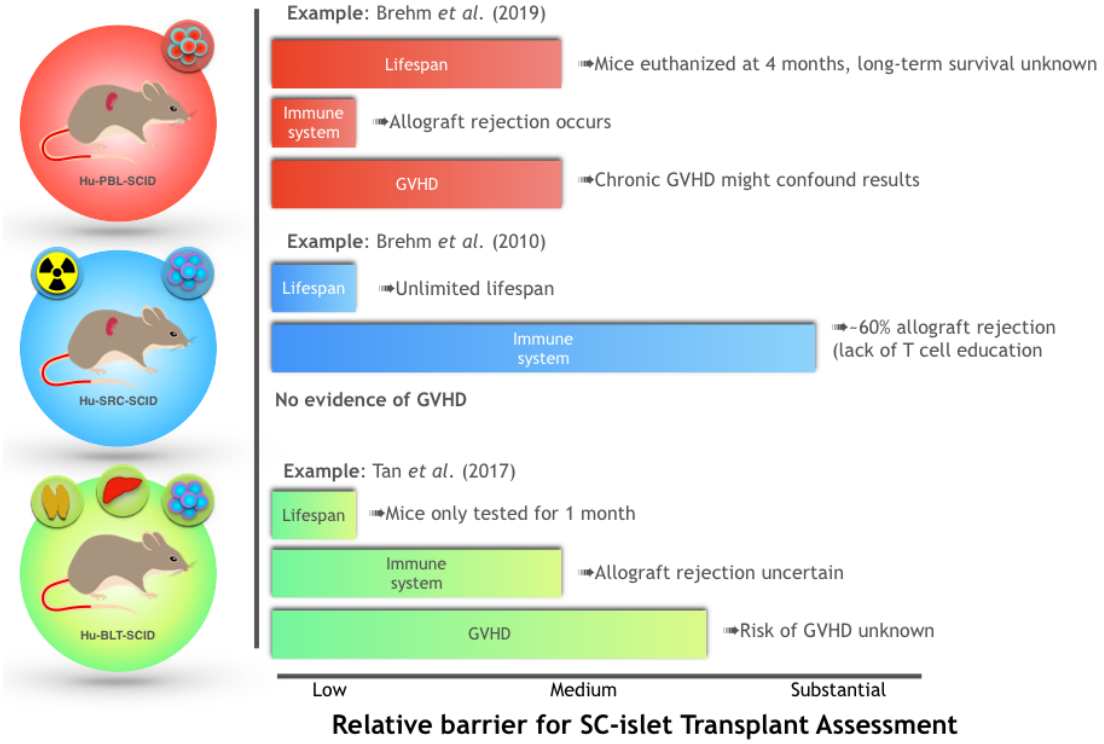

Figure 5.1.3 Relative barriers for SC-islet transplant assessment

Regardless of ongoing investigations to improve humanized mouse models, their use for evaluating stem cell-based ITx remains uncertain. The primary limitations of stem-based ITx remain off-target growth, allograft rejection in the case of allogeneic transplant, and recurrent autoimmunity for autologous iPSC directed grafts. Fortunately, islets can be implanted locoregionally and not systemically and initial evaluation in humans could be conducted subcutaneously. While subcutaneous ITx technologies have thus far not matched intraportal efficacy, they have demonstrated acceptable islet growth and islet cell function [53]. Even if these subcutaneous devices never achieve efficacy similar to intraportal transplantation, they already provide a safe, true human model with complete human immune system for clinical assessment

prior to intraportal transplant. In cases where aberrant cells or graft complications occur, removing them and eliminating risk is feasible. We believe this allows for safe in-human trials to be conducted, potentially within subcutaneous devices initially with subsequent intraportal infusion once safety is demonstrated. This may limit the need for humanized mouse models in this area of medicine entirely.

5.1.8 **Conclusion**

Stem cell-derived islet cells are generating optimism for a potential true cure for T1D. The question remains whether efficacy and safety demonstrated in rodent models is sufficient to implement this technique in clinical trials. Humanized mice models appear on the surface to offer an opportunity to further test these techniques prior to implementation. However, human immune system models remain limited; current models display only certain elements of the immune system. While utilization of hu-PBL-SCID models may allow evaluation of recurrent autoimmunity following transplant of autologous iPSC islets, they offer limited information regarding long-term efficacy or safety due to their brief lifespan. Unfortunately, other models do not appear useful for autologous iPSC ITx evaluation. The hu-SRC-SCID models fail to achieve adequate T-cell responses, while Hu-BLT-SCID models are limited primarily by their need for embryonic tissues, and their technical and resource heavy requirements. Work is ongoing to generate Hu-PBL-SCID mice that do not present GVHD to enable longer lifespans, and to produce Hu-SRC-SCID models with normal T-cell activity; however, efficacy of these models for evaluation of ITx remains unrealized. Thorough demonstration of efficacy and safety in current mouse models, potentially followed by human ITx within subcutaneous devices to

demonstrate safety currently offers a reliable pathway to obtain sufficient evidence to support stem cell-based ITx with intraportal infusion towards human clinical trials.

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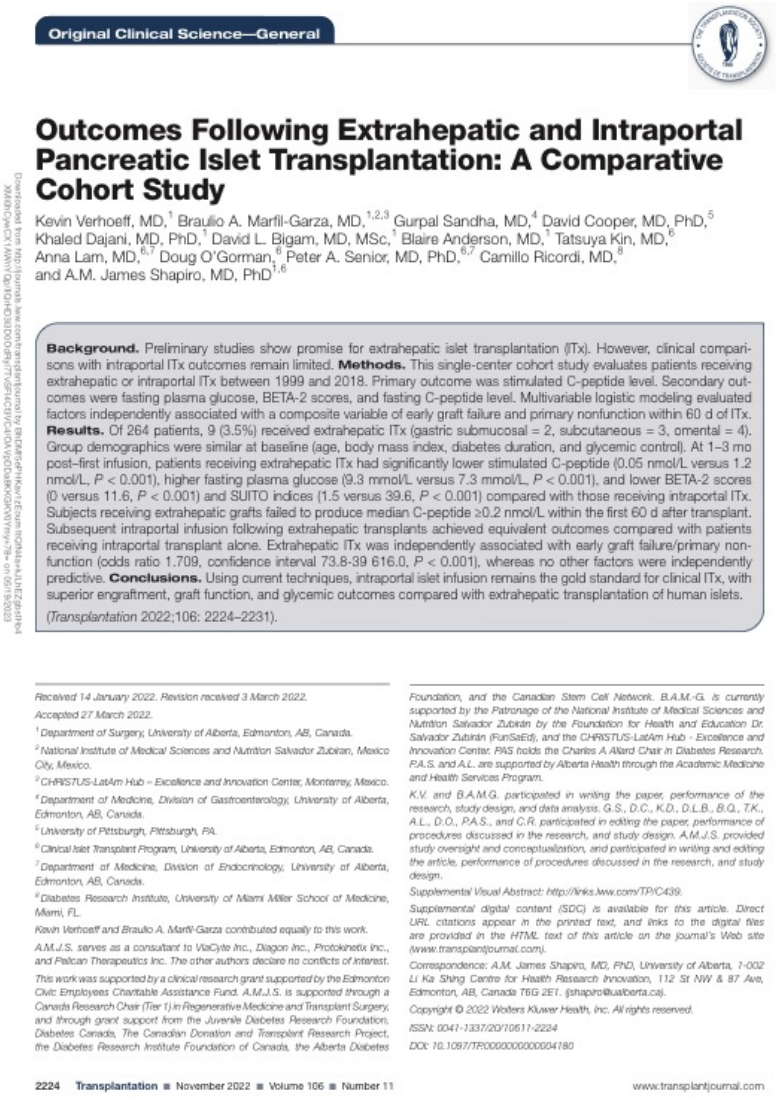
5.2 Chapter 5 subsection 2 – Outcomes Following Extrahepatic and Intraportal Pancreatic Islet Transplantation: A Comparative Cohort Study



Original Clinical Science—General

Outcomes Following Extrahepatic and Intraportal Pancreatic Islet Transplantation: A Comparative Cohort Study

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Background. Preliminary studies show promise for extrahepatic islet transplantation (ITx). However, clinical comparisons with intraportal ITx outcomes remain limited. **Methods.** This single-center cohort study evaluates patients receiving extrahepatic or intraportal ITx between 1999 and 2018. Primary outcome was stimulated C-peptide level. Secondary outcomes were fasting plasma glucose, BETA-2 scores, and fasting C-peptide level. Multivariable logistic modeling evaluated factors independently associated with a composite variable of early graft failure and primary nonfunction within 60 d of ITx. **Results.** Of 264 patients, 9 (3.5%) received extrahepatic ITx (gastric submucosal = 2, subcutaneous = 3, omental = 4). Group demographics were similar at baseline (age, body mass index, diabetes duration, and glycemic control). At 1–3 mo post–first infusion, patients receiving extrahepatic ITx had significantly lower stimulated C-peptide (0.05 nmol/L versus 1.2 nmol/L, $P < 0.001$), higher fasting plasma glucose (9.3 mmol/L versus 7.3 mmol/L, $P < 0.001$), and lower BETA-2 scores (0 versus 11.6, $P < 0.001$) and SUITO indices (1.5 versus 39.6, $P < 0.001$) compared with those receiving intraportal ITx. Subjects receiving extrahepatic grafts failed to produce median C-peptide ≥0.2 nmol/L within the first 60 d after transplant. Subsequent intraportal infusion following extrahepatic transplants achieved equivalent outcomes compared with patients receiving intraportal transplant alone. Extrahepatic ITx was independently associated with early graft failure/primary nonfunction (odds ratio 1.709, confidence interval 73.8-39 616.0, $P < 0.001$), whereas no other factors were independently predictive. **Conclusions.** Using current techniques, intraportal islet infusion remains the gold standard for clinical ITx, with superior engraftment, graft function, and glycemic outcomes compared with extrahepatic transplantation of human islets.

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5.2.1 **Abstract**

5.2.1.1 Background:

Preliminary studies show promise for extrahepatic islet transplantation (ITx). However, clinical comparisons with intraportal ITx outcomes remain limited.

5.2.1.2 Methods:

This single-center cohort study evaluates patients receiving extrahepatic or intraportal ITx between 1999 - 2018. Primary outcome was stimulated C-peptide level. Secondary outcomes were fasting plasma glucose (FPG), BETA-2 scores, and fasting C-peptide level. Multivariable logistic modelling evaluated factors independently associated with a composite variable of early graft failure and primary non-function within 60-days of ITx.

5.2.1.3 Results:

Of 264 patients, 9 (3.5%) received extrahepatic ITx (gastric submucosal = 2, subcutaneous = 3, omental = 4). Group demographics were similar at baseline (age, BMI, diabetes duration, and glycemic control). At 1-3 months post-first infusion, patients receiving extrahepatic ITx had significantly lower stimulated C-peptide (0.05 nmol/L vs 1.2 nmol/L, $p < 0.001$), higher FPG (9.3 mmol/L vs 7.3 mmol/L, $p < 0.001$), and lower BETA 2 scores (0 vs 11.6, $p < 0.001$) and SUITO indices (1.5 vs 39.6, $p < 0.001$) compared to those receiving intraportal ITx. Subjects receiving extrahepatic grafts failed to produce median C-peptide ≥0.2 nmol/L within the first 60-days after transplant. Subsequent intraportal infusion following

extrahepatic transplants achieved equivalent outcomes compared to patients receiving intraportal-transplant alone. Extrahepatic ITx was independently associated with early graft failure/primary non-function (OR 1,709, CI 73.8-39,616.0, $p < 0.001$), while no other factors were independently predictive.

5.2.1.4 Conclusions:

Using current techniques, intraportal islet infusion remains the gold-standard for clinical ITx, with superior engraftment, graft function and glycemic outcomes compared to extrahepatic transplantation of human islets.

5.2.2 **Introduction**

Clinical pancreatic islet transplantation (ITx) has evolved considerably since the groundbreaking Edmonton Protocol established 22-years ago[1]. Optimization of isolation techniques and clinical care has led to 10-year graft survival rates of nearly 80%, coupled with near complete abrogation from severe hypoglycemia, sustained improvements in glycemic control, and substantial reductions in insulin requirements[2-8]. Islet infusion into the intraportal hepatic circulation remains the gold-standard for clinical ITx. However, evaluation of alternative implantation sites continues to be explored, with promising preliminary experimental data supporting the gastric submucosa[9,10], omentum[11-14], and subcutaneous space[15,16]. As ongoing research with stem cell-derived β cell replacement progresses, recent interest into extrahepatic transplant sites has expanded due to its increased accessibility for monitoring of potential off-target growth, that simultaneously facilitates graft recovery, if required. Understanding comparative outcomes after extrahepatic ITx in humans is valuable before extra hepatic sites can be considered for use with stem cell therapies. While preliminary case reports suggest a degree of success[11], evidence remains scarce. Moreover, no comparative studies with intraportal ITx have been published to date.

Benefits from intraportal ITx include direct blood contact, which maximizes graft oxygenation, and insulin release into the portal circulation, which may facilitate a more physiologic glycemic response. However, caveats include islet damage from the instant blood mediated immune reaction, restrictions in packed cell volume, and rarely, procedural complications including portal venous thrombosis and bleeding[17-21]. Conversely, access for

limited graft biopsy in more localized sites such as the gastric submucosa or skin, and the ease of complete graft retrieval at least in the subcutaneous site may have some advantages over the liver. The omentum has been proposed as an attractive site due to easy operative accessibility, lack of volume restriction, and dense vascular supply with portal drainage, although it still involves a surgical (minimally invasive) procedure and its own unique risks including adhesive small bowel obstruction[11,13,19]. Similarly, gastric submucosal implantation allows for graft portal venous drainage, a large capacity for implantation, and offers the possibility to biopsy islet grafts endoscopically but has the least evidence evaluating efficacy in patients to date[9,10]. Finally, the subcutaneous space offers procedural safety, technically easy graft implantation, and facilitates ongoing graft monitoring[15,16]; unfortunately, this space releases insulin systemically and is substantially more hypoxic, which requires prevascularization strategies in order to support islet engraftment[16,22].

Herein, we report a large single-center experience with extrahepatic ITx and compare outcomes compared to intraportal ITx. We aim to evaluate graft survival, and glycemic outcomes for patients receiving extrahepatic ITx, including gastric submucosal, omental, and subcutaneous implants within devices, as compared to patients receiving intraportal ITx.

5.2.3 **Materials and Methods**

5.2.3.1 Study Design and Patient Selection

This is a single-center retrospective cohort study comparing individuals with type 1 diabetes (T1D) receiving allogeneic extrahepatic ITx with intraportal ITx between March 1999 and October 2018. The study protocol has been approved by the University of Alberta Health

Research Ethics Board (PRO00001120) and all patients have consented to use of their data for research purposes. All adult (≥18 years old) patients diagnosed with T1D undergoing allogeneic ITx were included. Patients receiving pancreas transplants, autologous ITx, stem cell-based ITx, and with type 2 diabetes were excluded. Both patients receiving islet alone and islet after kidney transplantation were included; these were grouped because only short term outcomes were evaluated and prior kidney transplantation was deemed unlikely to be a substantial contributing factor.

Patients in the extrahepatic ITx group included those receiving gastric submucosal (n=2), omental (n=4), and subcutaneous device islet implantation (n=3)[23]. Demographics, primary, and secondary outcomes were compared between groups to determine any differences. Patients receiving intraportal or extrahepatic ITx were analyzed as 'intention-to-treat' from their first procedure. Patients in the extrahepatic ITx group were further analyzed after they received subsequent intraportal ITx to assess the effect of extrahepatic grafts on the effectiveness of subsequent intraportal islet infusions. Data for patients receiving prevascularized subcutaneous ITx has previously been reported by our group and included in aggregate form in the current study[23]. Additionally, a secondary analysis comparing extrahepatic ITx to intraportal ITx occurring between January 2012-October 2018 was completed to enable evaluation of outcomes from contemporary groups. This was done to ensure any effects seen were not due to changes in treatment over time including changes in immunosuppression, transplant technique, or patient selection. All extrahepatic transplants were completed during the January 2012-October 2018 timeline.

Patient demographics were collected at time of first transplant and included sex, age at T1D diagnosis, T1D duration, age, and body mass index (BMI). Measures of pre-transplant diabetes control including HbA1c, insulin dose (units/kg/per day), and fasting C-peptide levels (nmol/L) were also collected, as were markers of glycemic lability (Lability Index), and hypoglycemia awareness (Clarke score)[24]. Transplant characteristics were also evaluated including number of islet infusions, timing of infusions, and total islet equivalents (IEQ)/kg of body weight received.

5.2.3.2 Outcome Variables

The primary outcome of this study was stimulated C-peptide levels 1-3 months after first ITx measured at 90 mins after a mixed meal tolerance test[1,25] Secondary outcomes include fasting plasma glucose (FPG), and BETA-2 score. The BETA-2 score incorporates insulin dose (insulin units/kg/day), FPG (mmol/L), HbA1c (%), and fasting C-peptide levels (nmol/L) and has been validated as a predictive tool for glycemic control and insulin independence[26,27].

Additionally, we evaluated graft survival measured by fasting C-peptide levels over time. In the immediate post-infusion period, fasting C-peptides levels were measured every 2-5 days for the first 60-days and reported as 10-day means with standard error of the mean (SEM). Subsequent C-peptide values for 5-years after first infusion were collected over 6-month intervals, and reported as means with SEM. Continuous data are described as medians and IQR, with discrete data reported as absolute frequencies and percentages. To further assess the cohorts, rate of primary non-function or early graft failure was determined for each group. Primary non-function was defined as C-peptide >0.1 nmol/L, and early graft failure was defined

as a return to C-peptide values <0.1 nmol/L (or baseline) prior to a subsequent infusion or within 60 days of first infusion. Multivariable logistic regression analyzed the entire cohort for patient and transplant factors independently associated with a composite variable of primary non-function and early graft failure. Finally, we evaluated allosensitization to extrahepatic grafts defined as any calculated panel reactive antibody (cPRA) increase or any *de novo* donor specific antibody development following transplant.

5.2.3.3 Transplant Procedures

Intraportal transplantation involved ultrasound and fluoroscopy-guided percutaneous cannulation of portal venous circulation and islet infusion as described previously[28]. Islet isolation procedures and release criteria have also been described. Omental transplant was completed via the biological scaffold "sandwich" technique previously described by researchers from the Diabetes Research Institute in Miami, Florida[11,12]. Patients were brought to the operating room, underwent general anesthesia and laparoscopy. The omentum was laid out flat islets suspended with the recipient's own plasma were dripped on to the omentum. Recombinant thrombin was then used to cover each of the islet droplets. The omentum was folded over to cover the implantation site and secured in place with ligaclips. Gastric submucosal transplants were completed as previously described by Echeverri et al[10]. Patients underwent conscious sedation with subsequent gastroscopy to evaluate the stomach; islets were then infused through a 19-gauge Boston Scientific Expect Slimline needle in eight submucosal locations throughout the stomach under direct vision. Procedures were performed by a gastroenterologist with advanced training in therapeutic endoscopy. Finally, prevascularized subcutaneous space

ITx was completed with islet implantation into a prevascularized non-immunoisolating polymer chamber device as previously described by Gala-Lopez et al.[23].

All extrahepatic transplants were completed as clinical trials intended to evaluate the potential of novel transplant sites. These sites were selected due to promising preliminary outcomes from others. Registered clinical trial protocols can be reviewed as follows: omental NCT02821026, gastric submucosal NCT02402439, and subcutaneous NCT01652911. In all cases, we collaborated with investigators who initially reported promising outcomes to optimally replicate their technique.

5.2.3.4 Immunosuppression

Patients receiving intraportal ITx received various induction, anti-inflammatory, and maintenance immunosuppression regimens. Induction was primarily alemtuzumab (n = 288 infusions, 47.1%), followed by daclizumab (n = 170 infusions, 27.8%), basiliximab (n = 77 infusions, 12.6%), and anti-thymocyte globulin (n = 77 infusions, 12.6%). A total of 287 infusions (47.0%) used etanercept and anakinra. All patients received tacrolimus (100%) during follow up and most had mycophenolate (n = 234, 91.4%) as a secondary maintenance immunosuppressant with the remainder receiving sirolimus combined with tacrolimus. Comparatively, induction immunosuppression for infusions into extrahepatic sites was primarily alemtuzumab (n = 6, 60%) and the others received anti-thymocyte globulin (n = 4, 40%). Most infusions were accompanied by both etanercept and anakinra (n = 6, 60%), and 4 (40%) received only etanercept. All patients with extrahepatic ITx had tacrolimus and mycophenolate for maintenance immunosuppression.

5.2.3.5 Statistical Analysis

For analysis of longitudinal C-peptide data, a mixed effects model using the maximum-likelihood method was fitted to determine differences over time and between groups, while accounting for missing data. For the analysis of categorical data, the X^2 tests were applied. To compare continuous variables in two independent groups, Mann-Whitney U tests were used. A value of $p<0.05$ is considered statistically significant.

To evaluate the independent effect of patient and transplant factors on early graft failure and primary non-function, a non-parsimonious multivariable logistic regression model was developed using hypothesis-driven selection methods. Variables with statistical significance in the multivariable model (Wald test $p < 0.05$) were evaluated for multi-collinearity using the variance inflation factors (VIF). Variables with VIF >10 were further explored for collinearity diagnostic tests and excluded if deemed collinear.

5.2.4 **Results**

Overall, 264 patients were included in this study. Of these, 9 (3.4%) patients received extrahepatic ITx for initial islet transplant before undergoing intraportal ITx. These patients were compared to 255 (96.6%) control patients receiving intraportal ITx. At baseline, patients were similar with regards to age at T1D diagnosis, BMI, and T1D duration (Table 5.2.1). Patients receiving extrahepatic ITx were more likely to be male (41.6% intraportal vs 77.8% extrahepatic, $p = 0.032$) and were older at time of first transplant (48.8 intraportal vs 59.8 extrahepatic, $p = 0.025$). Median fasting C-peptide levels, HbA1c levels and insulin requirements pre-ITx were similar (Table 5.2.1).

Regarding infusion characteristics of the first ITx, both groups received a similar islet preparation purity (60% intraportal vs 55% extrahepatic, p = 0.499) but patients receiving extrahepatic grafts received more islet equivalents (IEQs) per kg of body weight (6,100 IEQ/kg intraportal vs 7,000 IEQ/kg extrahepatic, p = 0.018). Overall, after groups received all of their ITxs, both groups received a similar number of infusions, and there was no difference in total infused IEQs/kg of body weight (14,300 IEQ/kg intraportal vs 22,500 IEQ/kg extrahepatic, p = 0.096). However, patients receiving extrahepatic infusions had a shorter delay between their third and fourth islet infusions (Table 5.2.1). Eight patients in the extrahepatic group received one extrahepatic implantation and one patient received two; patients receiving extrahepatic ITx were switched to the intraportal route if they failed to achieve clinical benefit from their initial graft including insulin reduction, improved glycemic lability, or reduced hypoglycemia. The decision to re-list patients for transplant was made following review by the ITx team and determination that late onset graft function was unlikely.

Table 5.2.1 Demographic and baseline characteristics of patients undergoing pancreatic islet transplantation according to implantation site

Variable	Intraportal (n=255)	Extrahepatic (n=9)	P value[a]
Demographics and clinical data at baseline, before 1st transplant			
Sex, M/F, n (%)	106 (41.6) / 149 (58.4)	7 (77.8) / 2 (22.2)	0.031
Age at diagnosis, yr (IQR)	14.0 (9 - 23)	15 (12 – 26)	0.417
Duration of DM, yr (IQR)	30.6 (22.6 – 40.2)	35.4 (25.3 – 46.1)	0.374
Age at transplant, yr (IQR)	48.8 (41.3 – 55.8)	59.8 (54.3 – 60.4)	0.025
Body-mass index (IQR)	25.0 (22.9 – 27.8)	25.4 (25.0 – 27.3)	0.751
Number of infusions per patient (IQR) Number of extrahepatic infusions per patient (IQR)	2 (2 – 3)	2 (2 – 4) 1 (1-2)	0.672
Time between infusions, mo (IQR) Time to 2nd infusion Time to 3rd infusion Time to 4th infusion Time to 5th infusion	 5.0 (2.1 – 11.1) 40.4 (16.6 – 70.9) 91.0 (68.5 – 140.5) 165.4 (143.6 – 181.5)	 4.3 (3.0 – 8.5) 5.5 (4.3 – 16.8) 15.1 (5.5 – 37.7) 14.6 (-)	 0.823 0.014 0.007 0.157
Total IEQs/kg of body weight, x 1,000 (IQR) 1st infusion, x 1,000 (IQR)	14.3 (11.2 – 18.6) 6.1 (4.8 – 7.0)	22.5 (13.3 – 27.2) 7.0 (6.5 – 9.1)	0.097 0.018
Purity (1st infusion)	60 (50 - 70)	55 (45 – 65)	0.477
Lability index (IQR)	449 (296 – 699)	566 (374 – 608)	0.520
Clarke score (IQR)	5 (4 - 7)	5 (3 – 5)	0.052
Laboratory values at baseline, before 1st transplant			
C-peptide (nmol/L) (IQR)	0.02 (0.02 – 0.03)	0.02 (0.02)	0.042
HbA1c % (IQR)	8.2 (7.5 – 9.0)	8.1 (7.5 – 8.1)	0.333
Insulin units/kg/day (IQR)	0.54 (0.46 – 0.68)	0.51 (0.46 – 0.60)	0.588

LDL: low-density lipoprotein, HDL: high-density lipoprotein
M: male, F: female, IEQ: islet equivalent.
Data are n (%) and median (IQR)
[a]X^2 was used to compare categorical variables, Mann-Whitney's test was used to compare continuous variables.
†Weighted averages were calculated as follows: weighted average= sum of weighted terms/total number of terms. For example, weighted average= $\text{purity}^{\text{infusion1}}(\text{islet number}^{\text{infusion1}})$ + $\text{purity}^{\text{infusion2}}(\text{islet number}^{\text{infusion2}})$ + /total number of islets infused.

Primary outcome assessment demonstrated that patients receiving extrahepatic ITx had significantly lower stimulated C-peptide levels 1-3 months after first ITx compared to patients

receiving only intraportal infusions (0.05 nmol/L, IQR 0.02-0.24 extrahepatic vs 1.26 nmol/L, IQR 0.95-1.59 intraportal; $p < 0.001$; Figure 5.2.1A). Secondary outcomes showed statistically higher FPG, and lower BETA-2 scores (Figure 5.2.1B-C) early after extrahepatic ITx compared to intraportal (Table 5.2.2). Once patients with initial extrahepatic ITx received subsequent intraportal islet infusions, they achieved similar stimulated C-peptide levels and FPG compared to those who initially received intraportal infusions (Figure 5.2.1A-B). Notably, BETA-2 scores were higher after intraportal transplant in the patients who initially received extrahepatic grafts (19.1, IQR 13.3-22.7; $p = 0.004$; Figure 5.2.1C). Similar differences in primary and secondary outcomes were observed in sub-analyses according to specific extrahepatic implantation sites compared to intraportal ITx with a suggestion that best outcomes may have been observed with the omental site (Figure 5.2.1D-F). Secondary analysis of patients receiving extrahepatic ITx to contemporary intraportal ITx (n = 106) also showed similar outcomes (**Appendix** Figure S5.2.3**)**

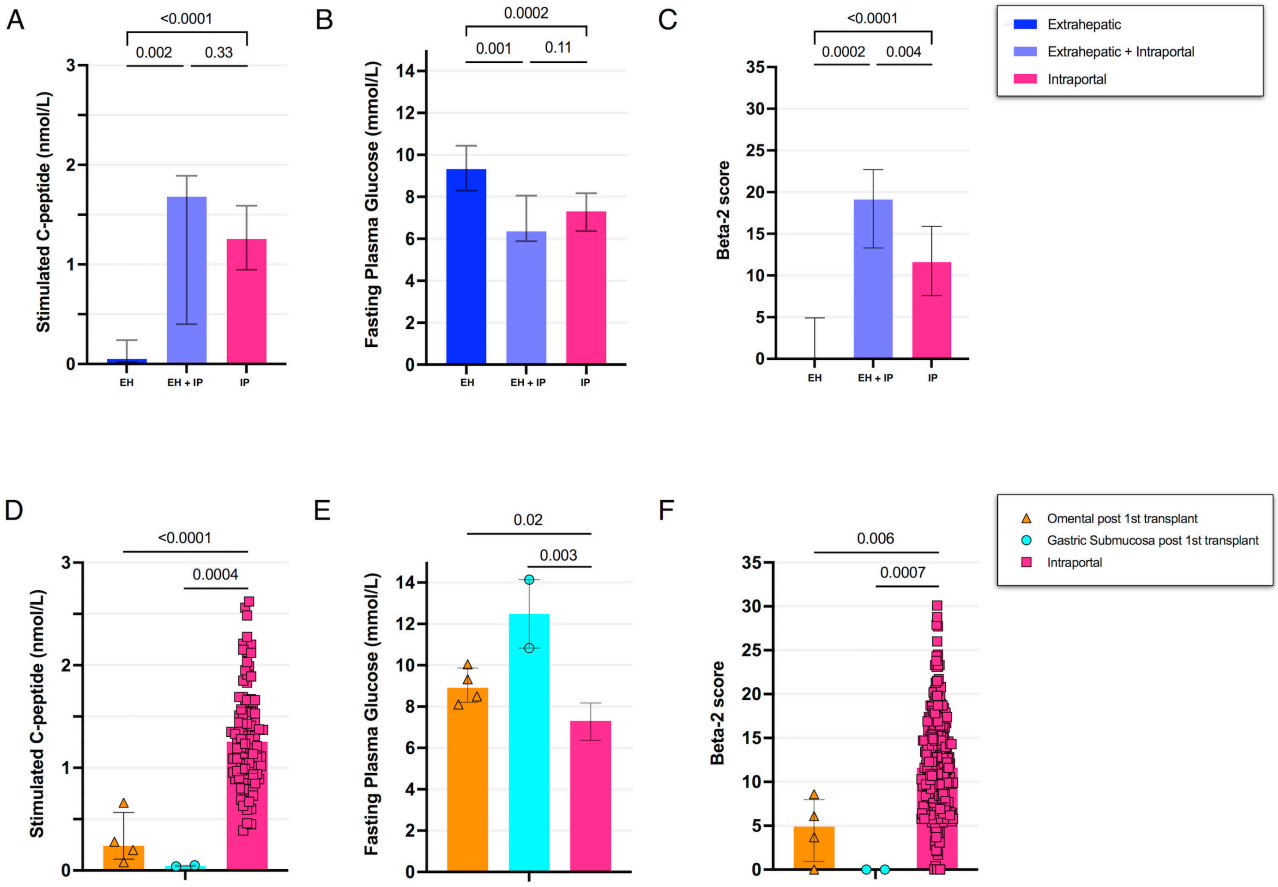

Figure 5.2.1 Stimulated C-peptide and secondary outcomes comparing extrahepatic islet cell transplant, intraportal islet cell transplant, and second (intraportal) transplant in patients who initially received extrahepatic implantation.
A) Stimulated C-peptide; B) Fasting plasma glucose; C) BETA-2 score; D) Stimulated C-peptide for individual extrahepatic sites; E) Fasting plasma glucose for individual extrahepatic sites; F) BETA-2 score for individual extrahepatic sites.
*EH: Extrahepatic transplant; EH + IP: Extrahepatic and intraportal transplant; IP: intraportal transplant. Data is presented as medians with error bars representing interquartile range. All measures are 1-3 months after implantation. A single stimulated C-peptide measure was included at 5-months after the patients second transplant.

Table 5.2.2 Primary outcomes following extrahepatic and intraportal pancreatic islet transplantation

Variable	Extrahepatic Alone	Extrahepatic + 1st intraportal	Intraportal Alone
Primary outcome			
Stimulated C-peptide (nmol/L)[a]	0.05 (0.02 – 0.24)	1.68 (0.4 – 1.89)	1.26 (0.95 – 1.59)
Secondary outcomes			
Fasting plasma glucose (mmol/L)	9.33 (8.3 – 10.44)	6.35 (5.89 – 8.06)	7.32 (6.39 – 8.18)
BETA-2 score	0 (0 – 4.9)	19.1 (13.3 – 22.7)	11.6 (7.55 – 15.7)

Data are median (IQR)
[a]All measures are 1-3 months after implantation. A single stimulated C-peptide measure was included at 5-months in the extrahepatic group, since this patient had a 2nd ITx in the gastric submucosa.

Assessment of fasting C-peptide levels over time showed that patients receiving extrahepatic transplants had significantly lower levels after their initial implants compared to those with intraportal infusions (mixed-effect model, group effect: $p < 0.001$; Figure 5.2.2A). Of the 9 patients undergoing extrahepatic ITx, 7 (77.8%) elected to proceed with subsequent ITx following failed extrahepatic graft. Only one patient proceeded with subsequent extrahepatic ITx, had similar early graft failure, and then proceeded with intraportal ITx. Patients receiving gastric submucosal, omental, or prevascularized subcutaneous transplant failed to produce a median fasting C-peptide level ≥0.2nmol/L in the first 60 days when compared to intraportal infusion, however, 3 of 4 subjects in the omental group had measurable C-peptide ≥0.2nmol/L at some point post extrahepatic transplant. Fasting C-peptide levels following intraportal ITx in patients who initially received extrahepatic implants was similar to those patients receiving whose initial transplants were via intraportal infusions (mixed effect model group effect: $p = 0.17$; Figure 5.2.2B). All patients with extrahepatic ITx responded similarly after receiving subsequent intraportal transplant (**Appendix** Figure S5.2.4). One subject receiving gastric

submucosal ITx developed *de novo* donor specific antibody, but no other patient had any cPRA increase after extrahepatic ITx.

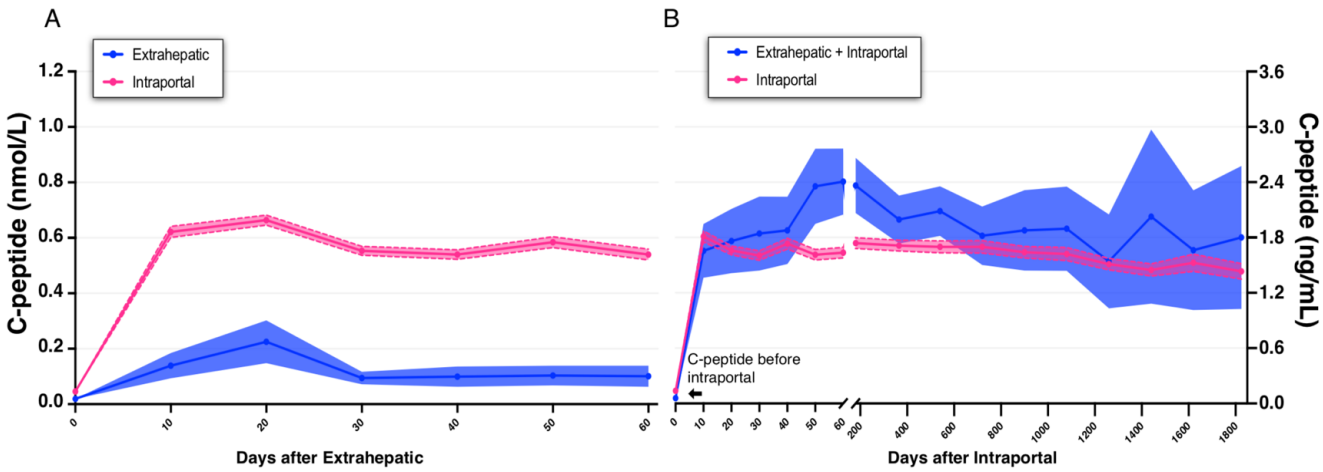

Figure 5.2.2 Fasting C-peptide levels following extrahepatic and intraportal pancreatic islet cell transplantation.
A) Fasting C-peptide 10-day medians for the first 60-days after implantation B) C-peptide after intraportal transplant and after intraportal transplant in patients who initially received extrahepatic implantation (extrahepatic group).
*Data is presented as mean (solid lines) and standard error of the mean (shaded area)

Primary non-function and/or early graft failure occurred significantly more following extrahepatic ITx than following initial intraportal transplant (88.9%, n = 8/9 extrahepatic vs 2.0%, n = 5/255 for intraportal, $p < 0.001$, **Appendix** Figure S5.2.5). Extrahepatic ITx was independently associated with graft primary non-function (OR 1,709, CI 73.8-39,616.0, $p < 0.001$). No other patient, transplant, or immunosuppression factors were independently predictive of primary non-function (Table 5.2.3). Evaluating patients with either early graft failure or primary non-function we see that only 14% (n = 1/7) with extrahepatic grafts experienced early graft failure after subsequent intraportal ITx; comparatively, in patients receiving initial

intraportal ITx who experienced either graft primary non-function, 80% (n = 4/5) experienced similar early graft loss following second intraportal transplant.

Table 5.2.3 Outcomes from multivariable logistic modelling evaluating factors independently associated with graft primary non-function (median C-peptide ≤0.1 nmol/L).

Risk Factor	Odds Ratio	95% confidence interval	p-value
Age	0.97	0.89-1.06	0.549
Male gender	1.23	0.21-7.22	0.816
IEQ per kg	1.00	1.00-1.00	0.292
Tacrolimus level (months 0-3)	0.76	0.48-1.20	0.236
***BMI (kg/m^2)**	1.01	0.98-1.02	0.108
Extrahepatic transplant site	1,709	73.80-39,616.00	<0.001

*BMI, body mass index

5.2.5 **Discussion**

This study demonstrates that administration of large numbers of high quality islets by an extrahepatic route failed to result in significant production of basal or stimulated C-peptide within the first three months post-transplant. Conversely, recipients of primary intraportal ITx demonstrated markedly superior C-peptide production in the first three months, as well as demonstrating sustained graft survival, and improved glycemic-related outcomes compared to extrahepatic ITx. However, individuals who had received extrahepatic ITx were able to achieve similar stimulated and fasting C-peptide levels, and similar glycemic outcomes once they subsequently received intraportal ITx, compared to those receiving initial intraportal ITx. Overall, when compared to intraportal ITx, extrahepatic implantation failed to show islet engraftment or improved diabetes outcomes in patients who subsequently received successful intraportal grafts, suggesting that the extrahepatic site and not patient or graft factors, was the

cause of these outcomes. Prior to future clinical evaluation of these extrahepatic sites, ongoing optimization of these innovative techniques is required.

The study's primary outcome analysis showed that patients with intraportal ITx had significantly higher stimulated C-peptide over the first three months after implantation compared to those receiving extrahepatic islet grafts. Notably, extrahepatic grafts produced a median stimulated C-peptide < 0.1 nmol/L despite receiving a greater islet mass. C-peptide production remains a primary outcome measure that correlates with glycemic control, insulin independence and resolution of glycemic lability, particularly hypoglycemia[29-32]. Differences in secondary outcomes were also evident, with significantly higher FPG after extrahepatic ITx, and worse graft function measured by BETA-2 scores. This is in contrast to some cases reported previously where substantial C-peptide production was observed in 2 of 3 patients undergoing omental ITx[11,14]; and pre-clinical models demonstrating successful gastric submucosal ITx in large animal models[10], and promising results for the prevascularized subcutaneous approach in mice [15,16]. However, while not clinically significant, C-peptide levels appeared higher in the omental when compared to the two other extrahepatic sites. Together, our data raise concerns about the feasibility of extrahepatic ITx, and emphasize a need to further optimize oxygenation, neovascularization and protection from fibrosis or other deleterious processes in extrahepatic sites to achieve clinical outcomes equivalent to intraportal ITx.

Importantly, patients who initially received extrahepatic transplant without success, and who subsequently underwent intraportal ITx achieved similar stimulated C-peptide levels, glycemic outcomes, and graft function to those who underwent initial intraportal transplant. Overall, similarities in our primary outcome and most secondary outcomes support the notion

that a failed extrahepatic ITx does not impact the success of subsequent intraportal ITx, and that improvement in techniques for extrahepatic ITx are necessary. However, an unplanned finding of this study demonstrated that patients receiving intraportal ITx who experience early graft failure or graft primary non-function may be at risk of subsequent graft failure and studies evaluating this patient cohort would be of interest. Additionally, although allosensitization was uncommon in patients receiving extrahepatic ITx, this risk remains a potential consideration in this patient population who are at risk of requiring future islet or solid organ transplants. The risk of allosensitization may have been mitigated in this series because maintenance immunosuppression was continued while on the wait list for a subsequent intraportal islet infusion.

Our findings contrast somewhat with the promising preliminary results with omental islet transplantation from Baidal et al, with outcomes that are similar to subject 2 in their study[14]. Our omental transplants did demonstrate sustained but low C-peptide production over time but remained inferior to intraportal infusion in our hands. Our experience is limited to only four omental ITxs and variance in technique or islet quality could explain this difference. Alternatively, it is likely that human-to-human variation in vascularity and fatty infiltration in the omentum between subjects, or age and species-specific differences in omental anatomy or immunological response could explain the discrepancy between preclinical experience and clinical translation of this approach in murine[13] and macaque[12] models. While omental cell composition remains similar between species[33], fat density and vascular distribution is highly variable with animal models often having substantially less adiposity, potentially increasing diffusion capacity[34]; the latter remains crucial to islet engraftment and may further contribute to

our findings[35]. Two recent studies evaluating omental ITx in humans have found similar results to ours, with >50% of patients having early graft failure, and others achieving marginal clinical benefit[36,37]. Of note, the Van Hulle et al. group evaluated graft biopsies and demonstrated that substantial foreign body reaction may have led to their outcomes[36]

Similarly, our results contrast with promising findings of allotransplantation of porcine islets within the gastric submucosa in immunosuppressed pigs reported by Echeverri et al.[10]. Importantly, the gross and microscopic gastric anatomy differs between humans and porcine models, with the porcine stomach being 2-3 times larger and having much more cardiac mucosa than humans[38]. These cardiac cells produce primarily mucus, while the human gastric submucosa contains parietal and chief cells that produce hydrolytic enzymes and acid[38]. Again, these small inter-species differences may account for the findings in our study.

Finally, our results also diverge from those shown with ITx into the prevascularized subcutaneous space in mice[15,22,23]. However, a recent oral presentation by Dr. Witkowski's group suggests that the prevascularized subcutaneous space (using similar techniques to the one employed in this study) can achieve engraftment and sustained C-peptide production and insulin dose reduction in humans when a lower islet tissue concentration is transplanted in the subcutaneous device within the rectus muscle fascia[39]. Our experience here only includes 3 patients and it remains possible that our observed failure with subcutaneous ITx is due to device capacity overload from the high islet masses that we implanted[23]. Alternatively, improved vascularization within intramuscular sites may offer potential improvements to current techniques[40]. Small iterative modifications may enable success of subcutaneous and other extrahepatic sites, and encourages ongoing refinements to further optimize these techniques.

Of note, for the gastric submucosal, subcutaneous and omental extrahepatic islet transplants we were more discriminatory in the selection of higher quality, higher purity preparations which should have lent favorably to improved islet survival and engraftment in these sites. Our aim was to reduce the amount of exocrine contamination in grafts placed in sites with more limited physical restraints. This may or may not have been wise in retrospect. We also selected young healthier recipients to optimize the conditions, which again should have lent favorably for extrahepatic sites.

A major limitation to the current study is the very small cohorts included in each of the extrahepatic sites, and the potential variability in our adoption of these new techniques. While this study presents only 9 subjects receiving extrahepatic grafts, it still represents the largest compilation of extrahepatic ITx to date. Additionally, the lack of substantial C-peptide production in every included patient suggests that even if additional patients were included, differences compared to intraportal infusion would still remain. Similarly, although we only present early outcomes following extrahepatic ITx, with the limited and relatively poor function observed in the extrahepatic sites, it is unlikely that these grafts would spontaneously gain further function beyond the 1-3 month timeframe evaluated in this study; the fact that these patients were promptly given intraportal islet infusions also precluded a longer term analysis. We cannot however completely rule out that late onset graft function would have occurred. Comparison of extrahepatic ITx that occurred since 2012 to intraportal from 1999-2018 also introduces the potential of era related effects. In order to limit that risk, we performed a secondary analysis comparing era matched cohorts and showed similar results. It should also be noted that this is a single center experience and subject bias cannot be ruled out. It remains to be

determined whether technical aspects have led to our negative results with extrahepatic ITx. Specialists assisted and trained our group with their omental transplant technique to minimize the likelihood that our findings are due to technical variability[11,14]. All endoscopic procedures were performed by a single experienced interventional gastroenterologist following previously described techniques that are easily reproducible[10]. As previously described, surgeons who were successful with animal model participated in the human procedures to ensure technical consistency[16,22,23]. Therefore, while technical differences could explain our findings, substantial efforts were made to reduce that likelihood. As discussed above, an additional variable that could contribute to the differences in our study compared to others is variance in islet cell preparations and transplanted islet tissue concentration. Herein, we report the IEQ/kg and purity of the extrahepatic and intraportal transplants but no data is available to compare our islet preparations to other centers. Mechanistic evaluation of the reasons for extrahepatic graft outcomes shown here are limited and may be beneficial to further improve these techniques. Nevertheless the successful outcomes with intraportal transplantation in both groups is consistent with the high quality islet preparations consistently provided by our islet isolation team. Ongoing optimization of the islet preparation and transplant techniques may enable future success of extrahepatic sites and ongoing work is encouraged.

We present a comparative cohort study evaluating patients receiving extrahepatic and intraportal ITx at a single islet transplant center. Patients who received extrahepatic ITx failed to achieve substantial C-peptide production when compared to intraportal transplantation. However, these patients did subsequently achieve similar graft function with a secondary intraportal ITx, suggesting that inadequate engraftment after extrahepatic transplant rather than

graft or patient factors are implicated in graft failure after extrahepatic ITx. As we consider alternative sites for engraftment of islets or stem cell-islets, it is clear from our preliminary experience that more refinements will be needed to substantially improve cellular engraftment and survival if these sites are to match the current efficiency of the intraportal approach. Intraportal ITx, despite all of its limitations, prevails as a current gold-standard as the only implantation site to have consistently demonstrated the capacity to support long-term islet engraftment, glucose-responsive C-peptide production, glycemic outcome improvements, and sustained insulin independence. While the concept of achieving clinical success with extrahepatic ITx remains attractive, substantial work is required to transform this concept into a reality.

5.2.6 Appendix: chapter 5 subsection 2

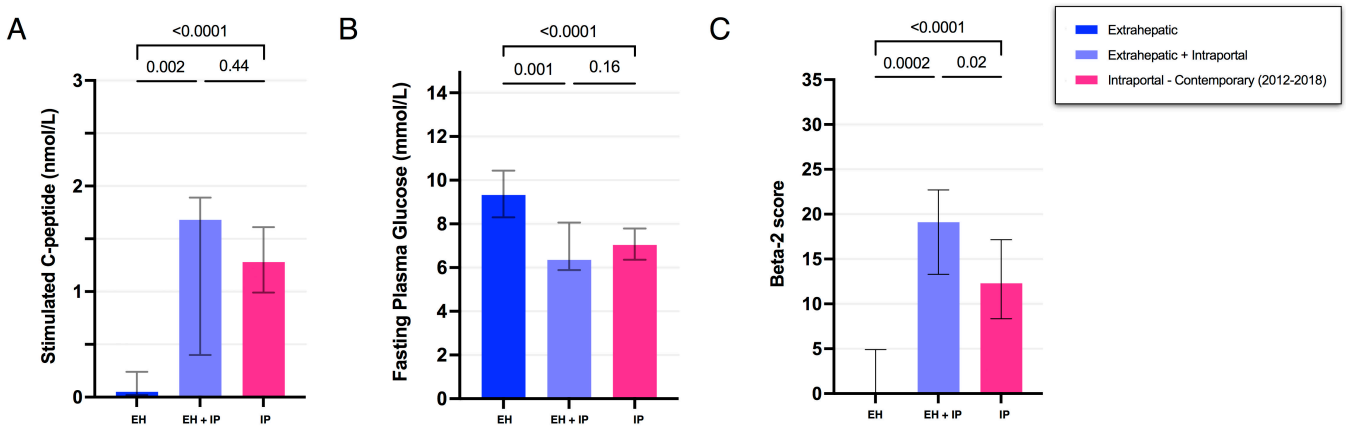

Figure S5.2.3 Era matched comparison of stimulated C-peptide and secondary outcomes comparing extrahepatic islet cell transplant, intraportal islet cell transplant (2012-2018), and second (intraportal) transplant in patients who initially received extrahepatic implantation.
A) Stimulated C-peptide; B) Fasting plasma glucose; C) BETA-2 score.

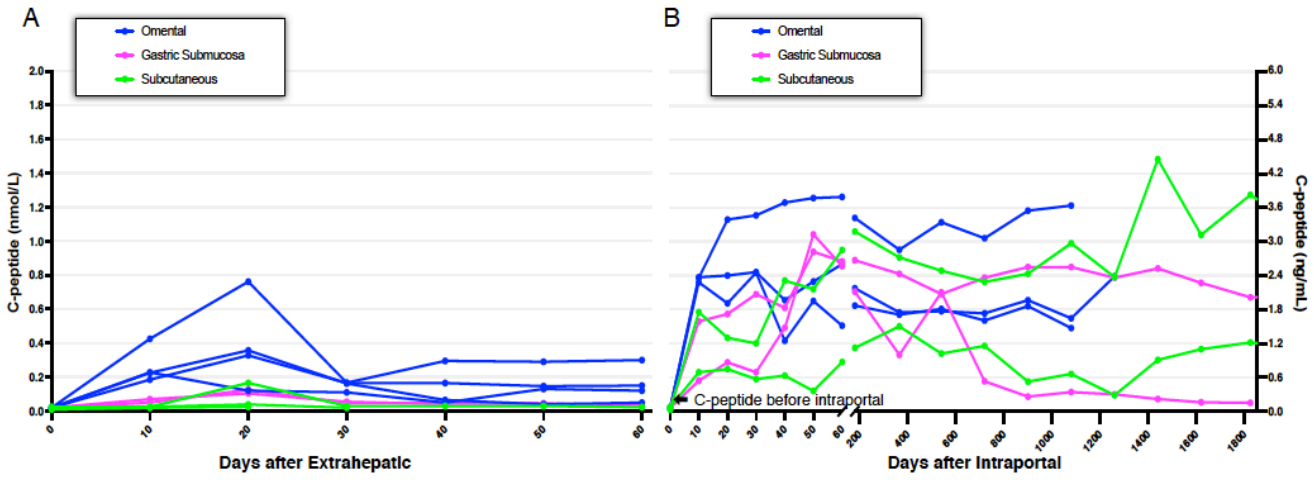

Figure S5.2.4 Individual C-peptide production after extrahepatic transplant for each extrahepatic transplant.

A) C-peptide 10-day averages for the first 60-days after implantation B) C-peptide after intraportal transplant in patients who initially received extrahepatic implantation (extrahepatic group).

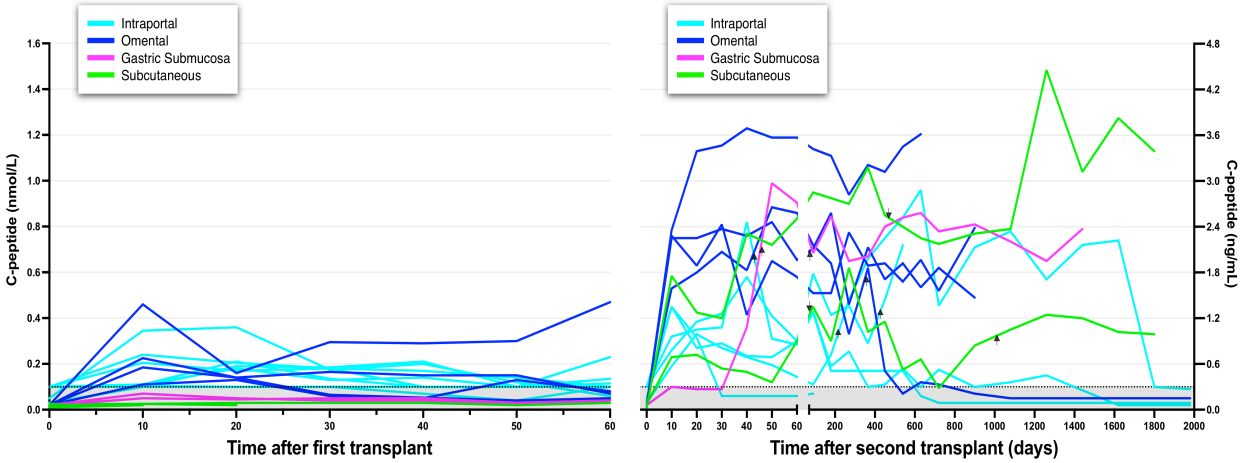

Figure S5.2.5 Fasting C-peptide values for patients with early graft failure and/or primary non-function following islet transplantation.
Primary non-function is defined as failure to achieve C-peptide >0.1 nmol/L, and early graft failure is defined as a return to C-peptide values <0.1 nmol/L (or baseline) prior to a subsequent infusion or within 60 days of first infusion. A) C-peptide 10-day averages for the first 60-days after implantation B) C-peptide after subsequent intraportal transplant in patients who initially had early graft failure and/or primary non-function.

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5.3 Chapter 5 subsection 3 – C-peptide Targets and Patient-Centered Outcomes of Relevance to Cellular Transplantation for Diabetes

Original Clinical Science—General

C-peptide Targets and Patient-centered Outcomes of Relevance to Cellular Transplantation for Diabetes

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Background. C-peptide levels are a key measure of beta-cell mass following islet transplantation, but threshold values required to achieve clinically relevant patient-centered outcomes are not yet established. **Methods**. We conducted a cross-sectional retrospective cohort study evaluating patients undergoing islet transplantation at a single center from 1999 to 2018. Cohorts included patients achieving insulin independence without hypoglycemia, those with insulin dependence without hypoglycemia, and those with recurrent symptomatic hypoglycemia. Primary outcome was fasting C-peptide levels at 6 to 12 mo postfirst transplant; secondary outcomes included stimulated C-peptide levels and BETA-2 scores. Fasting and stimulated C-peptide and BETA-2 cutoff values for determination of hypoglycemic freedom and insulin independence were evaluated using receiver operating characteristic curves. **Results**. We analyzed 192 patients, with 122 (63.5%) being insulin independent without hypoglycemia, 61 (31.8%) being insulin dependent without hypoglycemia, and 9 (4.7%) experiencing recurrent symptomatic hypoglycemia. Patients with insulin independence had a median (interquartile range) fasting C-peptide level of 0.66 nmol/L (0.34 nmol/L), compared with 0.49 nmol/L (0.25 nmol/L) for those being insulin dependent without hypoglycemia and 0.07 nmol/L (0.05 nmol/L) for patients experiencing hypoglycemia ($P < 0.001$). Optimal fasting C-peptide cutoffs for insulin independence and hypoglycemia were ≥0.50 nmol/L and ≥0.12 nmol/L, respectively. Cutoffs for insulin independence and freedom of hypoglycemia using stimulated C-peptide were ≥1.2 nmol/L and ≥0.68 nmol/L, respectively, whereas optimal cutoff BETA-2 scores were ≥16.4 and ≥5.2. **Conclusions**. We define C-peptide levels and BETA-2 scores associated with patient-centered outcomes. Characterizing these values will enable evaluation of ongoing clinical trials with islet or stem cell therapies.

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K.V. and B.A.M.-G. participated in writing the article, performance of the research, study design, and data analysis. K.D., D.L.B., B.A., T.K., A.L., D.O., and P.A.S. participated in editing the article, performance of procedures discussed in the research, and study design. A.M.J.S. provided study oversight and conceptualization and participated in writing and editing the article, performance of procedures discussed in the research, and study design.

INTRODUCTION

Pancreatic islet transplantation (ITx) continues to evolve, with growing evidence supporting its clinical value.[1-7] Considering promising reports of long-term outcomes, indications for ITx are likely to expand further in the coming decades. Novel implantation sites continue to be investigated,[8-17] and ongoing clinical trials evaluating stem cell-derived beta-cell replacement therapies offer hope to eliminate barriers such as the need for lifelong immunosuppression and donor supply limitations.[1,2,18,19] Following ITx, persistent graft function as measured by C-peptide is associated with reduced insulin requirements, improved glycemic control, reduced glycemic variability,

No additional data beyond the included figures and tables in this article are available or published elsewhere.

Supplemental Visual Abstract; http://links.lww.com/TP/C590.

Supplemental digital content (SDC) is available for this article. Direct URL citations appear in the printed text, and links to the digital files are provided in the HTML text of this article on the journal's Web site (www.transplantjournal.com).

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5.3.1 **Abstract**

5.3.1.1 Background:

C-peptide levels are a key measure of beta-cell mass following islet transplantation, but threshold values required to achieve clinically relevant patient-centered outcomes are not yet established.

5.3.1.2 Methods:

We conducted a cross sectional retrospective cohort study evaluating patients undergoing islet transplantation at a single center from 1999-2018. Cohorts included patients achieving insulin independence without hypoglycemia, insulin dependence without hypoglycemia, and those with recurrent symptomatic hypoglycemia. Primary outcome was fasting C-peptide levels at 6-12 months post-first transplant; secondary outcomes included stimulated C-peptide levels and BETA-2 scores. Fasting and stimulated C-peptide, and BETA-2 cut-off values for determination of hypoglycemic freedom and insulin independence were evaluated using receiver-operating characteristic curves.

5.3.1.3 Results:

We analyzed 192 patients, with 122 (63.5%) being insulin independent without hypoglycemia, 61 (31.8%) being insulin dependent without hypoglycemia, and 9 (4.7%) experiencing recurrent symptomatic hypoglycemia. Patients with insulin independence had a median (interquartile range) fasting C-peptide level of 0.66 (0.34) nmol/L, compared to 0.49

(0.25) nmol/L for those being insulin dependent without hypoglycemia, and 0.07 (0.05) nmol/L for patients experiencing hypoglycemia ($p<0.001$). Optimal fasting C-peptide cut-offs for insulin independence and hypoglycemia were ≥ 0.50 nmol/L and ≥ 0.12 nmol/L respectively. Cut-offs for insulin independence and freedom of hypoglycemia using stimulated C-peptide were ≥ 1.2 nmol/L and ≥ 0.68 nmol/L respectively, while optimal cut-off BETA-2 scores were ≥ 16.4 and ≥ 5.2.

5.3.1.4 Conclusions:

We define C-peptide levels and BETA-2 scores associated with patient-centered outcomes. Characterizing these values will enable evaluation of ongoing clinical trials with islet or stem cell therapies.

5.3.2 **Introduction**

Pancreatic islet transplantation (ITx) continues to evolve, with growing evidence supporting its clinical value[1-7]. Considering promising reports of long-term outcomes, indications for ITx are likely to expand further in the coming decades. Novel implantation sites continue to be investigated[8-17], and ongoing clinical trials evaluating stem cell-derived beta-cell replacement therapies offer hope to eliminate barriers such as the need for lifelong immunosuppression and donor supply limitations[1,2,18,19]. Following ITx, persistent graft function as measured by C-peptide is associated with reduced insulin requirements, improved glycemic control, reduced glycemic variability and protection from hypoglycemia[5,16]. Similarly, C-peptide secretion during a mixed meal tolerance test (MMTT) is a well-established primary outcome in immunotherapy trials designed to preserve beta-cell mass in new onset type 1 diabetes mellitus (T1D)[20-22]. However, it remains unclear what C-peptide level cut-off is associated either with clinically meaningful outcomes (how people feel, function, or survive), or appropriate minimal threshold levels that could meet regulatory approval of new cell replacement therapies.

We sought to examine whether we could identify optimal thresholds for fasting and glucose-stimulated C-peptide levels, and BETA-2 score, required to stabilize glycemic control, protect against severe hypoglycemia and/or achieve insulin independence. Thresholds required to achieve key patient-centered outcomes will help clinicians evaluate the relative graft function for patients following ITx. These thresholds will also provide a benchmark for ongoing and future clinical trials, including stem cell-derived beta-cell replacement therapies as these continue to

evolve, and potentially for comparison of efficacy in immune intervention trials with potential to preserve islet mass in new onset T1D.

5.3.3 **Methods**

5.3.3.1 Study Design, Patient Selection, and Outcome Variables

This is a single-center cross-sectional cohort study including adult (≥18 years old) patients with T1D undergoing allogeneic ITx at the University of Alberta Hospital from March 1999 to October 2018. Patients receiving extrahepatic ITx or stem cell-derived ITx were excluded. Additionally, patients with missing data for the primary outcome (C-peptide levels), insulin requirements or assessment of hypoglycemia were excluded (n = 68). A comparative analysis of three groups was conducted to evaluate C-peptide levels and their correlation with two patient-centered outcomes: insulin independence and freedom of hypoglycemia. The groups were divided as follows: 1) Patients with insulin independence and free of hypoglycemia, 2) Patients with insulin dependence but free of hypoglycemia, 3) Patients with insulin dependence with recurrent symptomatic hypoglycemia with unawareness[23,24].

Throughout this study, hypoglycemia refers to level 1, 2 or 3 hypoglycemia as defined by the joint position statement from the American Diabetes Association and the European Association for the Study of Diabetes[25]. Level 2 hypoglycemia refers to a glucose level of <3.0 mmol/L (<54 mg/dL) indicating a serious, clinically important event. Level 3 hypoglycemia denotes any event with severe cognitive impairment requiring external assistance for recovery[25]. Level 1 hypoglycemia (i.e. glucose level ≤3.9 mmol/L) was not included as the clinical severity of these events remain uncharacterized and according to the position statement, do not need to be

routinely reported[25]. The time point of assessment for C-peptide levels, BETA-2 scores, insulin use and the presence of hypoglycemia was done at 6-12 months after the first transplant with all four determinations made during the same month of follow up. All measures were collected >1 month before any supplementary ITx to exclude the effect from recently transplanted grafts – such that hypoglycemic events in the first month after transplant, when insulin doses are being titrated, were not included. For further validation, a secondary analysis was also completed to evaluate primary and secondary outcomes at 12-24 months after the first transplant. For this analysis, patients were categorized again into the three previously described groups. Similarly, all measures were collected >1 month before any supplementary islet infusion.

Demographics were collected at baseline (time of first transplant) and included sex, age at diagnosis of T1D, duration of disease, age at first transplant, and body mass index (BMI). Baseline measurements of diabetes control including HbA1c, insulin units/kg/per day, and fasting C-peptide levels (nmol/L) were also collected, as were markers of glycemic lability denoted by lability indexes, and Clarke scores[26,27]. We also report transplant factors including islet equivalents (IEQs) per kg transplanted, number of islet infusions, the purity of islet infusions, immunosuppression regimens, and oral anti-hyperglycemic agents used by patients. This study was approved by the University of Alberta Health Research Ethics Board (PRO00001120 and PRO00087040).

The primary outcome of this study was differences in fasting C-peptide levels between cohorts at 6-12 months following first ITx. Evaluation of fasting C-peptide levels as the primary outcome was chosen due to its ease of collection without requirements for stimulation, and their use by ITx centers worldwide. Secondary outcomes included comparison of stimulated C-peptide

and BETA-2 scores between cohorts. Stimulated C-peptide was obtained at 90-minutes after a MMTT, as previously described[28]. The BETA-2 score is a composite measure of insulin dose (insulin units/kg/day), fasting plasma glucose (mmol/L), HbA1c (%), and fasting C-peptide levels (nmol/L), which has been previously been validated as a predictive tool for patient relevant outcomes including glycemic control and insulin independence[29,30]. As discussed above, outcomes were secondarily assessed 12-24 months after ITx to further evaluate the validity observed findings after longer follow-up.

5.3.3.2 Transplant and Follow-up Procedures

All ITx were completed at the University of Alberta using standard intraportal infusion. This involves fluoroscopy-guided percutaneous portal venous cannulation and islet infusion as described previously[31]. Patients were admitted to hospital for post-procedural assessment and discharged when clinically well for at least 12-hours following transplant. All patients were followed weekly for the first month after any islet infusion, and every 3-6 months thereafter. During follow up, graft function, hypoglycemic episodes, glycemic control, and use of insulin or other glucose lowering therapies was evaluated.

5.3.3.3 Statistical Analysis

Statistical analysis was completed using STATA v17.0 (StataCorp, College Station, TX, USA). Categorical data are expressed as absolute values with percentages, while continuous data are expressed as medians with interquartile range (IQR). Additionally, 5th and 95th percentile values (5%-95%) are presented for the primary and secondary outcomes to characterize the

complete range of outcomes. Normality testing was performed with the D'Agostino-Pearson normality test to determine the need for non-parametric testing. Between-group comparisons of data were carried out using the Mann–Whitney U test or Kruskal–Wallis test with Dunn's Multiple Comparison Test. Alpha was set to < 0.05.

Analyses to determine the optimal fasting C-peptide, stimulated C-peptide, and BETA-2 score cut-off values for determination of freedom from recurrent symptomatic hypoglycemia and insulin independence were performed using the area under the curve of receiver-operating characteristics curves (AUC-ROC). A non-parametric estimation of the AUC-ROC was determined with Bamber and Hanley confidence intervals for the AUR-ROC curve. Optimal cut-off levels were determined by selecting the value with the greatest product of sensitivity and specificity (i.e., the Liu method)[32].

5.3.4 **Results**

5.3.4.1 Patient Demographics

A total of 192 patients were included in this study, with 122 (63.5%) being insulin independent without hypoglycemia, 61 (31.8%) being insulin dependent without hypoglycemia, and 9 (4.7%) being insulin dependent with hypoglycemia. Cohorts were similar with regards to sex, age at T1D diagnosis, glomerular filtration rate, duration of T1D, age at first transplant, and most measures of glycemic control prior to initial transplant (Table 5.3.1). However, patients with recurrent hypoglycemia had a higher lability index prior to their initial transplant (822, IQR 613, $p = 0.049$). Patients experiencing hypoglycemia had the lowest BMI (23.1kg/m^2, IQR 2.0), followed by those with insulin independence (24.4 kg/m^2, IQR 4.7), and those with insulin

dependence but without hypoglycemia (27.1 kg/m^2, IQR 4.5); these differences were statistically significant ($p < 0.001$). With regards to ITx characteristics, patients demonstrated similar islet preparation purity (60, IQR 24 vs. 60, IQR 18 vs. 52.5 IQR 20, $p = 0.558$), islet equivalents per kg (14,890.3, IQR 8,767.8 vs.15,642.7, IQR 5,938.7 vs. 11,555.5, IQR 3,397.7, $p = 0.124$) and number of transfusions (all 2, IQR 1, $p = 0.274$) for those with insulin independence, hypoglycemic freedom, and with hypoglycemia respectively (Table 5.3.1). Peri-procedural complications are not reported in this study; readers are directed to a recent study by Marfil-Garza et al. (2022), where our group reports on complications in this cohort along a 20-year period[16]. With regards to immunosuppression, most patients in this study received alemtuzumab as an induction agent and a combination of etanercept plus anakinra (**Appendix** Table S5.3.3). Maintenance immunosuppression included tacrolimus for all patients in combination with either mofetil mycophenolate or sirolimus. Analysis of immunosuppression levels showed that tacrolimus trough levels were significantly lower in recipients with insulin independence as compared to the other groups up to a year post-first transplant (**Appendix** Figure S5.3.3**)**. These immunosuppression and anti-inflammatory agents were also similar to those used in our center over the last 20-years[16]. Finally, patients with insulin independence were most likely to use non-insulin glucose lowering agents (n = 64, 52.5%), while relatively fewer patients with insulin independence but without hypoglycemia (n = 31, 50.8%), or with persistent hypoglycemia (n = 3, 33.3%) used these agents, which are further detailed in **Appendix** Table S5.3.4.

Table 5.3.1 Demographics of patients undergoing ITx categorized by insulin independence, insulin dependence but hypoglycemic freedom, and insulin dependence with hypoglycemia.

Variable	Insulin and Hypoglycemia Free (n = 122)	Insulin Dependent and Hypoglycemia Free (n=61)	Insulin Dependent with Hypoglycemia (n = 9)	P value[a]
Demographics and clinical data at baseline, before 1st transplant				
Sex, M/F, n (%)	55 (45.1) / 67 (54.9)	25 (41.0) / 36 (59.0)	3 (33.3) / 6 (66.6)	0.721
Age at diagnosis, yr (IQR)	13.5 (9.0 – 20)	15 (11 – 26)	21 (14 – 27)	0.187
Duration of T1D, yr (IQR)	33.9 (24 – 42)	30.7 (24.0 – 38.6)	26.6 (18.1 – 33.6)	0.274
Age at transplant, yr (IQR)	49.3 (41.8 – 56)	49.3 (44.4 – 56.8)	45.3 (43.0 – 57.1)	0.941
Body-mass index - kg/m^2 (IQR)	24.4 (22.3 – 27.0)	27.1 (24.5 – 29.0)	23.1 (22.0 – 24.0)	<0.001
eGFR, ml/min/m^2 (IQR)	86.0 (71 – 92.3)	81.4 (66.4 – 96.3)	96.5 (71.9 – 100.9)	0.371
Lability index (IQR)	426.5 (266 – 610-7)	467 (322 – 800.7)	822 (369 – 982)	0.049
Clarke score (IQR)	5 (4 – 7)	5 (4 – 6)	7 (6 – 7)	0.14
Laboratory values at baseline, before 1st transplant				
C-peptide (nmol/L) (IQR)	0.02 (0.02 – 0.06)	0.02 (0.02 – 0.03)	0.02 (0.02 – 0.03)	0.252
HbA1c % (IQR)	8.2 (7.4 – 9)	8.3 (7.7 – 9)	8.8 (8.4 – 9.4)	0.119
Insulin units/kg/day (IQR)	0.53 (0.46 – 0.67)	0.52 (0.43 – 0.71)	0.48 (0.43 – 0.50)	0.246
Transplant Characteristics Within the First-Year after Initial ITx				
Number of Islet Infusions	2 (2 – 3)	2 (2 – 3)	2 (2 – 3)	0.274
Total Islet Equivalents per kg (IEQ/kg)	14,890.3 (11,103.7 – 19.871.5)	15,642.7 (12,164.7 – 18,103.4)	11,555.5 (9,974.0 – 13,371.7)	0.124
Purity (1st infusion)	60 (50 – 74)	60 (50 – 68)	52.5 (50 – 70)	0.558

[a] Statistical comparison were done using X2 tests for categorical variables and Kruskal-Wallis tests for continuous variables.
eGFR = estimated glomerular filtration rate

5.3.4.2 Fasting C-peptide, Stimulated C-peptide Levels and BETA-2 Scores

Assessment of the primary and secondary outcomes showed significant differences with regards to fasting C-peptide, stimulated C-peptide, and BETA-2 scores at 6-12 months post-first

transplant (Figure 5.3.1A-C). Patients with insulin independence without hypoglycemia had a median fasting C-peptide level of 0.66 nmol/L (IQR = 0.34, 5%-95% = 0.41-1.24 nmol/L), compared to 0.49 nmol/L (IQR = 0.25, 5%-95% = 0.1-0.95 nmol/L) for those being insulin dependent and without hypoglycemia, and 0.07 nmol/L (IQR 0.05, 5%-95% = 0.02-0.11 nmol/L) in patients being insulin dependent with hypoglycemia ($p<0.001$). Regarding stimulated C-peptide levels, patients who were insulin free showed the highest production (1.52 nmol/L, IQR 0.66, 5%-95% = 0.76-2.35 nmol/L), followed by those requiring insulin but without hypoglycemia (1.19 nmol/L, IQR 0.71, 5%-95% = 0.44-2.48 nmol/L), and those with persistent hypoglycemia (0.24 nmol/L, IQR 0.28, 5%-95% = 0.2-0.6 nmol/L; $p<0.001$). Notably, only 94 (48.9%) patients underwent a mixed-meal tolerance test to determine stimulated C-peptide levels during the 6-12 month study inclusion period (insulin independent n = 55, insulin dependent but hypoglycemia free = 31, insulin dependent with hypoglycemia = 6). Finally, BETA-2 scores showed a similar trend, with patients who were insulin independent having the highest score (22.2, IQR 8.6, 5%-95% = 12.4-32.8), followed by those with insulin dependence but hypoglycemia freedom (12.60, IQR 8.64, 5%-95% = 3.6-21.5), and those with persistent hypoglycemia (3.40, IQR 1.99, 5%-95% = 0.4-6.6) with differences being statistically significant ($p<0.001$). Outcome measures were evaluated after a median of 219 (IQR 176) days since the last transplant for those with insulin independence, compared to 268.5 (IQR 146) days for those who were insulin dependent but hypoglycemia free, and 244 (IQR 205) for those with persistent hypoglycemia.

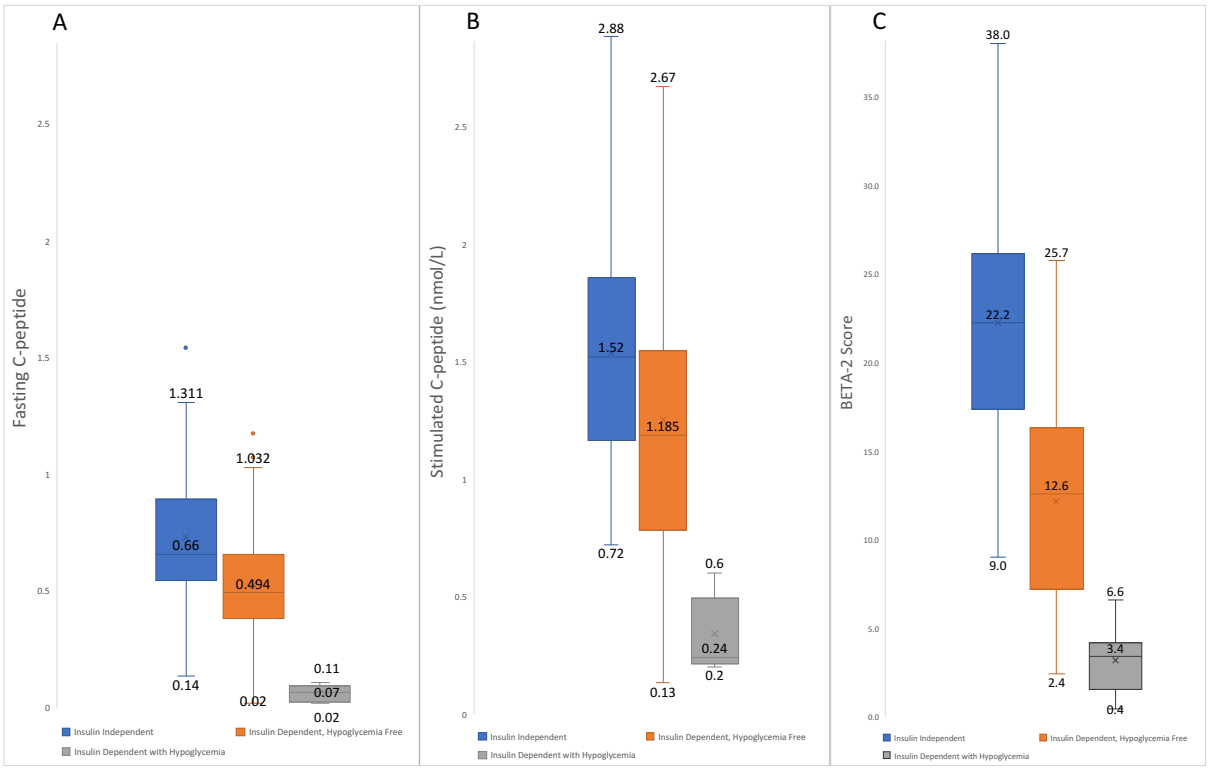

Figure 5.3.1 Fasting C-peptide, Stimulated C-peptide, and BETA-2 Score achieved for patients who are insulin independent without hypoglycemia, insulin dependent without hypoglycemia, and insulin dependent with recurrent symptomatic hypoglycemia with unawareness.
*Box-plot whisker x represent minimum and maximum values calculated as minimum = Q1 – (1.5xIQR) and maximum = Q3 + (1.5xIQR)

For secondary analysis of outcomes at 12-24 months after first transplant, there were 186 patients with adequate follow up and 6 (3.1% of initial population) that were lost to follow-up. Of included patients, 123 (66.1%) were insulin independent, 54 (29.0%) who were insulin dependent but free of hypoglycemia, and 9 (4.8%) with persistent hypoglycemia. Secondary assessment of primary and secondary outcomes after 12-24 months showed similar C-peptide, stimulated C-peptide, and BETA-2 scores for all cohorts (**Appendix** Table S5.3.5). Accordingly, all cut-off values also remained similar after 12-24 months of follow up (Table S3). Outcome

measurements at the 12-24-month data collection time point were obtained at a median of 546 (IQR 295) days from the last transplant for those with insulin independence, compared to 444 (IQR 288.8) days for patients with insulin dependence but free of hypoglycemia, and 590 (IQR 96.8) for patients with persistent hypoglycemia.

5.3.4.3 Optimal Cut-Off Values for Fasting C-Peptide, Stimulated C-peptide, and BETA-2 Score

AUC-ROC analysis determined that fasting C-peptide levels had an ROC area of 0.74 (95%CI, 0.67 – 0.82) for classification of insulin independence (Figure 5.3.2A). An optimal fasting C-peptide cut-off value of ≥ 0.50 nmol/L had a sensitivity of 80.3%, specificity of 57.1%, and correctly identified insulin independence in 71.9% of our patients (Table 5.3.2). For determining hypoglycemia freedom, fasting C-peptide levels had an ROC area of 0.99 (95%CI, 0.97 – 1.00, Figure 5.3.2B), and an optimal cut-off value of ≥0.12 nmol/L correctly identified 97.4% of patients (sensitivity = 97.3%, specificity = 100%,Table 5.3.2). ROC areas for determining insulin independence and hypoglycemic freedom using stimulated C-peptide were 0.72 (95%CI, 0.61 – 0.83) and 0.98 (95%CI, 0.95 – 1.0) respectively (Figure 5.3.2C-D); optimal cut-off values were ≥1.2 nmol/L and ≥0.68 nmol/L to characterize insulin independence and hypoglycemic freedom respectively. Finally, the BETA-2 scores determined insulin independence and hypoglycemia with ROC areas of 0.89 (95%CI, 0.84 - 0.94) and 0.98 (95%CI, 0.96 – 1.00) respectively (Figure 5.3.2E-F). Optimal BETA-2 score cut-off values were determined to be ≥16.4 and ≥5.2 for insulin independence and hypoglycemia respectively (Table 5.3.2) . When analyzing optimal cut-off values for data retrieved 12-24 months after the first transplant demonstrated similar values (**Appendix** Table S5.3.6).

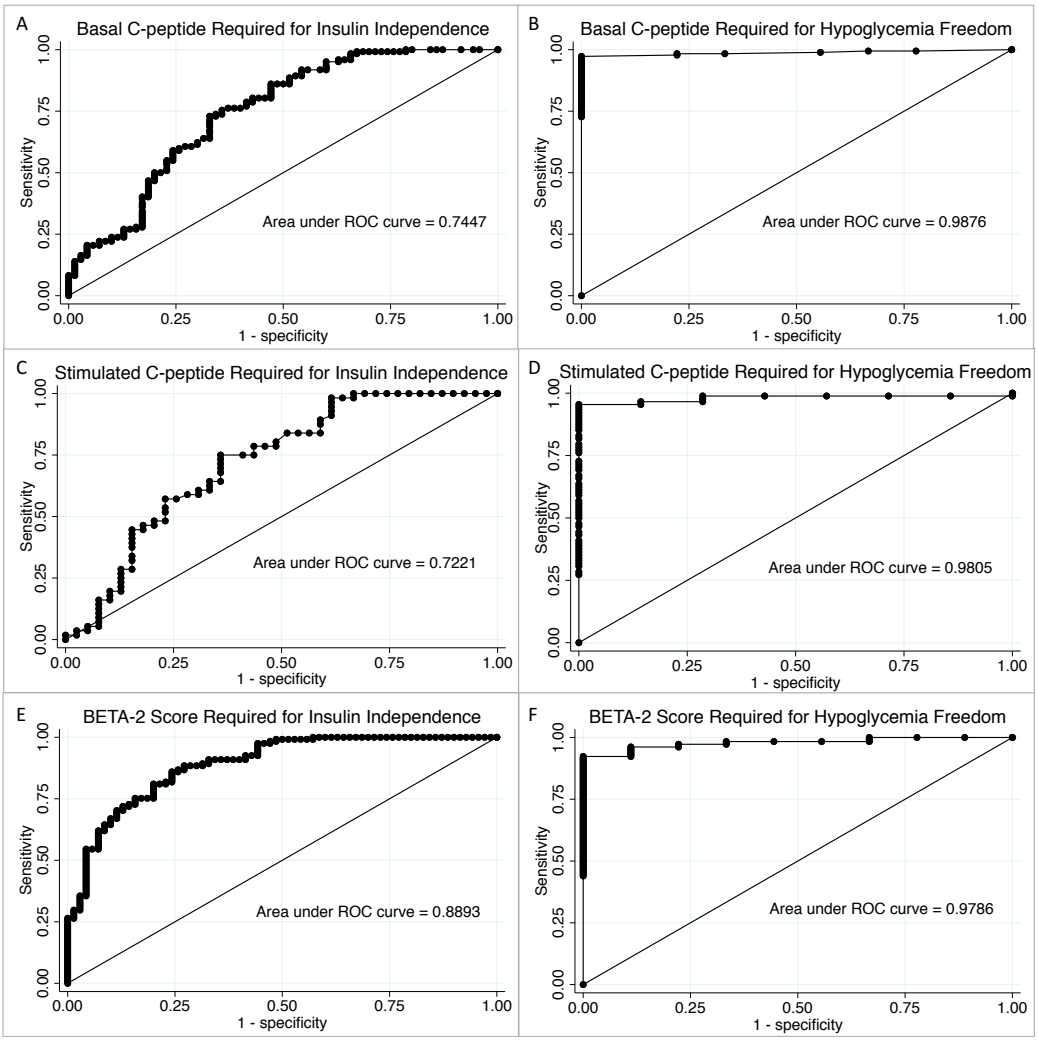

Figure 5.3.2 Receiver-operating characteristics (ROC) curves and area under the curves for C-peptide, stimulated C-peptide, and BETA-2 score to determine insulin independence and persistent symptomatic hypoglycemia in patients following islet transplantation.
A) ROC curve for fasting C-peptide as a determinant of insulin independence. B) ROC curve for fasting C-peptide as a determinant of recurrent symptomatic hypoglycemia. C) ROC curve for stimulated C-peptide as a determinant of insulin independence. D) ROC curve for stimulated C-peptide as a determinant of recurrent symptomatic hypoglycemia. E) ROC curve for BETA-2 score as a determinant of insulin independence. F) ROC curve for BETA-2 score as a determinant of recurrent symptomatic hypoglycemia.

Table 5.3.2 Optimal C-peptide cut-off values determined from area under the curve of receiver operating characteristics curves using the Liu method and the associated sensitivity and specificity of these values. Values represented here are from data collected 6-12 months after the first transplant.

	Optimal Cut-Off for Insulin Independence Without Hypoglycemia	Optimal Cut-Off for Hypoglycemia Freedom
C-peptide (fasting)	≥ 0.50 nmol/L	≥0.12 nmol/L
Sensitivity	80.3%	97.3%
Specificity	57.1%	100%
C-peptide (stimulated)	≥1.2 nmol/L	≥0.68 nmol/L
Sensitivity	75.0%	95.5%
Specificity	64.1%	100%
BETA-2 Score	≥16.4	≥5.2
Sensitivity	81.0%	96.2%
Specificity	80.0%	88.9%

5.3.5 **Discussion**

Herein, we demonstrate that the presence of even low levels (0.12 fasting C-peptide) of C-peptide, are associated with hypoglycemic stabilization. However, substantially higher C-peptide levels are required to achieve insulin independence, with 95% of patients achieving insulin independence having a fasting C-peptide value ≥0.41 nmol/L, with an optimal cut-off value of ≥0.50 nmol/L. Finally, we also show that the BETA-2 score was highly associated with insulin independence, with patients achieving a BETA-2 score of >5 reliably being hypoglycemia-free. These values provide important context for future studies reporting outcomes from stem cell replacement therapies to reverse diabetes, immunotherapies, ITx, and for transplant clinicians evaluating patient outcomes.

Potentially the most useful application of these findings will be to contextualize outcomes for patients included in future clinical trials evaluating novel beta-cell replacement strategies, and for current phase 2 or 3 immunomodulation studies for new-onset T1D[20-22]. Two notable

recent clinical studies evaluating macroencapsulated stem cell-derived ITx in patients with T1D indicate that circulating C-peptide levels can be achieved post-transplant with the use of these cell sources[19,33]. However, highly sensitive C-peptide assays are required to detect function. In these studies, while many patients achieved stimulated C-peptide values > 0.1 nmol/L, none achieved levels > 0.2 nmol/L[19,33]. Considering the findings of our study, those patients would require more C-peptide production to achieve clinical outcomes of interest. In this regard, a recent preliminary report by Vertex Pharmaceuticals, in which stem cell-derived, fully differentiated islets were infused in the intraportal circulation of a patient with T1D, showed that a fasting C-peptide level of 0.28 nmol/L and a stimulated C-peptide of 0.56 nmol/L were associated with an initial 91% decrease in insulin requirements and abrogation of severe hypoglycemia, and subsequent attainment of insulin independence[34]. Similarly, while several studies have reported failure to achieve insulin independence following extrahepatic ITx sites, many of those studies reported C-peptide production in some recipients[8-10,13-15]. Our results help define the clinical relevance of these reported C-peptide values and to understand how close or far those experimental approaches were to achieving important patient-centered outcomes.

Previously, studies have only demonstrated that a higher C-peptide values are associated with reduced diabetes complications, elimination of hypoglycemia, and improved glycemic control, but failed to specify target values to achieve specific outcomes[35,36]. While studies have demonstrated that fasting C-peptide levels >0.4-0.5nmol/L lead patients to be in target glucose range ≥75% of the time[37-39], and that C-peptide levels >0.2nmol/L following MMTT leads patients to be hypoglycemia-free >98% of the time[38], few have evaluated an optimal cut-off C-peptide level required to eliminate insulin or abrogate hypoglycemia. A recent oral presentation

at the latest congress of the International Pancreas and Islet Transplantation Association by Baidal *et al.*, which used data from the Collaborative Islet Transplant Registry (n=541), suggested lower values than previously reported, with a fasting C-peptide level of >0.26 nmol/L and stimulated C-peptide level of >0.97 nmol/L reliably correlating with insulin independence. For absence of hypoglycemia, an optimal cut-off of 0.07 nmol/L was found by these researchers[40]. While we observed slightly higher optimal C-peptide level cut-offs in our study, this may be due to differences in time of outcome evaluation, definition and techniques used to evaluate hypoglycemia, and/or the characteristics of the study populations. Regardless, considering these cut-off values from our study and others, and that our 5%-95% values for hypoglycemia include values <0.1, a question is raised regarding the utility of the typically used threshold to define graft failure (i.e., <0.1nmol/L), which has been adopted and reported globally by clinical islet transplant programs[41]. Consideration of a lower definition may be warranted to capture patients achieving hypoglycemic freedom, but with C-peptide values <0.1nmol/L, as introduced by Landstra et al. in a recent proposal to define graft function post-ITx, the Igls 2.0 criteria[42]. Overall, our study adds to the previous literature and helps to calibrate cut-off and ranges of fasting and stimulated C-peptide levels associated with relevant patient-centered outcomes. Future studies evaluating these cut-off values with regards to continuous glucose monitoring results would help further contextualize these findings and may help characterize the longitudinal and dynamic glycemic benefits we believe occur following islet transplantation.

Furthermore, because C-peptide levels are influenced by blood glucose levels and exogenous insulin, composite scores (incorporating these parameters) have also been created to quantify islet mass after ITx. Several groups, including our own, have demonstrated the utility of

the BETA-2 score to distinguish insulin independence and normal glucose tolerance after ITx[29,30]. Previous work has outlined a BETA-2 score cut-off of >15 to discriminate insulin independence, which is further supported with findings from this study. Additionally, we demonstrate that a BETA-2 score >5 reliably distinguishes patients who are free from severe hypoglycemia. Also, this BETA-2 score cut-off value had the greatest sensitivity and specificity for identifying patients without hypoglycemia compared to the other biochemical levels evaluated.

It is important to note that cohorts received similar islet transplant volumes and were managed by a single team with expertise in ITx, yet had variable C-peptide, BETA-2, and clinical outcomes. In our experience, this outcome heterogeneity is expected. Differences between groups with regards to induction immunosuppression and use of anti-inflammatories including etanercept and anakinra relate to previously described factors associated with sustained graft survival[16,43]. However, more studies evaluating donor and recipient factors including important immune characteristics are needed to better predict success post-ITx. Several limitations from this study require discussion. First, it should be noted that a large proportion of the patient glycemic data from this study represents finger-prick glucometer results; future studies correlating C-peptide levels with outcomes using continuous glucose monitors may better represent outcomes and is planned for future study. The cross-sectional design of this study also needs to be highlighted. While a consistent correlation between C-peptide and BETA-2 scores and patient-centered outcomes was observed at both 6-12 months and 12-24 months, it must be emphasized that these measurements represent a "snap-shot" in time and should not be interpreted in a predictive fashion. Further to this, the study's retrospective nature leaves the

potential that unmeasured confounding variables not assessed in this study led to the outcome differences. Hypoglycemic events, especially in patients with impaired awareness, may be under-reported in this study; however, the rate of level 2 and 3 hypoglycemia reported here coincide with the expected <0.5% rate of hypoglycemia following ITx[38]. In keeping with that, a large proportion of our sample were free of hypoglycemia, which may have inadvertently raised the cut-off values for C-peptide determined in this study. Clinical overlap between the insulin independent group and patients free of hypoglycemia but still requiring insulin should also be noted, especially as the dose of insulin or insulin reduction was not accounted for in this study. Additionally, while we demonstrated these outcomes 6-12 months following initial ITx, many patients received subsequent islet infusions. To reduce the impact of secondary infusions, and the potential for unreliable C-peptide results due to islet death or engraftment surrounding those infusions, all C-peptide levels were taken >1 month after any islet infusion and the time from last islet transplant has been reported, which is similar between groups. We have also assessed outcomes at 12-24 months following first islet transplant and shown similar results. Regardless of these considerations, we believe that characterizing the C-peptide levels required for clinically important outcomes will assist in clinical outcome evaluation, and planning of future clinical trials. Furthermore, we assume that islet and stem cell-derived islets will function in a similar manner with respect to C-peptide secretion, and this may not be the case with more immature differentiating pancreatic progenitor cell components. Future studies that systematically incorporate continuous glycemic monitoring with calculation of time in range would further strengthen our findings, but we do not have access to that data presently.

5.3.6 **Conclusion**

Herein, we define fasting C-peptide, stimulated C-peptide, and BETA-2 score values associated with patient-centered clinical outcomes after ITx. Characterizing these values will assist with assessment of ongoing clinical trials testing novel techniques for ITx. Future studies evaluating the predictive value of these and other markers for requiring subsequent islet infusions or additional treatment would be valuable to help direct clinical care.

5.3.7 **Appendix: chapter 5 subsection 3**

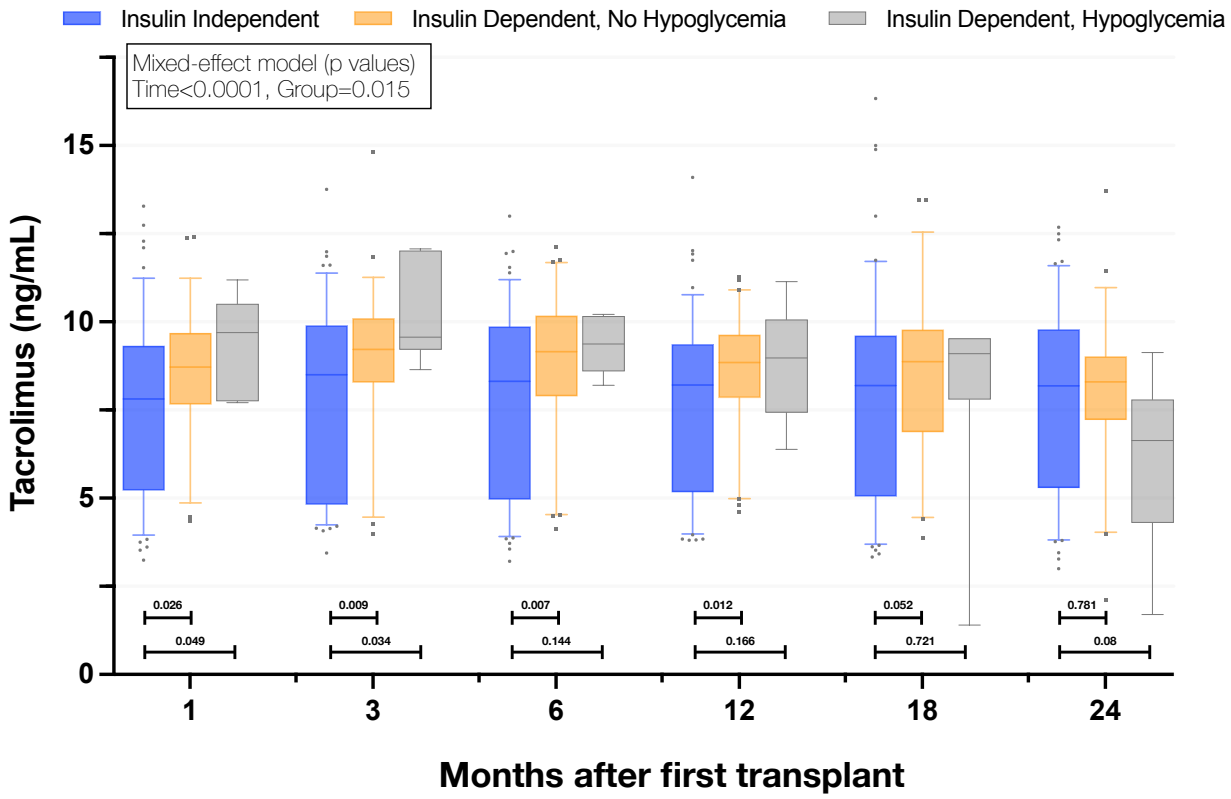

Figure S5.3.3 Tacrolimus trough levels post-transplant.
Data are shown as a box and whiskers plot, with outlier data points included. Boxplots represent interquartile ranges, and whiskers represent 5-95% percentiles. Statistics include a mixed-effect model using the maximum-likelihood method to analyze outcomes over time; time and group effects are reported. At each time point, Mann-Whitney tests were done comparing each group to recipients showing insulin independence and no hypoglycemia.

Table S5.3.3 Immunosuppression regimes for patient cohorts.

	Insulin Independent	Insulin Dependent Without Hypoglycemia	Persistent Hypoglycemia
Induction immunosuppression	**Total infusions=299**	**Total infusions=152**	**Total infusions=18**
Basiliximab, n (%)	39 (13.0)	19 (12.5)	6 (33.3)
Anti-thymocyte globulin, n (%)	40 (13.4)	16 (10.5)	2 (11.1)
Daclizumab, n (%)	92 (30.8)	22 (14.5)	0 (0)
Alemtuzumab, n (%)	126 (42.1)	94 (61.8)	11 (61.1)
Belatacept, n (%)	4 (1.3)	3 (2.0)	2 (22.2)
Anti-inflammatories			
Anakinra alone, n (%)	1 (0.03)	0 (0)	0 (0)
Etanercept alone, n (%)	51 (17.1)	18 (11.8)	1 (5.6)
Anakinra + etanercept, n (%)	126 (42.1)	101 (66.4)	13 (72.2)
Infliximab, n (%)	11 (3.7)	4 (2.6)	0 (0)
None, n (%)	110 (36.8)	29 (19.1)	4 (22.2)
Maintenance immunosuppression	**Total patients=122**	**Total patients=61**	**Total patients=9**
Tacrolimus, n (%)	122 (100)	61 (100)	9 (100)
Mofetil mycophenolate, n (%)	111 (91.0)	58 (95.1)	9 (100)
Sirolimus, n (%)	57 (46.7)	17 (27.9)	0 (0)

Note: Maintenance immunosuppression was with two immunosuppressant agents at a time. The Edmonton protocol involved maintenance immunosuppression using high-dose sirolimus (target 12-15 ng/mL during the first year, 7-10 ng/mL onwards) and tacrolimus (3-6 ng/ml), with substitution of MMF (up to 1g bid) and increased tacrolimus levels (8-10 ng/mL) in recipients experiencing side effects from sirolimus. Modifications to our protocols included increased tacrolimus levels (10-12 ng/mL during the first 3 months, 8-10 ng/mL onwards) and MMF (up to 1g bid). Tacrolimus targets were adjusted (6-8 ng/mL or lower) in case of adverse effects or renal dysfunction.

Table S5.3.4 Use of non-insulin glucose-lowering agents post-transplantation

	Insulin Independent	Insulin Dependent Without Hypoglycemia	Persistent Hypoglycemia
Use of non-insulin glucose-lowering agents, n (%)	64/122 (52.5)	31/61 (50.8)	3/9 (33.3)
SGLT-2 inhibitors, n (%)	15/64 (23.4)	8/31 (25.8)	1/3 (33.3)
GLP-1 agonists, n (%)	10/64 (15.6)	6/31 (19.4)	2/3 (66.6)
DPP-4 inhibitors, n (%)	44/64 (68.8)	25/31 (80.6)	1/3 (33.3)
Thiazolidinediones (glitazones), n (%)	17/64 (26.6)	2/31 (6.5)	0/3 (0)
Metformin, n (%)	25/64 (39.0)	4/31 (12.9)	0/3 (0)
Other (acarbose, glyburide, repaglinide), n (%)	17/64 (26.6)	5/31 (16.1)	0/3 (0)

Table S5.3.5 Primary and Secondary Outcomes for Each Cohort at 6-12 months After First Transplant and 12-24 Months After First Transplant

Cohort	Fasting C-peptide Median *IQR* *5%-95%*	Stimulated C-peptide Median *IQR* *5%-95%*	BETA-2 Score Median *IQR* *5%-95%*
6-12 month follow-up			
Insulin Independent Hypoglycemia Free	0.66 nmol/L *0.34* *0.41-1.24 nmol/L*	1.52 nmol/L *0.66* *0.76-2.35 nmol/L*	22.2 *8.6* *12.4-32.8 nmol/L*
Insulin Dependent but Hypoglycemia Free	0.49 nmol/L *0.25* *0.1-0.95 nmol/L*	1.19 nmol/L, 0.71 0.44-2.48 nmol/L	12.60 *8.64* *3.6-21.5 nmol/L*
Persistent Severe Hypoglycemia	0.07 nmol/L *0.05* *0.02-0.11 nmol/L*	0.24 nmol/L *0.28* *0.2-0.6 nmol/L*	3.40 *1.99* *0.4-6.6 nmol/L*
12-24 month follow-up			
Insulin Independent Hypoglycemia Free	0.69 nmol/L *0.26* *0.02-0.94 nmol/L*	1.52 nmol/L *0.73* *0.69-2.74 nmol/L*	20.7 *8.7* *6.9-36.8 nmol/L*
Insulin Dependent but Hypoglycemia Free	0.50 nmol/L *0.32* *0.37-1.22 nmol/L*	0.95 nmol/L *0.56* *0.07-2.23 nmol/L*	10.0 *8.6* *0.45-20.2 nmol/L*
Persistent Severe Hypoglycemia	0.04 nmol/L 0.1 0.02-0.13 nmol/L	0.14 nmol/L *0.13* *0.02-0.2 nmol/L*	2.5 *1.6* *0.7-7.6 nmol/L*

Table S5.3.6 Optimal C-peptide cut-off values determined from area under the curve of receiver operating characteristics curves using the Liu method and the associated sensitivity and specificity of these values.
Values represented here are from data collected 12-24 months after the first transplant.

	Optimal Cut-Off for Insulin Independence Without Hypoglycemia	Optimal Cut-Off for Hypoglycemia Freedom
C-peptide (fasting)	≥ 0.52 nmol/L	≥0.15 nmol/L
Sensitivity	88.6%	97.7%
Specificity	61.9%	100%
C-peptide (stimulated)	≥1.14 nmol/L	≥0.55 nmol/L
Sensitivity	79.0%	98.4%
Specificity	70.3%	100%
BETA-2 Score	≥16.4	≥5.0
Sensitivity	80.4%	94.2%
Specificity	90.6%	77.8%

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5.4 Chapter 5 subsection 4 –Stem Cell-Derived Islet Transplantation in Patients with Type 2 Diabetes: Can Diabetes Subtypes Guide Implementation?

JOURNAL ARTICLE ACCEPTED MANUSCRIPT

Stem Cell–Derived Islet Transplantation in Patients with Type 2 Diabetes: Can Diabetes Subtypes Guide Implementation?

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5.4.1 **Abstract**

Historically, only patients with brittle diabetes or severe recurrent hypoglycemia have been considered for islet transplantation (ITx). This population has been selected to optimize the risk-benefit profile considering risks of long-term immunosuppression and limited organ supply. However, with the advent of stem cell-derived ITx and the potential for immunosuppression-free ITx, consideration of a broader recipient cohort may soon be justified. Simultaneously, the classical categorization of diabetes is being challenged by growing evidence to support a clustering of disease subtypes that can be better categorized by the All New Diabetics in Scania (ANDIS) classification system. Using the ANDIS classification 5 subtypes of diabetes have been described, each with unique causes and consequences. We evaluate consideration for ITx in the context of this broader patient population and the new classification of diabetes subtypes.

In this review, we evaluate considerations for ITx based on novel diabetes subtypes, including their limitations, and elaborate on unique transplant features that should now be considered to enable ITx in these “unconventional” patient cohorts. Based on evidence from those receiving whole pancreas transplant and our more than 20-year experience with ITx, we offer recommendations and potential research avenues to justify implementation of SC-derived ITx in broader populations of patients with all types of diabetes.

5.4.2 **Introduction**

Islet transplantation (ITx) continues to advance as a viable therapy for patients with brittle, hard-to-control, life-threatening type 1 diabetes (T1D), with growing data supporting promising long-term outcomes[1-5]. With the introduction of stem cell (SC)-derived ITx, and clinical trials underway evaluating this novel approach[6,7], the possibility of a renewable islet cell source is within sight. Furthermore, through genetic manipulation of embryonic stem cells (ESCs) generate hypo-immune islets, or autologous ITx from induced pluripotent stem cell (iPSCs), immunosuppression free ITx is emerging as a future potential[5,8,9]. Ensuring unlimited access to cell sources for transplantation while eliminating lifelong immunosuppressive requirements would improve clinical benefit and reduce risks, which is likely to expand the potential patients eligible for SC-derived ITx, including some patients living with type 2 diabetes (T2D), where transplants may benefit from no autoimmunity to damage transplanted beta-cells. The latter has become more relevant as growing evidence supports phenotypic heterogeneity amongst patients with T2D, with variable disease clinical characteristics, etiology, progression, and therapy responses[10-13]. Ahlqvist et al. (2018) categorizes patients into five distinct diabetes subtypes that are discussed below, with numerous studies validating these criteria clinically, genomically, and based on therapeutic responses[10-14]. With these aspects in mind, a reassessment of the clinical considerations for ITx in patients with T2D is warranted to ensure optimal preliminary implementation and outcome evaluation with future studies involving SC-derived ITx.

We herein review novel diabetes subtypes previously presented by Ahlqvist et al. (2018) and discuss their pathophysiologic and clinical characteristics in context with potential cell replacement therapies. Importantly, we elaborate on specific aspects and unique transplant features that need to be considered to enable ITx in these "unconventional" patient cohorts. Finally, based on evidence from those receiving whole pancreas transplant and our more than 20-year experience with ITx[1], we offer recommendations and potential research avenues to guide implementation of SC-derived ITx in patients in patients with subtypes of T2D.

5.4.3 **Diabetes Subtypes**

Diabetes represents a clinical disease marked by the presence of hyperglycemia. While the disease has historically been classified into T1D and T2D, increasing evidence is demonstrating that diabetes exists as distinct subtypes. Using the All New Diabetics in Scania (ANDIS) categorization system five unique clinical subtypes of diabetes have now been isolated and validated across several cohorts[10,11,15,16]. While some overlap exists between subtypes, each of these diagnostic categories has a unique clinical presentation, disease progression, therapeutic response, and complications[12,16,17]. These categories exclude patients with monogenic diabetes and secondary diabetes, which will not be discussed in this review. These categories are being increasingly applied due to their potential to guide future personalized treatments, which could be critical for efficacy of SC-derived ITx in these patients.

ANDIS categories of diabetes include severe autoimmune diabetes (SAID), severe insulin deficient diabetes (SIDD), severe insulin resistant diabetes (SIRD), mild obesity-related diabetes (MOD), and mild age-related diabetes (MARD) (Figure 5.4.1). Diabetes subtypes can

be categorized using six clinical parameters: the presence of glutamate decarboxylase autoantibodies (GADA), age at diabetes onset, hemoglobin A1c (HbA1c), body mass index (BMI), and measures of insulin resistance and secretion using the updated homoeostatic model assessment estimates of β-cell function (HOMA2-B) and insulin resistance (HOMA2-IR). The most distinct of these subtypes is SAID, which represents approximately 6% of patients and is delineated by the presence of GADA. These patients have early disease onset, autoimmune islet destruction, typically have higher HbA1c levels, and have absolute loss of insulin production (historically categorized as T1D). This definition is drawn from Ahlqvist et al. (2018) who did not measure other classically described antibodies (including IA-2 or ZnT8) associated with T1D and therefore may under-represent those with autoimmunity against islets. Many of the non-GAD autoantibody positive patients are likely categorized as SIDD. The remaining subtypes were classically categorized as T2D and are the focus of this review. SIDD includes ~18% of patients and is categorized by early onset, relatively low BMI, low insulin secretion (low HOMA-B), elevated HbA1c. As such, patients with SIDD frequently progress to requiring insulin, typically experience higher HbA1c levels, and have the highest prevalence of retinopathy and neuropathy[11,13,15,18]. Notably, SIDD is genetically and clinically distinct from T1D and SAID and lacks key single nucleotide polymorphisms associated with autoimmune diabetes[19]. On the other hand, SIRD (representing ~15% of patients) is the least heritable form of diabetes, and also has distinct genetic characteristics as it is not associated with the well-established T2D locus in *TCF7L2,* which is clinically associated with inadequate insulin production[*10,20*]. SIRD occurs later in life, and is unique in that high plasma C-peptide levels are notable, and it occurs in patients with central obesity, metabolic syndrome, and insulin resistance (high HOMA2-IR). Particularly,

these patients present with central, rather than peripheral, obesity and ectopic fat including substantial risk of fat deposition within the liver[10,11]. As such, these patients experience the highest risk of diabetic nephropathy and non-alcoholic fatty liver disease (NAFLD)[11,13,14]. MOD describes ~22% of patients with diabetes and is characterized by an association with genetic obesity clusters and is clinically represented by early onset and obesity but without insulin resistance[10,21]. Finally, MARD is characterized by old age and overall mild clinical phenotypes (i.e. modest HbA1c elevation and HOMA2 indices), coupled with a reduced risk of nephropathy compared to other subtypes, and limited benefit from treatments including with oral antihyperglycemic agents[16].

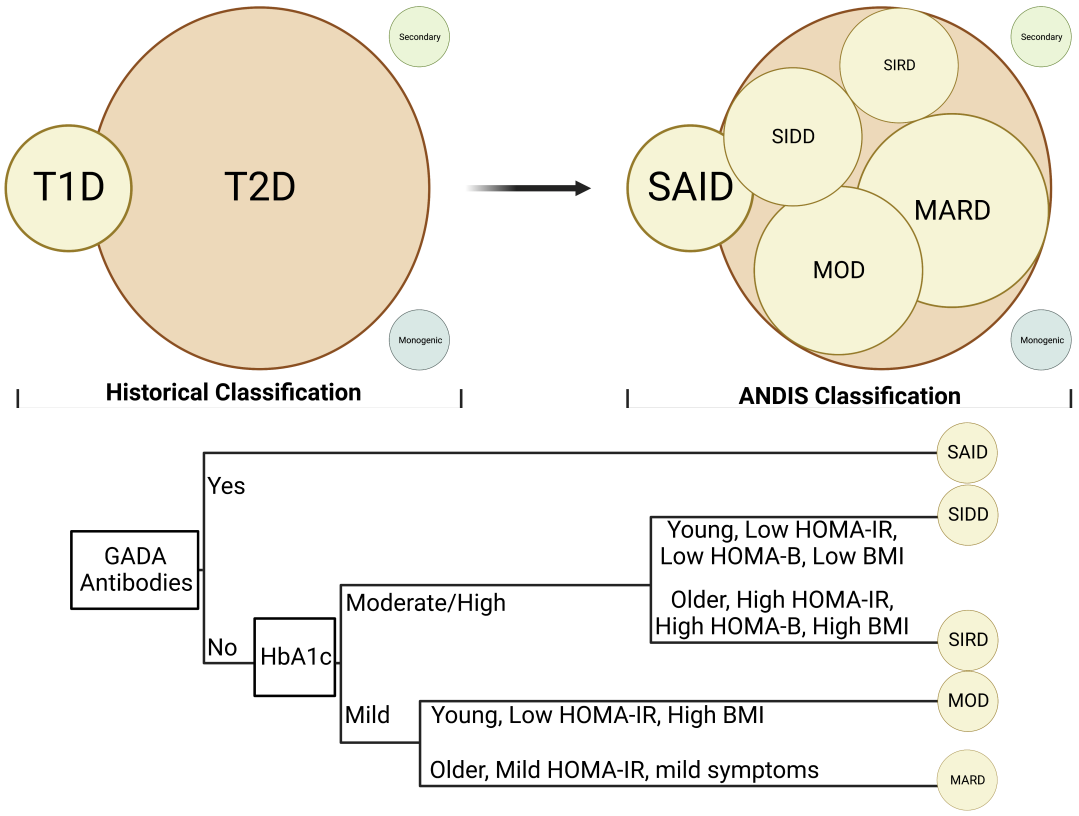

Figure 5.4.1 Categorization of diabetes based on historical classification systems and updated ANDIS classification.
Figure reproduced/adapted from Ahlqvist et al (2020)[10] with permission from the American Diabetes Association.

5.4.4 Relevance of Diabetes Subtype on Stem Cell-Derived Islet Transplantation

Each diabetes subtype has demonstrated unique therapeutic responses due to their different pathophysiologic mechanisms. Therefore, we hypothesize that adequately classifying patients will be crucial for implementation and evaluation of SC-derived ITx. Considerations and relevance of factors associated with T1D/SAID for SC-derived ITx have been thoroughly described elsewhere [5,8], and therefore, this review focuses primarily on SIDD, SIRD, MOD, and

MARD. The utility of cell replacement therapies in the setting of different T2D subtypes will depend on the degree of insulin resistance and diabetes risk based on hyperglycemic risks and projected progression of secondary complications to justify a cell transplant. Since there is limited but positive experience of diabetes reversal and insulin independence in highly selected patients with T2D (the 'lean type 2's') receiving whole pancreas transplants, it is anticipated but not yet known with certainty that repletion of insulin-producing islets of sufficient quantity could potentially overcome insulin resistance. Dosing of islets to this degree is rarely possible with human cadaveric-derived cells, but could be with more limitless dosing from stem cell manufactured products.

5.4.4.1 SIDD

Patients with SIDD have a clinical presentation resembling T1D/SAID, including severe hyperglycemia, increased risk of diabetic ketoacidosis, severely elevated HbA1c at diagnosis, and low C-peptide secretory capacity. These patients also experience a rapid progression to insulin dependence with a moderate risk of hypoglycemia[10-13]. Currently, these patients are typically diagnosed with T2D as they lack evidence of islet autoimmunity (GAD positivity). Importantly, genetic evaluation demonstrates that SIDD is not associated with SAID loci, but remains highly associated with *TCF7L2* and genetically similar to other classical T2D subtypes [14]. Others have suggested that due to their tendency to rapidly progress towards insulin requirement and the observed high risk of neuropathy, retinopathy, and other diabetes-related complications, these patients benefit from early, intensive treatment[10]. Together, the severe lack of insulin and rapid disease progression prompts us to hypothesize that these patients should be

the primary focus of efforts attempting to “expand” the classical indications for ITx to include selected people diagnosed with T2D characterized by insulin deficiency (Figure 5.4.2). In fact, because of the clinical similarity to T1D but without the risk of recurrent autoimmune destruction, we hypothesize that patients with SIDD could achieve superior outcomes following SC-derived ITx compared to any other patient group.

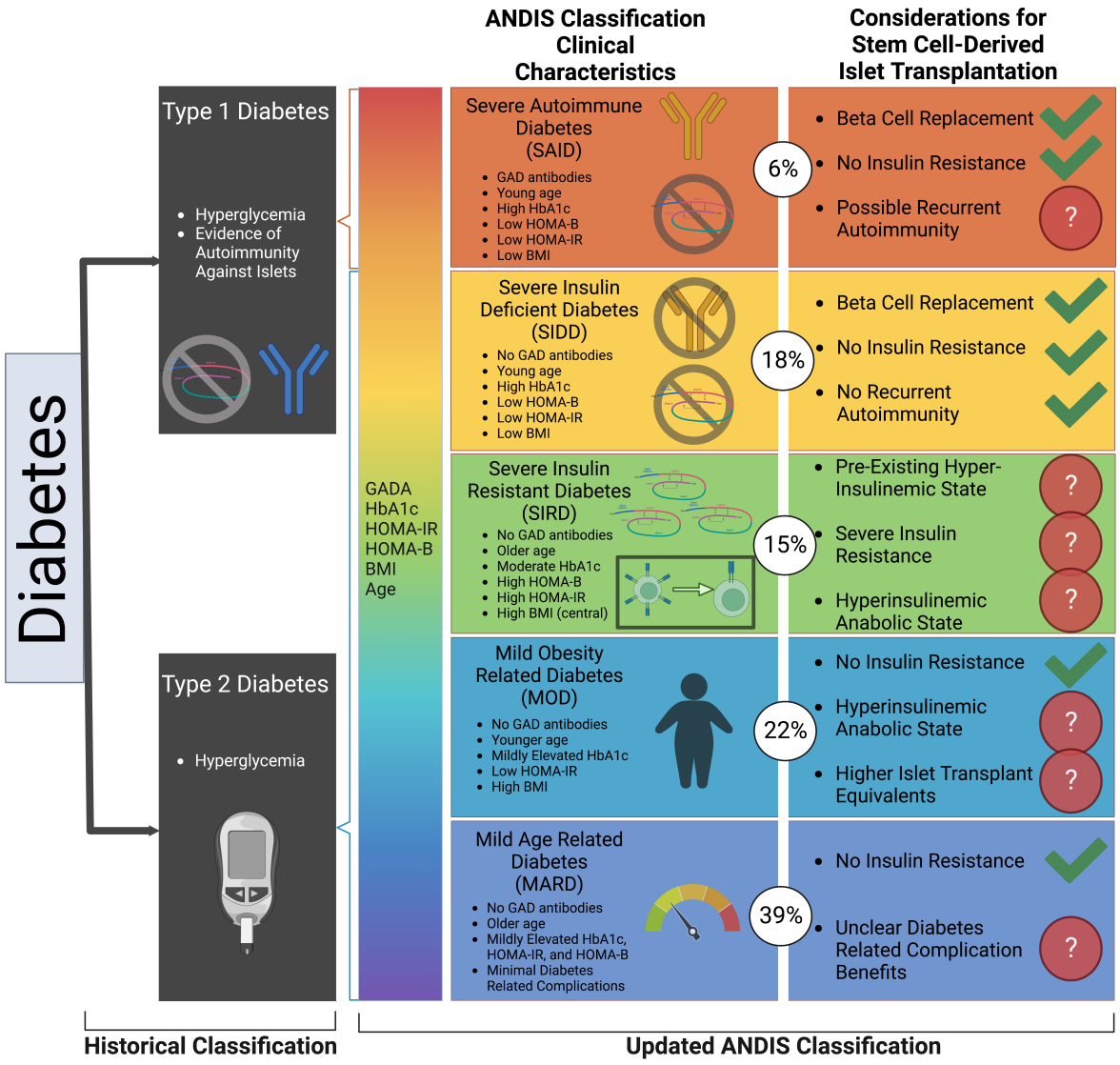

Figure 5.4.2 Classification of diabetes, clinical characteristics for ANDIS classification, and classification associated considerations for stem cell-derived islet transplantation.

Importantly, the etiology of insulin deficiency in these patients remains uncertain and requires further investigation to ensure that islets do not become dysfunctional or destroyed following transplant. As discussed above, many patients without GAD antibodies but with IA-2 or ZnT8 antibodies are likely categorized as SIDD when applying the ANDIS classification.

Considering the growing recognition of latent autoimmune diabetes of adults (LADA), recommendations suggest that newly diagnosed patients with risks factors for LADA should also be tested for at least one other antibody[22]. Other studies have shown that up to 7% of patients initially classified as SIDD have been found to be GADA negative, yet have alternative islet or insulin autoantibodies, suggesting they may still require therapies to combat recurrent autoimmunity[13]. Further investigation of these patients for zinc transporter 8 (ZnT8), IA2, and insulin autoantibodies (IAA) would provide further characterization of SIDD patients. Additionally, for patients without antibodies who present with modest HbA1c should be evaluated with the MODY clinical risk calculator or genetic testing as per recommendations[23,24]. While investigation for non-GADA is required and may limit the number of true non-autoimmune SIDD, we hypothesize that these patients are likely to benefit most in response to SC-derived ITx. For those with non-GADA autoimmune markers, further investigation into the timeline for loss of islet function will be important to determine whether combative therapies to eliminate recurrent autoimmune destruction (in the case of accelerated loss) or repeat SC-derived ITx would be most beneficial and cost-effective.

As an additional note, for patients who are found to have an autoantibody and are diagnosed with LADA, C-peptide quantification is likely to enable an optimal personalized approach to therapy[22]. Patients can be categorized as low (< 0.3 nmol/L), intermediate (0.3-0.7 nmol/L), and high (<0.7 nmol/L) based on their euglycemic serum C-peptide measurement. In these cases, we hypothesize that patients with low or intermediate C-peptide, and those who's C-peptide deteriorates over time to these levels and without insulin resistance, are likely to benefit most for SC-derived ITx. This is supported by a recent study evaluating patients with T2D

undergoing whole pancreas transplant, whereby those with high C-peptide had higher incidence of graft dysfunction[25]. Although limited ITx specific evidence on this topic currently exists, evaluation of these outcomes following ITx will be of interest in the future.

5.4.4.2 SIRD

Patients with SIRD have clinical central obesity, metabolic syndrome, hyperinsulinemia, and severe peripheral tissue insulin resistance (recognized with increased HOMA2-IR indices). This subtype presents frequently with chronic kidney disease at baseline and shows a relatively fast progression of their nephropathy[26,27], as well as an increased risk of NAFLD[10,14,28]. Studies evaluating this subtype have demonstrated that SIRD has a distinct etiology of diabetes, without any association to *TCF7L2* or other loci related to insulin secretion and with limited familial inheritance[14]. In fact, whole genome association studies have suggested that β cells plays only a minor role in the etiology of SIRD and that unique alleles within the *PNPLA3* gene may account for the severe insulin resistance in these patients[28]. As such, we hypothesize that patients with SIRD are unlikely to have a substantial clinical benefit from SC-derived ITx, as larger cell volumes would be required to overcome their insulin resistance, thus increasing their risks for any treatment-related complications (Figure 5.4.2). The supratherapeutic insulin levels that would be required to achieve an effect could potentially worsen obesity and associated complications in this subpopulation. Additionally, evidence shows that, even with improved glycemic control, patients classified within the SIRD subtype experience progressive nephropathy and NAFLD due to their severe insulin resistance[28,29]. Thus, therapies aimed at

weight loss and improving insulin sensitivity are more likely to confer overall clinical benefits, and if effective, perhaps cell replacement could be considered as a therapeutic adjunct if at all.

5.4.4.3 MOD

The MOD subtype is characterized by early onset diabetes with obesity but without insulin resistance. These patients experience less metabolic derangement compared to those with SAID or SIDD and therefore achieve less absolute HbA1c reduction with oral antihyperglycemic agents but experience fewer diabetes-related complications[16]. However, they experience the second highest rate of progression to insulin dependence, suspected to be caused by their early disease onset and prolonged diabetes course [10-13]. Currently, limited information with regards to clinical outcomes and disease status is available for patients with MOD and will certainly be of interest in the future. Considering the lack of insulin resistance, the addition of insulin producing SC-derived islets could theoretically prove beneficial in this population. However, generating a hyperinsulinemic state, with its associated anabolic effects, could again potentially worsen obesity in this subgroup too. Considering the wide array of musculoskeletal, metabolic, cardiac and other obesity-associated complications and ongoing optimization of weight loss therapies including glucagon-like peptide-1 analogues such as semaglutide[30], SGLT2 inhibitors[31], and bariatric surgery[32], SC-derived ITx likely represents a secondary, tertiary, or adjunctive therapy in patients with MOD. Additionally, as these patients experience obesity, higher insulin equivalents would be required to achieve therapeutic benefits, which may impact the cost of SC-derived ITx in this cohort. Overall, current knowledge remains limited but suggests that although

patients with MOD lack substantial insulin resistance, weight loss therapies are likely to provide superior benefit than SC-derived ITx (Figure 5.4.2).

5.4.4.4 MARD

Finally, the MARD subtype is characterized by older age at diagnosis, relatively mild diabetes symptoms, and minimal diabetes-related complications (Figure 5.4.2)[10,13]. While these patients have a relative insulin deficiency and limited insulin resistance, suggesting they might benefit from SC-derived ITx, patient selection will be critical to optimize any potential benefits and avoid unnecessary harm. For elderly patients with mild hyperglycemia that is well controlled with diet or single anti-hyperglycemic agents, SC-derived ITx is unlikely to offer further long-term benefits. Furthermore, from a technical perspective, generating iPSCs from elderly patients has been demonstrated to have decreased efficiency and increased mutagenic risk, which must be considered at the onset of autologous iPSC ITx implementation[33,34]. On the other hand, in a highly selected group of patients with MARD with other renal, cardiac, or neuropathic comorbidities that could be worsened with diabetes, or for patients who experience difficulty managing their medications (including polypharmacy), SC-derived ITx could potentially offer benefits. Overall, for most patients with MARD SC-derived ITx would likely outweigh the benefits, however, individualized evaluation is likely necessary.

5.4.5 **Indirect Evidence for Islet Transplantation in Type 2 Diabetes**

While ITx is currently limited to patients with brittle T1D and those with recurrent severe hypoglycemia, evidence from patients with T2D receiving whole pancreas transplant provides

insight into potential efficacy of ITx for T2D[35]. While most of those patients receive simultaneous kidney-pancreas or pancreas after kidney transplants, outcomes have consistently been shown to be similar between patients with T1D and T2D[36-39]. Most notably, the capacity to achieve similar HbA1c levels in patients with T1D and T2D suggests that beta cell replacement with adequate mass can effectively provide long-term glycemic control to patients with T2D[36-39]. In those patients, HbA1c has been shown to remain consistently <6%, with the only difference being a higher C-peptide level in those with T2D[39]. Unfortunately, none of these studies have evaluated patients based on the ANDIS classification of diabetes. However, many of these studies excluded patients with obesity and in one study the HOMA-IR indices were similar between patients with T1D and T2D, suggesting a possible bias toward selection of patients that would be classified as having SIDD[39]. However, a recent systematic review on pancreas transplant also highlighted a need to better characterize patients with T2D who may benefit from transplant[35]. Future studies categorizing patients by the ANDIS classification prior to pancreas transplant may be of interest to better characterize outcomes and are needed to inform the hypotheses in this review. Regardless, current evidence from whole pancreas transplant provides strength to the potential for ITx in patients with T2D.

5.4.6 **Summary of Recommendations and Limitations**

Although novel diabetes subtype classifications offer insight into potential therapeutic response to SC-derived ITx, limitations must be recognized. First, these considerations and recommendations remain purely theoretical at this point and will need to be continuously reevaluated as SC-derived ITx emerge into clinical practice. Additionally, it should be noted that

when using the ANDIS classification up to 20% may require reclassification over the next 5 years following diagnosis[13]. The largest ANDIS classification changes occur due to changes between MOD, SIDD, and MARD, whereby patients would still achieve benefits from ITx, whereas fewer patients are expected to transition to the SIRD subtype[13]. In addition to categorical fluidity, substantial overlap exists between subtypes making clinical categorization difficult. Future research to determine the best diabetes classification system will certainly be of benefit for ongoing optimization of personalized therapeutic approaches. As an alternative to the ANDIS classification, utilization of the clinical categorization suggested by Dennis et al (2019) or potentially a more pragmatic approach to categorize patients as low or high C-peptide may facilitate clinical application and may help identify patients with clinical T1D or LADA that are miscategorized as T2D[16,22,40]. If categorizing patients based on serum C-peptide, those with lower serum values are likely to benefit most from SC-derived ITx. Regardless of the categorization system, we introduce aspects of SC-islet therapies for additional consideration as we better understand the nuances of these different T2D subtypes. Overall, despite limitations with these early subtype classifications, we believe that understanding and characterizing patients based on clinical presentation, pathophysiology, and natural history remains critical to avoid limiting potential therapeutic options that could substantially impact disease progression, such as ITx and eventually SC-derived ITx.

Although current experience with ITx in patients with T2D is limited, we offer several recommendations and hypotheses based on our experience with ITx over the past 22-years. First, we suggest that when considering ITx for people with T2D, these candidates should be evaluated and characterized according to the updated diabetes subtypes with a particular emphasis on

identifying those with greater degrees of insulin deficiency [10,11,13]. Additionally, we suggest that outcomes following ITx or SC-derived ITx be reported according to diabetes subtypes to improve our understanding and identify ideal scenarios where these therapies can best thrive, as response to other therapies has been variable across subtypes[10-13]. As highlighted above, we also hypothesize that initial efforts should focus on people with T2D classified within the SIDD subtype or those with insulin deficiency, as they are most likely to achieve the greatest benefit from ITx or SC-derived ITx, while those classified within the MOD, MARD, or SIRD subtypes are less likely to achieve benefit and will require careful case-by-case patient selection.

5.4.7 **Conclusion**

Pancreatic ITx has been typically limited to patients with T1D experiencing severe and recurrent hypoglycemia, extreme glycemic lability or in those having rapid progression of diabetes-related complications. These limitations are driven by the limited organ supply and risks of lifelong immunosuppression for risk-benefit balance. We anticipate that the introduction of SC-derived ITx, particularly cell products that eliminate immunosuppression requirements, will open the possibility to extend treatment to other patient populations, including those diagnosed with T2D. However, a thorough understanding of the spectrum of disease and a thoughtful characterization of the distinct T2D subtypes remains crucial to optimize and evaluate future efforts with SC-derived ITx.

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Chapter 6: Lessons learned and future directions for preclinical and clinical studies to advance stem cell-derived islet transplantation clinically

6.1.1 **Introduction**

If there is something to be learned from the last 5-years, with the rapid evolution and translation of SARS-CoV-2 vaccines and therapies, it is that clinicians, scientists, and legislators possess the capability to advance therapies when driven by need. With that in mind, it is hard to argue against diabetes mellitus (DM) as the single greatest health care need being faced globally, with approximately 400 million affected patients, rapidly growing prevalence, and substantial associated morbidity and mortality [1]. Diabetes should be attacked with equal determination and innovation as seen with SARS-CoV-2. It behooves us, as clinicians and scientists, to strive for more than just iterative advances and push for research that drives clinically impactful therapies. As discussed in Chapter 1, stem cell-derived islet transplantation (SC ITx) offers a real potential for a cell-based cure for this cell-based disease. While SC ITx remains in its comparative infancy compared to other diabetes therapies, its novelty should not preclude excitement to frame preclinical work in the context of clinical translation. As a clinician, this thesis presents preclinical and clinical studies with specific goals to advance SC ITx. The introductory chapters highlight success of translating ITx clinically over the last twenty-years, including numerous preclinical optimizations such as the administration of anti-inflammatories anakinra and etanercept following ITx. Chapter 1.2 then introduces SC ITx, specifically touching on key barriers to the field that the rest of this thesis aims to resolve including the need to scale therapies (Chapter 2), optimize SC islet generation to eliminate off-target cells (Chapter 3), and combat inflammatory and immune destruction of transplanted islets (Chapter 4). Finally, Chapter 5 evaluates how we can transition these preclinical studies clinically including the utility of

humanized mouse models (Chapter 5.1), the optimal transplant location (Chapter 5.2), ensuring we can evaluate and implement therapies clinically (Chapter 5.3), and expanding the pool of potential islet recipients (Chapter 5.4).

Together, the studies presented in this thesis advance SC ITx across numerous battle fronts. Despite these advancements, the next 10-years hold promise to take current studies and make a difference to millions of patients worldwide. In this final chapter, I discuss future for both preclinical and clinical studies needed to translate SC ITx clinically. This challenge should be approached with rigor and excitement, and as previously stated in Chapter 3.2, "it is no longer sufficient to simply report protocols that generate insulin producing SC-islets." Never has it been more poignant to accelerate the advancement of bioengineering for clinical practice.

6.1.2 **Remaining preclinical study priorities**

Despite substantial advancement of the field from producing partially impure pancreatic progenitors [2,3], to now characterizing cells throughout differentiation and producing increasingly pure SC islet-like cells [4-8], several areas of focus remain. This list is not exhaustive, and indeed better understanding the metabolic transition, differentiation pathways, and characteristics of cells as they differentiate will remain important to improve our biological understanding of the differentiation process and ensuing product. However, here, we focus on direct barriers currently being faced by clinical trials limiting in-human implementation. In my opinion, these include the need to eliminate off-target growth, advance encapsulation technologies including bioprinting and alternative transplant sites, and ensuring the scalability of this evolving biotechnology.

6.1.2.1 Off-target elimination

Despite numerous approaches to positively or negatively select highly pure SC islets, off-target growth remains a significant barrier to SC ITx. In chapter 3.2, even with nearly 100% of cells being C-peptide$^+$ cystic off-target growth persists. As discussed in that study, co-expressing Cpep$^+$/SOX9$^+$ cells likely matured *in vivo* into ductal off-target tissues. As such, simple selection of C-peptide$^+$ cells or elimination of SOX9$^+$ cells with cell sorting is likely not possible at a clinically significant scale. For this reason, although they are being evaluated, chemical and mechanical approaches to reduce off-target cell are likely to be inadequate or to reduce yield of the SC islet product [9-21]. Instead, it is likely that further SC islet maturation is required to reduce the risk of off-target cells. Balboa et al. (2022) demonstrated *in vitro* maturation and improved function of islets following 6-weeks of culture after a seven stage differentiation protocol[8]. Unfortunately, the authors of this study did not report outcomes related to their grafts or off-target growth. It would be interesting to know whether this prolonged in vitro maturation, associated with improved islet maturity, also reduced the off-target growth within grafts. Regardless, the prolonged culture reported by Balboa et al. (2022) would complicate scalability and increase the cost of SC islet generation; as such, methods to improve SC islet differentiation would be most beneficial. However, I would propose the following set of experiments stemming from their results as an approach to eliminate off-target growth. First, because Balboa et a. (2022) demonstrated islet maturation with *in vitro* culture, I would propose repeated these trials with graft characterization. Characterizing cells following 1 to 6 weeks of *in vitro* maturation and evaluating their *in vivo* safety may allow the field to define the cell characteristics needed to

achieve a product without off-target growth. I would hypothesize prolonged *in vitro* maturation eliminates off-target or cystic growth. If successful, I would propose several techniques that could be evaluated to rapidly mature SC islets, rather than prolonged *in vitro* maturation. In our lab, we have trialed the AKT inhibitor AT7867, demonstrating that it improved pancreatic progenitor maturation and reduces *in vivo* maturation time (see Appendix A for manuscript details). This provides a conceptual basis for small molecules to potentially increase islet maturation timelines. As such, I would suggest evaluating alternative "maturation molecules". For example, we know from Chapter 3.2 that the primary difference between immature SC islets and human islets is related to their relatively immature metabolism Other studies evaluating islet embryology and development have shown similar metabolism in fetal islets, demonstrating that a transition from mTORC1 to AMPK pathways induces their metabolic maturation [22]. Therefore, treating metabolically immature Stage 6 SC islets with rapamycin, a potent mTORC1 inhibitor, may offer a potential mechanism to induce metabolic maturation. Harmine, a molecule that can induce β cell proliferation and increase their glucose stimulated insulin secretion may also offer a potential molecule to improve SC islet function or maturation [23]. Alternatively, we also know that if islets are persistently exposed to hyperglycemic conditions their maturation and function is inhibited [24,25]. Therefore, I would alter the *in vitro* glycemic environment, evaluating hypoglycemic, euglycemic and variable glycemic culture media during the last stage of differentiation, to evaluate whether is supports SC islet maturation. Also somewhat related to their metabolism, I would attempt to alter the *in vitro* oxygen tension of cultured immature SC islets to determine if hypo-oxygenation could drive metabolic maturation. Overall, evaluating

differential maturation agents and conditions may enable rapid generation of comparatively mature SC islets.

Perhaps a less studied concept is that the transplant site and environment may play a critical role in SC islet differentiation, proliferation, and maturation *in vivo*. For example, it has been demonstrated that human islets transplanted into the renal subcapsular space of mice proliferate after contralateral nephrectomy is performed[26]. Certainly, considering the relative immaturity of transplanted SC islets, the potential for these cells to inadvertently transdifferentiate into off target tissues because of their local transplant environment remains a possibility. This includes the renal subcapsular space in mice, where grafts will be affected by several hormones associated with volume status and electrolyte changes, but also grow within a milieu primarily composed of renal tubules and ducts. Whether these environmental factors play a substantial role in SC islet maturation, and whether it influences cystic growth, will be critical to evaluate. A relatively straightforward study, where SC islets are transplanted into the renal subcapsular space, subcutaneous devices, prevascularized spaces, or intraportally to evaluate the effect on the final mature product would be tremendously informative. It remains possible that current SC islet products are in fact safe without off target growth if transplanted into the intraportal system, however, further evaluation of this consideration in a preclinical setting is warranted.

6.1.2.2 Potential for encapsulation and alternative transplant sites

Despite proof-of-concept offered by ITx for a cell-based diabetes therapy, clinical trials evaluating SC ITx continue to be difficult to implement. The risk associated with off-target

growth combined with the irretrievability of an intraportal site presents a high risk — high reward ethical dilemma limiting clinical trials. As such, nearly all current SC ITx clinical trials utilize a micro or macro encapsulation technique with transplant into the subcutaneous space. Despite transplanting cells into the subcutaneous space, the Shapiro et al. 2021 and Ramzy et al. (2021) reports both demonstrated circulating C-peptide in stem cell islet recipients[27,28]. Only one clinical trial (NCT05210530) currently allows intraportal transplant, offering only a single cell product to a highly select patient cohort that require ongoing immunosuppression. Chapter 5.2 provides the largest cohort study evaluating extrahepatic sites, highlighting the difficulty of achieving clinical benefit in those settings. This has also led current Vertex clinical trials to transition towards implantation of SC islets into the intraportal system or the preperitoneal space. However, to expand clinical trials and demonstrate efficacy of SC ITx, optimization of these encapsulation approaches or implementation of novel transplant sites would simplify and speed up clinical translation.

The first and most studied approach to this dilemma is encapsulation or optimization of a subcutaneous transplant site. Encapsulation within the subcutaneous site offers a retrievable, monitorable, and easily accessible site that would allow large volume ITx, hitting the mark for several favorable aspects of SC ITx. However, the subcutaneous space remains a relatively avascular setting, while encapsulation faces foreign body response that has previously limited islet survival. For a recent review on encapsulation devices the reader is directed to Marfil-Garza (2020) and Zhang et al (2022)[29,30]. Our lab has trialed approaches to harness the neovascularization induced by foreign body responses to create prevascularized site for ITx, which is currently under clinical evaluation[31]. Combining this prevascularized site with

encapsulation devices to prevent immune islet destruction may offer a potential approach for future clinical trials[32]. One potential approach includes three-dimensional bioprinting to encapsulate and protect islets, with Aspect biosystems providing one such technique that will be important to evaluate in the coming years. However, the list of studies demonstrating success with encapsulation of subcutaneous transplant in murine models that failed to translate into humans is substantial. Determining whether a similar foreign body response will prevascularize the subcutaneous space in primates or human models will be critical to the success of such approaches. Alternatively, prevascularizing SC islets themselves may offer a potential approach to enable encapsulation or subcutaneous transplant[33]. Regardless of the approach, ensuring SC islet vascularization, while potentially protecting them from immune destruction, would allow rapid uptake and expansion of SC ITx trials. Alternatively, devices that fail in human settings with SC islets could wrongly be attributed to SC islet failure, and should be cautiously evaluated. Considering that the subcutaneous space and encapsulation has been evaluated for decades, this challenge remains substantial.

6.1.2.3 Scalability and commercialization

In addition to eliminating off-target cell populations, it is incumbent on us, as the scientists developing and optimizing these SC islets to ensure that the ensuing product is financially and technically scalable. Without considering this, like many therapies before this, SC ITx faces a certain future of being limited to few recipients with specific indications, or to only those who can afford such therapies. It is important to highlight that diabetes does not distinguish, with broad populations and socioeconomic backgrounds being affected; this

challenge is perhaps best highlighted by the reduction in life-years ranging from 7-70 years based primarily on access to therapies [1]. Therefore, these SC islet therapies must be applicable across a breadth of socioeconomic and health care systems.

First, yield and scalability should be highlighted as key outcomes for future islet differentiation or purification protocols to ensure clinical applicability. These considerations must be made now, during preclinical development. As such, all future studies should report yield and be driven to demonstrate scalability of their results. Chapter 2 highlights some approaches for addressing scalability that require further investigation. For autologous iPSC ITx generating, selecting, and expanding an optimal starting iPSC product is critical. Recent research has demonstrated the applicability of artificial intelligence, in combination with automated techniques to select ideal iPSC colonies[34,35]. Improving upon this work to determine improved selection processes[36], or to reduce the time required to select optimal iPSC colonies (currently 20+ days) would greatly improve the translatability of iPSC therapies. Beyond iPSC selection, our work in Chapter 2.2 and Chapter 3.2 demonstrates the utility of iPSC expansion and differentiation within Vertical-Wheel® bioreactors. Scaling those approaches to larger 3 L or 15 L formats, will certainly be of interest. These larger format vessels may require different rotational mixing speeds or alternative media changing schedules. We used 60 rotations per minute speed due to previous work that reported this as an optimal speed for stem cell expansion rather than differentiation [37,38]; however, the optimal speed of reactors may be different during differentiation and may change from stage-to-stage, offering a method to potentially improve cell differentiation efficiency or yield. Additionally, these vessel formats allow control of mixed gasses within the bioreactor and continuous media replacement, that will require ongoing

optimization to improve cell expansion and differentiation. However, potentially most important, these suspension based protocols must be tested in mass production formats for scale up including CellFactory™, TreeFrog Therapeutic's C-Stem™, and Lonza Coccon® platforms. Proof of concept within scalable and personalizable formats such as these are critical to enable widespread application of both iPSC and embryonic stem cell islet therapies.

In addition to optimizing protocols to ensure scalability, the field also needs to recognize the cost reduction that SC ITx promises. In Alberta, the current interprovincial cost associated with a single ITx is approximately $70,000, with costs up to $200,000 reported in American centers; however, these costs require significant contextualization. Unlike current diabetes therapies SC ITx is a one-time treatment, rather than continuous therapy and the costs must be contextualized as such. Approaching SC ITx with not just the cost, but the long term patient and financial benefit will be imperative to ensuring an accurate representation of this therapy. For example, a year of insulin therapy costs up to 28 billion dollars in the United States, approximately $3,500 per year per patient. The costs associated with monitoing glycemic control including newer continuous glucose or bioartificial pancreas systems is equally as restrictive. Additionally, the cost of diabetes associated complications including renal, cardiovascular, opthalmalgic and more contribute an unknown but massive cost. In public health care settings new renumeration approaches are considering paying providers or commercial suppliers per year of benefit. As such, considering a payment model where the supplier is paid a specific amount per year for the first 10-years if patients had glycemic control and an increased rate following years 10 onwards if the patient did not develop complications may be appropriate for this therapy. Similar approaches could also be considered for private health care structures. Overall,

such innovative therapies such as these will require unique cost conceptualization and funding approaches to ensure patients can benefit.

Finally, cryopreservation offers an important avenue to evaluate in order to improve the clinical applicability of these cell products. Zhan et al. (2022) recently demonstrate the capacity to cryopreserve islets and stem cell-derived islets with an optimized scalable vitrification protocol that achieves impressive post-recovery yield and cell quality. This data offers the potential to dramatically improve opportunities for islet transplantation and alter the pathway to transplant for recipients. Cryopreservation such as this may allow islet banking and prolonged preservation to match ideal recipients over temporal barriers. Additionally, in the case of SC ITx this would allow for evaluation, *in vivo* testing, and approval of a cell product followed by transplant of the same cell product that is cryopreserved and available to patients. These are just some of the benefits that cryopreservation may offer in the future of these cell products.

Overall, the commercialization of SC ITx should be seen directly and indirectly as our responsibility. As discussed in Chapter 2.1, even during early preclinical work, we must consider the potential of generated protocols for scale-up and scale-out. Reporting yield and scalability remains underreported in current literature, yet should be considered key outcome measures and reported in all future studies. Additionally, as we frame these therapies in the literature and to industry it will be imperative to not only focus on the cost of therapy, but the overall long-term cost savings and benefit to patients. Generating an accessible product for millions of patients remains equally important as ensuring the efficacy and safety of SC ITx and it is our responsibility to consider this key outcome for success.

6.1.3 **Advancing stem cell-derived islets into clinical practice**

In order to keep up with the rapid preclinical advancement of SC ITx, clinical studies must also push forward. As with many therapies under trial, clinical studies will be uniquely positioned to provide important answers that are not evaluable in the preclinical setting. Namely, as discussed in Chapter 5.1, the preclinical models to evaluate the impact of immunity on SC islets remain limited. Most notably, the impact of immune destruction will provide guidance on whether an autologous iPSC islet or modified or immune protected embryonic stem cell (ESC) islet therapy will be best. Additionally, with the introduction of these therapies, clinicians must be aware about, and continue to support patient enrollment into SC islet clinical trials to inform future use. We must also improve the breadth of our outcome evaluation in order to capture not only physician-important outcomes such as HbA1c, but key patient centered-outcomes including quality of life and mental distress, that ITx may improve but that has not been well captured or reported to date. Using this data, and the significant body of recent long-term outcomes from historical ITx[39], clinicians must push for updated legislation to improve accessibility. This includes broadening recipient pools to consider those with earlier T1D and those with T2D as potential ITx recipients to capture the full potential of SC ITx.

6.1.3.1 Universal Cell Product vs. Personalized Cell Product

As perhaps the most uncertain aspect of SC ITx, it remains highly unclear whether autologous iPSC ITx or allogeneic SC ITx will prevail as the dominant approach. The two therapies are thoroughly reviewed, compared, and contrasted in Chapter 1. Both approaches

continue to be optimized, evaluated, and will continue to be debated in a preclinical setting, but it will be the clinical results that truly determine the preferred modality. In my opinion, two clinical outcomes will determine the success of each therapy. First, determining the success of genetically modified ESC islets to remain functional and evade immune destruction remains imperative to success of allogeneic SC ITx. On the other hand, determining the presence and characteristics of recurrent autoimmunity in autologous iPSC ITx will be critical for success in patients with T1D. Although humanized mouse models may be able to inform specific immune responses, these remain limited as discussed in Chapter 5.1. Complete recapitulation of the immune system to mimic in-human responses will likely require in-human evaluation.

While allogeneic SC ITx offers a single islet source with easier scalability as reviewed in Chapter 1, the allograft rejection is certainly its greatest barrier and may be its downfall. The ViaCyte/Vertex VCTX210 (previously PEC QT) offers the first ESC-derived islet product with genetic modification to reduce allogeneic immunoreactivity. Certainly the results from its ongoing clinical trial will be critical, not just in terms of efficacy, but to determine the potential of genetic modification to eliminate immune destruction. If islet immune destruction persists, alternative CRISPR modifications to increase interleukin 10 expression [40-42], or eliminate human leukocyte antigens [43-45] could be trialed and would represent the most likely next steps. More recently, evidence has suggested that upregulation of genes including CXCL10, SUM01, CD274, IL32, ICAM1, and PRDX1 may offer potential targets to reduce immune destruction of SC islets [46]. Alternative approaches such as encapsulation could be trialed but would delay clinical implementation and may represent a significant barrier as previously discussed. Finally, combinatorial approaches with immune reset or regulatory T cell therapies could represent an

option, but may limit cost effectiveness and arguably represent immune modulation strategies that simply replace immunosuppression, and carry potential risks of their own.

While autologous iPSC ITx faces cynics due to concerns regarding scalability or cost, the true per patient long-term costs are unlikely to be dramatic barriers if it offers an efficacious therapy. Critics of autologous iPSC ITx often cite the cost of iPSC generation and expansion as dramatic barriers. However, we must consider that iPSC generation and expansion costs only a few thousand dollars (Chapter 2). With artificial intelligence, automation, and engineering advancements those costs will be marginal. Again, as discussed above, diabetes is a tremendously costly disease and the up-front one-time cost of iPSC ITx should not preclude its application. A more substantial barrier to autologous iPSC ITx is likely to be the recurrent autoimmunity that islets face following transplant. Very little data exists to even develop a hypothesis regarding the relative impact this may have. It remains unclear whether the autoimmune destruction will be consistent across age groups, sexes, or heterogeneous patients with T1D with variable autoantibody presence. Similarly, each patient's T1D onset has a variable second phase and whether that will relate to the degree of autoimmunity against SC islets will be critical to evaluate. As discussed in Chapter 4, immune reset techniques or low dose immunosuppressants may be required. Alternatively, combining autologous iPSC ITx with novel drugs like teplizumab, that delay the second phase of T1D, may be beneficial to maintain graft survival in these patients; however, this will only be evaluable in the clinical setting and will be critical to determine the viability of autologous therapies.

Immune destruction and combatting immunity with lifelong immunosuppression has remained the primary barrier to deceased donor allogeneic ITx for the last twenty years.

Similarly, combatting the immune response likely remains the primary barrier to SC ITx. Outcomes from the current Vertex clinical trial with CRISPR modified ESC islets will certainly be informative. Similarly, determining the presence, timing, and severity of recurrent autoimmunity to iPSC islets will inform preventative approaches. The last twenty years have demonstrated that ITx with immunosuppression is limited to highly select recipients and has substantial islet destruction over time. This would limit commercialization, cost, and perhaps most importantly the relative benefits to patients from SC ITx. For both allogeneic and autologous SC ITx the immune tolerance and response will likely dictate the therapy that continues forward and will be critical to determine in the next 5-years.

6.1.3.2 Clinical Trial Enrollment and Evaluation

Although current clinical trials are ongoing several important factors will predict their success. Considering the strict inclusion and exclusion criteria of current trials, enrolling patients is likely to be a challenge. Once enrolled, the field must determine what outlines success both from a clinician perspective, but also from a regulatory and patient perspective.

The limitations of current clinical trials remain under evaluated and reported. Currently, due to the relative novelty of SC ITx, few patients meet inclusion into active clinical trials. Nearly 20,000 patients were screened for the current Vertex VX-880 trials in order to include only a handful. It is not reasonable nor informative if these trials require 10-years to enroll sufficient patients. As such, clinicians and stakeholders must raise awareness about these trials to ensure ongoing advancement. Currently, few if any, patients are referred for consideration of islet transplantation or for such trials, despite potential benefits for many patients. This likely

originates from lack of education about islet transplantation and availability of accurate information. Considering this, we must ensure that information is adequately disseminated to clinicians. This can be achieved marginally through publication, but will involve discussions, presentations, and divulgation at local, regional, national, and international meetings and conferences to ensure that physicians, scientists, and perhaps most importantly the diabetes community are aware of such opportunities.

In terms of evaluating the success of SC ITx, chapter 5.3 provides some preliminary work outlining C-peptide levels that correlate with patient important outcomes. However, the field continues to lack understanding of the benefit that ITx provides to patients quality of life, mental, and overall wellbeing. In discussing these therapies with patients, the freedom and relief from mental distress often arises but has not been captured in the current research. Perhaps, some of the quality of life data can be collected from recipients of deceased donor ITx to better understand long-term effect. Indeed, better understanding and characterizing outcomes like this will be important for patients and for regulators as we push for broader application of SC islet therapies.

6.1.3.3 Broadening Recipient Pools

First, for patients with type 1 diabetes, the indications and guidelines required to be considered for an islet transplant should be re-evaluated. These have remained in place for over 20-years and have become antequated considering the advancements of ITx. Reassessment of these indications using recently published long-term outcomes from several centers is necessary. Furthermore, considering a growing trend towards patient centered care, a comparatively patient

centered approach whereby potential recipients can select to undergo ITx after consultation and education may be beneficial.

Additionally, we cannot continue to consider ITx as solely a type 1 diabetes therapy. This is relevant to all of the above sections including the preclinical and clinical work that remains. As covered in Chapter 5.4, if successful, SC ITx is likely to provide a robust therapy for patients with type 1 diabetes, and targeted subgroups of patients with type 2 diabetes (T2D). Some may raise concerns that this broader recipient pool could raise further issue with scalability, safety, or clinical trials. However, this should be viewed as an opportunity to establish funding and lobby support for these therapies. As above, there are over 400 million patients with diabetes and growing[1], yet of all patients with diabetes 90% have T2D. Raising awareness and demonstrating the clinical potential of ITx in patients with T2D would allow a united and strengthened diabetes innovation voice. I would propose that clinical trials evaluating SC ITx should include matched patients with T2D, particularly those with relative insulin deficiency rather than insulin resistance. These patients will be critical to determine the relative effect of recurrent autoimmunity on islet destruction but also serve as a proof of concept for islet mass replacement in T2D. Indeed, those with T2D and end stage renal disease likely offer an ideal candidate for such trials. Currently, ITx is viewed solely as a treatment for T1D and it is our responsibility to broaden the scope of this therapy to offer potential benefits to those patients.

6.1.4 **Concluding Remarks**

It has been a privilege to contribute preclinical and clinical studies that advance SC ITx. It is my hope and prediction that regenerative cell therapies for diabetes will become

commonplace during my career as a clinician scientist. The field has pushed forward rapidly over the last decade but to achieve those goals, we must remained focused on the responsibility and goal at hand. Innovators, both scientific and clinical, must continue to overcome the remaining key barriers to clinical implementation. Preclinically, we must continue to strive for an optimal and potentially immune protective transplant device or location. Simultaneously, we must ensure absolute safety of SC islet cell therapies with elimination of off-target cells and ensure that the products we generate are scalable to ensure accessibility and commercialization. With the help of courageous and hopeful patient partners, implementing these therapies clinically we must investigate the immunogenic, both allo- and autoimmune, is controlled and that awareness ensures enrollment and success of current and future clinical trials. With these success, and by empowering the voice of all patients with diabetes, changing the future of diabetes therapy and fulfilling the promise of a cell-based cure remains possible. Of course, this road is fraught with challenges to overcome, but should be pushed forward with excitement to change the lives of millions with innovation, something that I have witnessed at times of needs even in the early stages of my career.

6.1.5 **References**

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Appendix A: other published works

In addition to the above work on stem cell-derived islet transplantation I have also contributed to several related studies that may be of interest to readers of this thesis. These are summarized here with a brief overview of the study's findings and importance in relation to this thesis.

1. Marfil-Garza, B.A; Imes, S; Verhoeff, K; Hefler, J; Lam, A; Dajani, K; Anderson, B; O'Gorman D; Kin, T; Bigam, D; Senior, P.A; Shapiro, A.M.J. Pancreatic Islet Transplantation in Type 1 Diabetes: 20-year Experience from a Single Centre Cohort in Canada. The Lancet Diabetes and Endocrinology. May 2022. DOI: https://doi.org/10.1016/S2213-8587(22)00114-0
   - This study offers the largest single center cohort study of long-term outcomes following islet transplantation. The study found robust control of glycemic lability and hyperglycemia in patients with preserved graft function and nearly complete abrogation of hypoglycemia in all patients. Additionally, we found that the combined use of anakinra plus etanercept and the BETA-2 score were associated with improved outcomes following islet transplantation. Data such as this provides key information to hopefully expand islet transplantation, but also serves as a proof of concept for stem cell-derived islet therapies.
2. Cuesta-Gomez, N; Verhoeff, K; Jasra, I; Pawlick, R; Dadheech, N; Shapiro, A.M.J. Characterization of stem-cell-derived islets during differentiation and after implantation. Cell Reports. August 2022, 40(8). DOI: 10.1016/j.celrep.2022.111238

- This review manuscript the most thorough evaluation of off-target populations that may arise during islet differentiation. Importantly, we hypothesize proposed methods to evaluate differentiation efficiency and clinical release criteria that may be used during clinical evaluation and implementation.

3. Cuesta-Gomez, N; Verhoeff, K; Dadheech, N; Pawlick, R; Marfil-Garza, B; Razavy, H; Shapiro, A.M.J. AT7867 promotes pancreatic progenitor differentiation of human iPSCs and accelerates diabetes reversal. Manuscript submitted for publication.
    - This preclinical study demonstrates the utility of a small molecule, AT7867, for induction of improved pancreatic progenitor differentiation. Studies like this demonstrate the potential to further improve differentiation efficiency and maturation without prolonging in vitro culture, offering a method to reduce off-target populations as discussed in Chapter 6.
4. Marfil-Garza, B.A; Hefler, J; Verhoeff, K; Lam, A; MD, Dajani, K; Anderson, B; O'Gorman, D; Kin, T; Senior, P.A; *Bigam, D; *Shapiro, A.M.J. Whole Pancreas and Pancreatic Islet Transplantation: Comparative Outcomes of a Single–centre Cohort over 20–years. Annals of Surgery. December 2022. DOI: 10.1097/SLA.0000000000005783

This clinical study provides the largest study to date comparing outcomes and safety of islet transplantation to whole pancreas transplant. Results demonstrate that islet transplantation is comparatively safer, but has a lower rate of insulin independence and graft survival.