Manufacturing and Transplantation of Stem Cell Derived Beta Cells

by

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Table of Contents

Acknowledgements **ii**

List of Tables **vii**

List of Figures **viii**

Abstract **xvii**

Chapter 1. Introduction **1**

1.1 State of Type I Diabetes Cell Replacement Therapy 1

1.2 Manufacturing Platforms for Cell Transplantation 5

1.3 Transplantation 7

1.3.1 Enhancing Survival 8

1.3.2 Enhancing Integration 10

1.3.3 Enhancing Function 13

1.4 Immune Protection 14

1.4.1 Local Immune Modulation 14

1.4.2 Antigen-Specific Immune Modulation 19

1.5 Final Thoughts 22

1.6 Structure of Thesis 22

Bibliography 24

Chapter 2. Microporous Polymer Scaffolds for the Transplantation of Embryonic Stem Cell Derived Pancreatic Progenitors to a Clinically Translatable Site for the Treatment of Type I Diabetes **40**

2.1 Introduction 40

2.2 Methods and Materials 41

2.2.1 Microporous PLG Scaffolds 41

2.2.2 In Vitro Differentiation of PSCs into Pancreatic Progenitor Cells 42

2.2.3 qRT-PCR 43

2.2.4 Streptozotocin Induced Diabetic Mouse Model 43

2.2.5 Transplantation of Pancreatic Progenitors to Streptozotocin-Induced Diabetic NSG Mice 44

2.2.6 Serum Collection and Human C-Peptide Analysis 45

2.2.7 Immunostaining 45

2.2.8 Statistics 46

2.3 Results 46

2.3.1 In Vitro Differentiation of Pancreatic Progenitors and Seeding on Scaffolds 46

2.3.2 Transplantation of PSC-Derived Pancreatic Progenitors to Epididymal Fat Pad 48

2.3.3 Exendin-4 Delivery from Scaffold Enhances Pancreatic Progenitor Function 51

2.4 Discussion 52

Bibliography 55

Chapter 3. Normoglycemia Achieved in Diabetic Mice Following Islet Organoid Maturation in the Peritoneal Fat 60

3.1 Introduction 60

3.2 Methods 61

3.2.1 Fabricating Microporous PLG Scaffolds 61

3.3 hPSC-Derived β Cells 62

3.3.1 qRT-PCR Analysis 62

3.3.2 Streptozotocin Induced Diabetic Mouse Model 63

3.3.3 Transplantation of hPSC-Derived β Cells 64

3.3.4 Intraperitoneal Glucose Tolerance Test and Human C-peptide Analysis 65

3.3.5 Immunohistological Staining 65

3.3.6 Statistics 65

3.4 Results 66

3.5 Discussion 73

Bibliography 76

Chapter 4. Enhanced Pancreatic Endocrine Induction of Pancreatic Progenitors Following Culture within Microporous Scaffolds **79**

4.1 Introduction 79

4.2 Methods 82

4.2.1 Microporous Scaffold Fabrication 82

4.2.2 Designing sfGFP-Cpep Fluorescent Insulin Reporter hPSC Line 83

4.2.3 Differentiation of hPSCs and Scaffold Seeding 83

4.2.4 qRT-PCR Analysis 84

4.2.5 Statistics 84

4.3 Results 85

4.3.1 Pre-Pancreatic Progenitor Scaffold Culture 85

4.3.2 SC-derived β-Cell Scaffold Culture 86

4.3.3 Endocrine Progenitor Scaffold Culture 87

4.3.4 Planar Seeded SC-derived β-Cell and Endocrine Progenitor Scaffold Culture 87

4.4 Discussion 91

Bibliography 94

Chapter 5. Conclusions and Future Directions 96

5.1 Conclusions 96

5.2 Future Directions 98

5.2.1 Proposed Cell Culture Opportunities 99

5.2.2 Considerations for the In Vivo Diabetic Model 101

5.2.3 Promotion of Survival During Early Engraftment 103

5.2.4 Promotion and Characterization of Long-Term Maturation 105

Bibliography 107

List of Tables

Table

2.1. Primers of Pancreatic Differentiation Markers Used for qRT-PCR 43

3.1. Primers of pancreatic differentiation markers used for qRT-PCR 63

List of Figures

Figure

1.1. **Survival and function of islets or β-cell clusters on microporous scaffolds post-transplantation.** Following seeding, islets or hPSC-derived β-cells settle into the pores of the microporous scaffold. Three of the major challenges to survival and function include vascularization, inflammation, and adaptive immune responses. Scaffold modifications can be used to enhance vascularization or to attenuate the impact of inflammation or adaptive immune responses, with the arrows indicating that scaffold modifications can increase vascularization, transition a pro-inflammatory to an anti-inflammatory environment, or induce Tregs that can attenuate Teff responses at the graft. Vascularization: Release of growth factors such as VEGF or PDGF and/or co-transplant of supportive cells can enhance vascularization within the scaffold.[39,64,65] Inflammation: Release of anti-inflammatory cytokines such as CXCL, IL-4, and IL-10 can be used to transition from a pro-inflammatory to an anti-inflammatory state.[16,99] Adaptive Immune Response: Modifying the scaffold with FasL108 or CCL22105 microparticles have been shown to induce regulatory T-cells upon release, as has systemic treatment with ECDI treated splenocytes.[124] (Schematic illustrated by Jennifer Harley) 4

1.2. **Porous Scaffolds used for Islet/β-cell Transplantation.** Biocompatible materials used to fabricate microporous scaffolds (A-D): A) Poly (lactide-co-glycolide) (PLG) with 250-425 μm pores - scale bar 500 μm. Adapted from Gibly et. al 2011[131] with permission from Elsevier, Polydimethylsiloxane (PDMS) – scale bar 500 μm. Adapted from Jiang et. al 2017[132] with permission from Elsevier, C) Silk Fibroin 200-400 μm pores – scale bar 50 μm. Adapted from Mao et. al 2017[68] with permission from Elsevier, D) Polyethylene glycol (PEG) Hydrogel - scale bar 1 mm. Adapted from Rios et. al 2018[133] with permission from John Wiley and Sons. PLG scaffold used to culture pancreatic progenitors into insulin producing β-cells (E): *Top* ECM deposition within pores of the scaffold by hPSC derived β-cells, *Bottom* Effect of pore size on insulin gene expression. Adapted from Youngblood et. al 2019[31] with permission from Elsevier. 4

1.3. **Strategies for enhancing survival post transplantation.** A) Fibrin-heparin and VEGF loaded collagen scaffolds containing oxygen generating microparticles (MPs) enhanced islet revascularization and improved restoration of euglycemia. Adapted from Montazeri et. al 2016[54] with permission from Elsevier. B) Layered microporous PLG scaffolds loaded with the trophic factor exendin-4 enhance restoration of euglycemia compared to blank scaffolds. Adapted from Hlavaty et. al 2014[34] with permission from John Wiley and Sons. 10

1.4. **Immunomodulation strategies: CCL22 released microparticles in VCA model and Fas-L attached microgels in T1D model.** A) Characterization and 40-day release profile of recruitment-MP. Treatment with Recruitment-MP (50 18

mg) prolongs allograft survival indefinitely. Hindlimb allograft survival curve showing indefinite survival (>200 days) in six of eight rats treated with Recruitment-MP (50 mg). Adapted from Fisher et. al[107] 2020 with permission from Creative Commons Attribution-Non Commercial license. B) Flow-focusing microfluidics were used to generate biotinylated microgels from biotin-functionalized PEG-4MAL macromers. Islet graft survival: SA-FasL-presenting or control microgels and unmodified BALB/c islets were co-transplanted under the kidney capsule of chemically diabetic C57BL/6 recipients. Adapted from Headen et. al 2018[111] originally published in Nature Materials, with permission from author Dr. Lonnie D. Shea.

1.5. **Immunomodulation strategies: ECDI-treated donor lymphoid cell infusion and ECDI-attached PLG nanoparticles in T1D model.** A) Glycemic control posttransplant: four ECDI-DLI experimental group and two control group diabetic NHPs each promptly achieved normoglycemia post-allogeneic islet transplant. Adapted from Lei et. al 2015[134] with permission from John Wiley and Sons. B) STEM image and size of PLG particles prior to lysate coupling with ECDI. A short course of low dose rapamycin with PLG-dAg demonstrated significantly greater islet allograft survival (n = 11) compared with mice treated with rapamycin alone (n = 9) **p < 0.01. Adapted with permission from Bryant et. al 2014[128] with permission from Elsevier. 21

2.1. In vitro differentiation of PSCs to pancreatic progenitor cells. H1 pluripotent PSCs were seeded to Matrigel-coated 6-well plates at a density of 1.5 million cells per 47

well, and pluripotent cells were imaged for sox17 (green) and oct3/4 (red) prior to beginning the differentiation (A). Definitive endoderm cells were imaged for sox17 (green) and oct3/4 (red) following stage 1 of the differentiation (B). Primitive gut tube cells were imaged for FoxA2 (green) and Hnf4 (red) (C), and pancreatic progenitor cells were stained for insulin (green) and Nkx2.2 (red) (D) as well as Pdx1 (green) and glucagon (red) (E) following the fourth stage of differentiation. All cells were counterstained with DAPI (blue). Scale bars for all images = 200 μm.

2.2. qRT-PCR of in vitro differentiation of human PSC derived pancreatic progenitors. 48

2.3. Microporous PLG scaffold morphology (A) and live/dead stain of seeded pancreatic progenitors (B). Schematic of experimental workflow (C). PSCs were differentiated in vitro to pancreatic progenitors and then seeded to either microporous PLG scaffolds without exendin-4 or to scaffolds containing an encapsulated exendin-4 microparticles within an inner layer disk. Scaffolds were subsequently transplanted to the epididymal fat pads of STZ-treated, diabetic NSG mice. 48

2.4. Blood glucose measurements of mice transplanted with pancreatic progenitor cells, seeded to blank PLG scaffolds or transplantation of cells under the kidney capsule (A, arrow indicates administration of insulin pellet). Blood glucose measurements of mice transplanted with pancreatic progenitors seeded to PLG scaffolds containing exendin-4 or transplantation of cells under the kidney capsule (B, arrow indicates administration of insulin pellet). Human C-peptide in serum 49

for a cohort of mice receiving microporous nonlayered PLG scaffolds containing PSC-derived pancreatic progenitors (n = 4), a cohort of mice receiving layered PLG scaffolds containing exendin-4 (n-4), and a cohort of mice transplanted with PSC- derived pancreatic progenitors under the kidney capsule (n = 4) (C). Histology of a blank scaffold (D) and exendin-4 layered scaffold (E) removed at week 4, stained for c-peptide (green), human mitochondria (red), and counterstained with DAPI (blue). All values reported as the average ± standard error; * indicates $p < 0.05$, ** indicates $p < 0.01$, † indicates $p < 0.05$ difference between week 4 and week 8 among a cohort of mice. Arrows on images (D and E) indicate regions of C-peptide/human mitochondria coexpression. All scale bars for images D and E = 100 μm.

2.5. Image quantification of monohormonal insulin producing cells and polyhormonal insulin/glucagon producing cells for blank scaffolds at weeks 4 and 8 (A). Histology of blank layered scaffolds (B and C) removed at weeks 4 and 8, stained for insulin (green), glucagon (red), and counterstained with DAPI (blue). All values reported as the average ± standard error; ** indicates $p < 0.01$. Arrows indicate regions of monohormonal insulin production. Scale bars = 200 μm. 50

2.6. Image quantification of monohormonal insulin producing cells and polyhormonal insulin/glucagon producing cells for exendin-4 layered scaffolds at weeks 4 and 8 (A). Histology of exendin-4 layered scaffolds (B and C) removed at weeks 4 and 8, stained for insulin (green), glucagon (red), and counterstained with DAPI (blue). All values reported as the average ± standard error; ** indicates $p < 0.01$; 51

**** indicates p < 0.0001. Arrows indicate regions of monohormonal insulin production. Scale bars = 200 μm

3.1. **Comparison of In Vivo graft function between fat pad and kidney capsule:** A) blood glucose, B) weight, C) intraperitoneal glucose tolerance test, and D) serum human C-peptide. No significant differences in C-peptide or IPGTT, as determined by comparing the cohort's means across timepoints via 2-Way repeated measures ANOVA with Bonferroni multiple comparisons. 67

3.2. **Graft function from subcutaneous implantation:** A) blood glucose, B) weight, C) intraperitoneal glucose tolerance test, and D) serum human C-peptide. 68

3.3. **Changes in hPSC-derived β cell gene expression following 160 day maturation period in fat pad, subcutaneous site, and kidney capsule.** Gene expression was analyzed via PCR. Values were normalized to the house keeping gene GAPDH and shown relative to pluripotent H1 expression. **P*<0.05, ***P*<0.01, ****P*<0.001, *****P*<0.0001. 69

3.4. **Comparison of graft function from the fat pad in grafts exposed to diabetic or non-diabetic environments:** A) blood glucose, B) weight, C) intraperitoneal glucose tolerance test, and D) serum human C-peptide. **P*<0.05, ***P*<0.01, Two-Way repeated measures ANOVA with Bonferroni test multiple comparisons was used to compare the cohort's means at each timepoint. 71

3.5. **Changes in hPSC-derived β cell gene expression following 210 days of In Vivo maturation, with 12-week period of diabetic or non-diabetic environment exposure:** Gene expression was analyzed via PCR. Values were normalized to 72

the house keeping gene GAPDH and shown relative to pluripotent H1 expression. *P<0.05, **P<0.01.

3.6. **Immunofluorescent staining of surviving cells:** A) Explanted tissue from cohorts that received subcutaneous, fat pad (diabetic maturation), or fat pad (non-diabetic maturation) scaffolds, was sectioned and stained for insulin (green), human-mitochondria (red). B) The images were analyzed for colocalization of human mitochondria and insulin. The Fat Pad cohorts (diabetic and non-diabetic maturation) both showed significantly higher percentage of insulin positive human mitochondria area compared to subcutaneous stained tissue. The diabetic and non-diabetic maturation cohorts did not show a significant difference in the percentage of insulin positive human mitochondria area. 72

4.1. Early Stage (S0) Cluster Seeding On Scaffolds: (A)Schematic of seeding timing and analysis. (B) Clusters were cultured in suspension, or seeded onto scaffolds prior to stage 1, and cultured to completion of stage 1 definitive endoderm, (C) Clusters were cultured in suspension, or seeded onto scaffolds prior to stage 1, and cultured to completion of stage 4 pancreatic progenitor. Statistical significance was determined based on unpaired T-test for each gene pair *P<0.05, **P<0.01, ***P<0.001. 86

4.2. Late Stage (Stage 6 Day 1) Cluster Seeding on Scaffolds: (A)Schematic of seeding timing and analysis. Clusters were cultured in suspension, or seeded onto scaffolds prior to stage 6, and cultured to completion of stage 6. (B) Gene expression analysis of end of stage 6 cells cultured in suspension or on scaffolds. Statistical 87

significance was determined based on unpaired T-test for each gene pair *P<0.05, **P<0.01.

4.3. Pre-endocrine (Stage 5 Day 1) cluster seeding on scaffolds: (A)Schematic of seeding timing and analysis. Clusters were cultured in suspension, or seeded onto scaffolds prior to stage 5, and cultured to completion of stage 5. (B) Gene expression analysis at Stage 5 Day 7 for cells cultured in suspension or on scaffolds. Statistical significance was determined based on unpaired T-test for each gene pair *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001. 88

4.4. sfGFP-C-peptide reporter activity during culture and glucose stimulated insulin secretion: (A) sfGFP-C-peptide reporter signal for suspension clusters and pre-endocrine (Stage 5 Day 1) cluster seeded scaffolds during stage 5 and stage 6. (B) Florescent signal from C-peptide reporter within scaffolds measured during GSIS assay. 89

4.5. Planar culture seeding on scaffolds at Stage 5 Day 1 and Stage 6 Day 1: (A)Schematic of seeding timing and analysis. Clusters were cultured in suspension, or seeded onto scaffolds prior to stage 6, and cultured to completion of stage 6. (B) Gene expression analysis of end of stage 6 cells cultured in suspension or on scaffolds. Statistical significance was determined based 1-Way ANOVA with Tukey Multiple Comparisons. Significance detected in expression for NGN3. 90

4.6. sfGFP-C-peptide reporter activity during planar culture, and scaffolds seeded at Stage 5 Day 1 and Stage 6 Day 1: (A) Stage 5 Day 1 Planar Culture, (B) Stage 6 Day 1 planar culture, (C) Stage 6 Day 4 Planar Culture, (D) Scaffold culture, seeded with Stage 5 Day 1 planar culture, at Stage 6 Day 4 and Stage 6 Day 7, (E) Scaffold Culture, either seeded with Stage 5 Day 1 or Stage 6 Day 1, at Stage 6 Day 4. 90

Abstract

Type 1 diabetes is caused by the autoimmune destruction of insulin-producing β-cells. Even with advanced insulin delivery systems, blood glucose levels deviate outside of the range maintained by native islets, which places the individual at risk for vascular complications and life-threatening hypoglycemic events. Islet replacement therapy has demonstrated the capacity to tightly control blood glucose, but its wide adoption is limited by the availability of cadaver donors and the lack of effective methods to support these cells within a clinically accessible site. The results presented in this thesis address these limitations through studying maturation of human pluripotent stem cell (hPSC) derived β cells within a transplantable biomaterial platform, and evaluating novel approaches to the implantation and support of these cells during their continued maturation in vivo.

First, I present a study that examined delivery of hPSC-derived pancreatic progenitors within microporous PLG scaffolds into the epididymal fat pad, the murine surrogate for the clinically relevant omental pouch. Kidney capsule injection was the comparison condition. We observed that the microporous scaffolds supported cell engraftment, however secreted levels of circulating C-peptide were lower than from the kidney capsule. The scaffolds were subsequently modified to provide sustained release of exendin-4, a glucagon-like peptide-1R analog, which led to significantly increased C-peptide production. Image analysis revealed that exendin-4 releasing scaffolds enhanced the proportion of pancreatic progenitors that matured to monohormonal insulin producing cells.

Next I present findings from studying how hPSC-derived β cells mature and function within three transplantation sites: the i) scaffold delivery into the epididymal fat pad, ii) scaffold delivery into the subcutaneous space, and iii) the kidney capsule injection (control). Additionally, we investigated the impact of blood glucose levels on maturation of the hPSC-derived β cells by transplanting mice with pre- or post-engraftment diabetes induction. Hyperglycemia was ameliorated in the cohorts of mice that received scaffolds into the epididymal fat pad, following a period of in vivo maturation. The function of these cells was demonstrated by the reduction in blood glucose levels, healthy increase in weight, therapeutic levels of circulating human insulin, and healthy responses to glucose challenge tests. The function from the epididymal fat pad was superior to the subcutaneous space and was observed to be comparable to the kidney capsule.

Many of the current differentiation protocols culture the cells above a feeder layer in monolayer, or in suspension within a bioreactor. Typically, these protocols require the disruption of the cell niche during key differentiation stages or pre-transplantation handling. Biomaterial scaffolds maintain the integrity of cell-to-cell and cell-to-matrix connections by providing both a space for cell niche development as well as a vehicle for transplantation into the body. Herein, I present results from testing the developmental stage in which progenitors are seeded into the 3D niche, and two differentiation strategies prior to seeding: monolayer and suspension culture. Maturation was characterized via gene expression analysis, glucose stimulated insulin secretion assay, and nondestructive microscopy utilizing a sfGFP-C-peptide cell line that reports C-peptide production and secretion. We observed that seeding clusters during the key transition phase from pancreatic progenitor to pancreatic endocrine enhanced commitment to the final beta cell fate.

This work enhances our understanding of hPSC-derived beta cell manufacturing within scaffolds, and delivery to an extrahepatic site to achieve normoglycemic blood glucose levels.

Chapter 1. Introduction

A majority of the following introduction corresponds to the published peer-reviewed paper:

Integration of islet/beta-cell transplants with host tissue using biomaterial platforms

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1.1 State of Type I Diabetes Cell Replacement Therapy

Type I diabetes (T1D), which affects an estimated 1.6 million Americans, is caused by the autoimmune destruction of the pancreatic β-cells within islets of Langerhans. Individuals with T1D must currently rely on life-long exogenous insulin therapy. Advanced insulin pumps and continuous glucose monitoring and feedback systems have given individuals with T1D tighter control of their blood glucose levels in recent years.[1,2] Despite the use of this technology and advanced algorithms, blood glucose levels are not maintained within the normal range as effectively as native islets, which puts individuals at risk for vascular complications and dangerous hypoglycemic events.[1–3] The Edmonton Protocol, first published in 2000, has been utilized clinically to transplant islets via hepatic vein infusion in conjunction with immunosuppressive therapy.[4] Individuals receiving islet transplantation have shown lower levels of HbA1c, more time spent in the healthy glycemic range, and a reduced risk for hypoglycemic events in comparison to recipients of continuous subcutaneous insulin infusion or multiple daily insulin injections.[5] The

Edmonton Protocol has shown the enormous promise for long term, tightly controlled glucose levels without exogenous insulin, however, several drawbacks prevent wide adoption of this strategy.[6] Hepatic vein infusion exposes cells directly to the blood without protection, resulting in an instant blood-mediated inflammatory reaction (IBMIR). The required systemic immunosuppression brings harsh side effects, putting patients at risk for infection, organ damage, and developing cancer. The harsh environment and need for lifelong immunosuppression provide a strong motivation to develop extrahepatic delivery strategies.

The limited supply of acceptable islets from cadaver donors requires alternative cell sources to be investigated and developed for T1D cell replacement therapy. Alternative cell sources that have shown promise include xenogeneic sources and human pluripotent stem cell (hPSC) sources, which include embryonic stem cells (hESCs) and adult induced pluripotent stem cells (iPSCs). While xenogeneic sources, such as porcine islets, have shown positive results in pre-clinical non-human primate (NHP) studies, the xenogeneic immune response is considered an additional barrier to an already challenging immune response.[7] Numerous studies have demonstrated the ability to generate pancreatic progenitors, cells committed to the pancreatic lineage, from pluripotent cells.[8–11] These hPSC-derived pancreatic progenitors provide a means of overcoming the shortage of allogeneic donor tissue for this therapy.[12]

Biomaterial scaffolds are being developed as a platform for delivering islets or hPSC derived β-cells to extrahepatic sites. Extrahepatic sites are desired to avoid IBMIR, which introduces new challenges. Immediately following transplantation, transplanted cells face multiple challenges: they must rely on diffusion for oxygen, nutrients, and waste exchange during the period prior to revascularization, and they must overcome or avoid the inflammatory response and host autoimmune and allogeneic response (Figure 1.1). Biomaterials have been widely used for

delivering islets, largely in an encapsulation approach that creates a physical barrier between therapeutic cells and the host immune system.[13] Innovation with encapsulation technologies continues such as novel materials to limit fibrosis[14], or thinner coatings[15], to reduce the transplant volume. Nevertheless, this approach restricts access to vascular ingrowth that is present within native islets and contributes to their function. Non-encapsulating scaffolds, in contrast to encapsulating hydrogels, allow for integration of transplanted cells with host tissue, which can support long term survival and function.[16–19] These scaffolds have included microporous or fibrous scaffolds that allow for cell infiltration following cell transplantation, as well as degradable hydrogel systems that initially encapsulate cells, with subsequent degradation allowing for host tissue infiltration and integration. Scaffolds can provide support for cell adhesion and signals through functionalization of the material with peptides or proteins, or localized delivery of trophic factors. Collectively, scaffolds provide the opportunity to engineer the local microenvironment at the site of transplantation to support the integration, survival, and long-term function of transplanted cells.

Herein, we provide an overview of scaffold platforms that are being employed for manufacturing hPSC derived β-cell products and can deliver islets or c-cells to extrahepatic locations for integration with the host (Figure 1.2 A-D). We review strategies towards enhancing the maturation and function of therapeutic β-cells, supporting survival of transplanted insulin-producing cells amid revascularization and inflammation, and the application of immune engineering methods to allow transplanted cells to avoid autoimmune or allogeneic host immune responses.

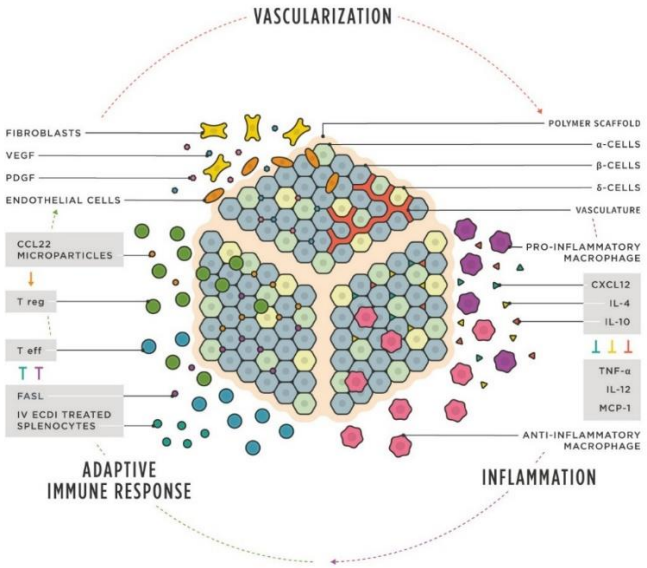

Figure 1.1. Survival and function of islets or β-cell clusters on microporous scaffolds post-transplantation. Following seeding, islets or hPSC-derived β-cells settle into the pores of the microporous scaffold. Three of the major challenges to survival and function include vascularization, inflammation, and adaptive immune responses. Scaffold modifications can be used to enhance vascularization or to attenuate the impact of inflammation or adaptive immune responses, with the arrows indicating that scaffold modifications can increase vascularization, transition a pro-inflammatory to an anti-inflammatory environment, or induce Tregs that can attenuate Teff responses at the graft. Vascularization: Release of growth factors such as VEGF or PDGF and/or co-transplant of supportive cells can enhance vascularization within the scaffold.[39,64,65] Inflammation: Release of anti-inflammatory cytokines such as CXCL, IL-4, and IL-10 can be used to transition from a pro-inflammatory to an anti-inflammatory state.[16,99] Adaptive Immune Response: Modifying the scaffold with FasL108 or CCL22105 microparticles have been shown to induce regulatory T-cells upon release, as has systemic treatment with ECDI treated splenocytes.[124] (Schematic illustrated by Jennifer Harley)

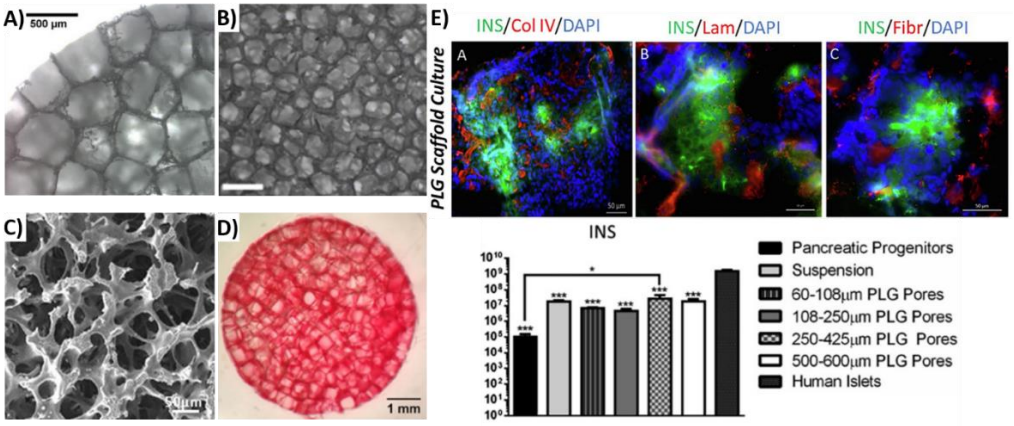

Figure 1.2. Porous Scaffolds used for Islet/β-cell Transplantation. Biocompatible materials used to fabricate microporous scaffolds (A-D): A) Poly (lactide-co-glycolide) (PLG) with 250-425 µm pores - scale bar 500 µm. Adapted from Gibly et. al 2011[131] with permission from Elsevier, Polydimethylsiloxane (PDMS) – scale bar 500 µm. Adapted from Jiang et. al 2017[132] with permission from Elsevier, C) Silk Fibroin 200-400 µm pores – scale bar 50 µm. Adapted from Mao et. al 2017[68] with permission from Elsevier, D) Polyethylene glycol (PEG) Hydrogel - scale bar 1 mm. Adapted from Rios et. al 2018[133] with permission from John Wiley and Sons. PLG scaffold used to culture pancreatic progenitors into insulin producing β-cells (E): *Top* ECM deposition within pores of the scaffold by hPSC derived β-cells, *Bottom* Effect of pore size on insulin gene expression. Adapted from Youngblood et. al 2019[31] with permission from Elsevier.

1.2 Manufacturing Platforms for Cell Transplantation

While hPSC-derived pancreatic progenitors have shown promise as allogeneic donor tissue, further development of manufacturing methods is required prior to successful clinical application. Large-scale production of hPSC-derived insulin-producing cells requires strict control of aggregate formation and differentiation factors for weeks of culture. The traditional culture system for cell-based therapies involves bioreactors for large scale manufacturing, in which the cells are provided reagents at distinct stages and the cells self-assemble into clusters. Upon the completion of culture, these clusters are collected for direct infusion or embedded within materials for transplantation.[10,14] Biomaterial platforms provide a tool that can assist with assembly of cells into structures of tunable size, which is controlled by the material properties rather than the shear rate of the culture media.[20] Additionally, as cells are cultured within the platform, the factors they secrete are deposited within a niche that may support their long-term function. Finally, upon completion of culture, the material platform can be directly transplanted with minimal cell manipulation and without disruption of the cellular niches.

Degradable hydrogels have been widely used for 3D differentiation of hPSCs.[21–23] Matrigel based materials have successfully produced islet organoids, as well as increased functional insulin secretion compared to 2D culture.[24] Recent work has shown that functional β-cells can be produced from 2D culture under specific conditions. A recent protocol reproducibly generated cells with comparable or better functionality compared to suspension cultures.[9] The condition optimized for this protocol was the addition of a 24-hour treatment with latrunculin A, which is a factor that depolymerizes the actin cytoskeleton, and increases endocrine induction.[9] While promising, Matrigel is sourced from murine tumors and generally not considered a translatable

platform due to its batch to batch variability, undefined composition, potential to transfer pathogens and facilitate tumor formation and growth.[25–27] Collagen scaffolds and fibrin gels are two naturally occurring hydrogels that have been shown to support 3D hPSC differentiation into β-cells.[22] A number of hydrogels have been employed for organoid culture with the ability to promote the formation of 3D structures that contain multiple cell types found in islets.[23,24,28] These materials provide mechanical support for the formation of structures, and are modified with biological cues such as growth factors, cell adhesion ligands, immunomodulatory drugs, or enzyme degradation sites.[29,30] Proteomic and RNA sequencing analysis was used to probe regulatory pathways during pancreatic differentiation of hPSCs, and the results suggested 3D confinement of the cells promoted integrin signaling and β-cell differentiation.[21] These hydrogels can generate cells with a greater expression for β-cell genes, and enhanced glucose-responsive function compared to 2D culture.[21,24]

Microporous scaffolds are also being developed as a platform for manufacturing hPSC-derived β-cells.[31] Microporous scaffolds have commonly been used in regenerative medicine to allow seeded cells to form into tissues for subsequent transplantation. Specific to islets, the size of transplanted islets impacts insulin secretion and viability, and the pore size can regulate the size at which islet organoids form.[32] After settling into pores, hPSCs form cell-cell interactions and mature into β-cells with transcription marker expression and glucose stimulated insulin secretion comparable or higher in scaffold cultured cells compared to suspension culture.[31] Additionally, hPSCs differentiated in microporous scaffolds were shown to form an extracellular matrix through deposition of collagen IV, laminin, and fibronectin (Figure 1.2E). ECM proteins could also be deposited onto the material surface in order to provide cues that promote the differentiation to islet organoids. Multiple biomaterials have been investigated for their potential to support islet organoid

formation, such as polyethylene glycol or the copolymer of lactide and glycolide (PLG), both of which have shown promise for islet transplantation in diabetic mouse models.[17,33–35] Importantly, the scaffolds that initially function as a support for organoid maturation, can then be directly transplanted. Transplanting β-cells differentiated on a microporous scaffold avoids disruption of the existing cell-cell and cell-matrix interaction that may occur during the handling of suspension cultured cells for transplantation.

1.3 Transplantation

Pancreatic islets and β-cells are highly metabolically active with oxygen and glucose requirements that are greater than many other cell types, and the extrahepatic transplantation site must support these metabolic demands for a relatively large number of cells (≈500,000 IEQ per human transplant recipient).[36] In preclinical models, the kidney capsule is widely used, though this site is challenging to surgically access, cannot readily be altered as with the liver, and has the potential co-morbidity of nephropathy, which would likely reduce healthy vasculature.[37] The intraperitoneal and omental pouch have been widely used in preclinical and for clinical transplantation due to its large capacity for transplanting cells, which can also be delivered on scaffolds or with biologics that create a supportive environment. The subcutaneous space has an advantage of easy surgical access and could have sufficient space for large numbers of cells, though this site is less vascularized than other sites.[38] The reduced vasculature in the subcutaneous tissue can lead to cell necrosis at the center of unmodified scaffolds as early as 24 hours after implantation.[39] The intramuscular site offers a rich vascularization,[40,41] yet choosing an appropriate method of delivery at an intramuscular site is key to avoiding a detrimental fibrotic response.[41] The native home of islets, the pancreas, has been considered as a transplant site, but it is widely considered less clinically promising due to the site requiring an invasive procedure.[37] Collectively,

a major challenge for clinical translation is providing an environment at the transplantation site that is able to support the metabolic needs of a large number of metabolically active cells, with hPSC derived β-cells also requiring an environment that supports their maturation.

1.3.1 Enhancing Survival

The initial challenge following transplantation is survival of the transplanted cells, and the properties of the transplantation site are critical. Oxygen is considered a limiting factor following transplantation, and a robust vasculature in close proximity to the graft can supply oxygen by diffusion. Scaffolds allow for oxygen and nutrient diffusion through the pores, yet passive diffusion is often insufficient to support the entire cell population for significant times. Alternatively, pre-vascularizing of the transplant site to enhance the local vascularity can enhance survival following the addition of islets. The temporary placement of a biomaterial to promote a pro-angiogenic inflammatory response successfully pre-vascularized a subcutaneous transplantation site and supported restoration of euglycemia following syngeneic islet transplantation.[42] Fingolimod (FTY720), which targets sphingosine-1-phosphate (S1P) receptors on endothelial cells and smooth muscle cells to increase the density of surrounding microvascular networks, was loaded in scaffolds to prime an extrahepatic transplant site, and increased the formation of microvessels in diabetic mice, which supported islet function post-transplantation.[43] Scaffolds loaded with VEGF and fibrinogen have effectively pre-vascularized the site prior to islet transplantation.[44–46] However, the efficacy of VEGF to induce vascularization is site dependent.[47] A PEG hydrogel loaded with VEGF enhanced vascularization and reduced blood glucose when implanted in the epididymal fat pad, yet had no significant effect when implanted in the subcutaneous space or small bowel mesentery.[47] While the epididymal fat pad, analogous to the

omentum in humans, is considered a clinically translatable site, other methods to promote vascularization may be needed at alternative sites in order to support the needs of the cell graft. At a subcutaneous site, poly(D,L,-lactide-co-ε-caprolactone) (PDLLCL) scaffolds were allowed to pre-vascularize for 4 weeks prior to islet delivery. All mice in the diabetic cohort receiving 1200 islets into pre-vascularized PDLLCL scaffolds achieved normoglycemia, while none of the mice in the cohort receiving the same islet dose into an unmodified subcutaneous pocket achieved normoglycemia.[45] The Sernova Corporation has developed a device for islet transplantation called The Cell Pouch™, which is currently in a clinical trial, and is based on allowing six weeks for pre-vascularization in the subcutaneous space prior to islets being transplanted within cylindrical immune-isolating chambers.[48] However, the islet loading levels and the diameter of cylinders within The Cell Pouch™ may contribute to hypoxia induced cell death, and future devices must limit the thickness of avascular tissue requiring oxygen diffusion.[49] A complementary strategy to pre-vascularization involves drug delivery to inhibit apoptosis. Diabetic mice receiving islet transplants co-delivered with V5, a BCL2-associated X protein (BAX)-inhibiting peptide, had grafts with significantly enhanced function and a larger number of surviving islets.[50] Collectively, pre-vascularizing the transplantation or blocking apoptosis are emerging strategies aimed at enhancing graft survival at early time points.

Oxygen has been directly delivered at the transplantation site to enhance survival prior to vascularization of the islets or islet organoids. Beta O_2 has developed an implantable device called βAir, which has a refillable oxygen chamber designed to provide oxygen to encapsulated islets. This system has successfully restored euglycemia in diabetic rats and minipigs.[51,52] However, significant functionality was not yet achieved in a phase I clinical trial.[53] Alternatively, oxygen generating compounds can be embedded within materials that can immediately provide oxygen to

the transplanted cells for days to weeks, which spans the critical time prior to integration with the host vasculature (Figure 1.3A).[54–56] Incorporation of hydrogen peroxide or calcium peroxide into scaffolds has been developed to locally provide oxygen release following transplantation and has been shown to improve graft function.[54–56] Once the oxygen generating compound is depleted, hypoxia will return unless sufficient integration with the host vasculature has occurred. Increasing compound loading onto scaffolds may increase the length of oxygen delivery, yet the loading and release must be balanced to provide the necessary oxygen without the excessive production of harmful reactive oxygen species.[56]

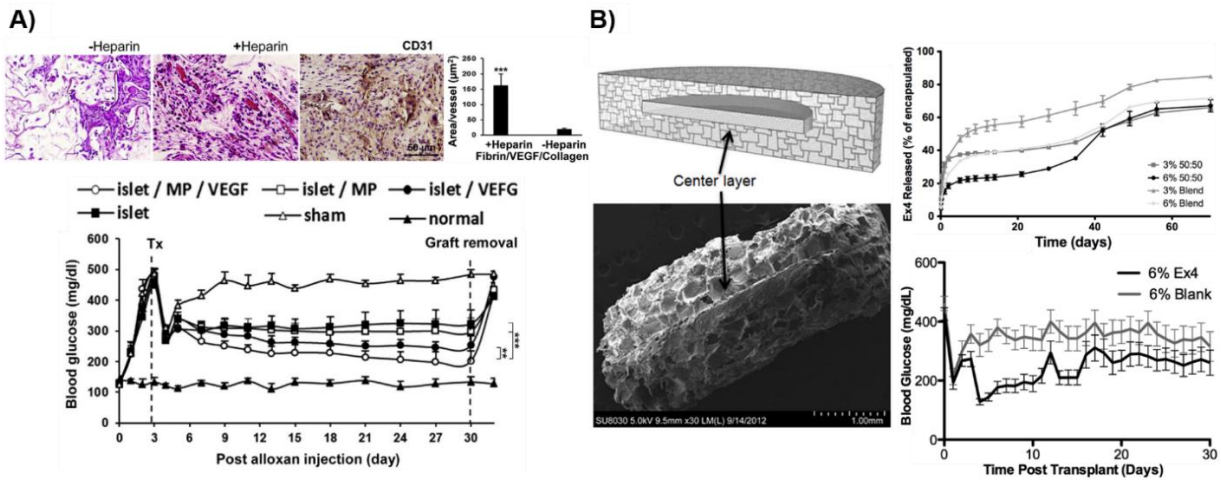

Figure 1.3. Strategies for enhancing survival post transplantation. A) Fibrin-heparin and VEGF loaded collagen scaffolds containing oxygen generating microparticles (MPs) enhanced islet revascularization and improved restoration of euglycemia. Adapted from Montazeri et. al 2016[54] with permission from Elsevier. B) Layered microporous PLG scaffolds loaded with the trophic factor exendin-4 enhance restoration of euglycemia compared to blank scaffolds. Adapted from Hlavaty et. al 2014[34] with permission from John Wiley and Sons.

1.3.2 Enhancing Integration

Long-term function of the transplanted cells is enabled by integration of the cells with the host vasculature, which can provide the necessary nutrients and allow for the sensing of blood glucose and the distribution of secreted insulin. Long term islet function is maintained in part through a capillary network among the islets, indicating the need to integrate the cells with the vascular network post-transplantation.[57] One of the major barriers to successful integration is the foreign

body response. Viacyte's cell replacement product VC-01 is an encapsulating device used to subcutaneously deliver hPSC-derived pancreatic progenitors. The recent clinical trial of VC-01 showed surviving cells out to 24 months, yet the function and integration of the device were limited by the foreign body response.[58] The choice of biocompatible materials and the use of degradable platforms can influence the fibrotic response.[59,60] Microporous scaffolds, and degradable hydrogels support engraftment and revascularization.[33,61,62] Microporous scaffolds typically have a large surface area to volume ratio, so that the distribution of cells near the surface minimizes the distance to host vasculature and the competition for nutrients. Additionally, the pores of the scaffold are generally sufficiently large to support vessel ingrowth; whereas the degradable hydrogels must undergo local degradation to create space for vessel ingrowth. Collectively, these examples illustrate that multiple strategies are being developed to enhance vascularization and engraftment, including the release of angiogenic factors and cell co-transplantation.

Angiogenic growth factors have been incorporated into scaffolds with the goal of enhancing the rate of vascularization and graft survival and function. The formation of robust vasculature networks depends on many aspects of the microenvironment, and the specific factors needed may differ depending on the properties of the site and the transplantation platform.[63] Growth factors such as vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF-BB), and acidic fibroblast growth factor (aFGF) have all been shown to increase vascularization of transplanted scaffolds and decrease time to euglycemia.[39,64,65] The identity of the angiogenic factors, as well as its loading and release rate can impact both vascularization and function. A collagen scaffold utilizing fibrin-conjugated heparin affinity based release of VEGF showed increased formation of new microvessels compared to control scaffolds containing only fibrin and VEGF (Figure 1.3A).[54] Local release of these angiogenic factors increases the rate of

vascularization, and importantly used factors that are endogenous to islets and pose minimal risk to the transplanted cells. As mentioned above, FTY720 was able to enhance pre-vascularization of scaffolds; however, the delivery of cells concurrently with FTY720 decreased islet function and failed to restore euglycemia.[66,67] Furthermore, not all growth factors result in long term improvement of function. aFGF, in particular, resulted in worse long-term function compared to control groups.[65] The scaffold itself may also be modified to induce the expression of angiogenic factors by the transplanted or infiltrating cells. Heparin modified scaffolds upregulated VEGF expression and decreased time to restore euglycemia in diabetic mice when compared to control scaffolds.[68] This release or induction of angiogenic factors can be combined with oxygen generating compound, with the coupling of these two approaches enhancing function following depletion of the oxygen generators.[54] Combination strategies increase complexity of scaffold design, and require careful consideration of possible negative interaction. However, note that incorporation of multiple elements is likely to increase the regulatory burden.

Co-transplantation of support cells with β-cells can enhance integration with the host, and may provide cues that are beneficial to β-cells.[69–71] Co-transplant of islets or hPSC-derived β-cells have been conducted with a variety of cell types, including endothelial cells (ECs), parathyroid glands (PTGs), and fibroblasts.[72–75] The mechanism of enhanced engraftment occurs through stimulation of growth factor release, (PTGs and ECs stimulate VEGF), and by providing the cells needed to generate new vasculature (ECs).[73,74] Providing multiple cell types has the potential to recapitulate native pancreatic tissue, which may lead to enhanced function and engraftment. ECs have enhanced the maturation of hPSC-derived pancreatic progenitor cells *in vitro.*[28,75] An important consideration when designing an EC/ β-cell co-transplantation strategy is that ECs experience increased cell death and dysregulation in elevated glucose environments.[76] In order to support

engraftment of hPSC-derived β-cells *in vivo*, ECs must be able to survive the post-transplant environment. Furthermore, the co-transplantation approach may differ for islets, which are already functional for restoring euglycemia, relative to hPSC-derived β-cells that may require further *in vivo* maturation.

1.3.3 Enhancing Function

Local delivery of trophic factors to transplanted β-cells can stimulate receptors and signaling pathways known to regulate survival, proliferation, differentiation, and insulin secretion. The glucagon-like peptide-1 (GLP-1) agonist exendin-4 stimulates glucose-dependent insulin secretion in β-cells, promotes β-cell proliferation, and protects against apoptosis.[77,78] In vitro culture of cells in a supporting hydrogel and receiving Exendin-4 had higher insulin secretion and less apoptosis compared to controls.[79] The local and sustained release of exendin-4 allowed for up to a 25% decrease in the number of human islets in order to enhance glycemic control (Figure 1.3B).[34] Scaffolds similarly releasing exendin-4 were used to support hPSC derived pancreatic progenitors, and enhance maturation and c-peptide secretion.[80] Alternatively, nerve growth factor (NGF) has promoted glucose responsive insulin secretion in β-cells through activation of calcium channels.[81] Islets were transplanted into diabetic mice on a gelatin sponge, known as Gelitaspon, with or without NGF.[82] Mice receiving islets transplanted on Gelitaspon soaked in 100 ng/ml NGF had significantly lower blood glucose and an improved response to intraperitoneal glucose tolerance test compared to the control group. A number of other trophic factors (Prolactin[83], Growth hormone (GH), also known as somatotropin,[16,34,77,78,84] Hepatocyte growth factor[85], IGF-1[86,87], Betacellulin (BTC) and Activin-A[88]) have been identified with potential to influence β-cell

responses post-transplantation, yet these factors have not been investigated with scaffold based transplantation.

1.4 Immune Protection

The process of immune recognition and immune destruction of transplanted cells for an allogeneic transplantation occurs in a 4-step manner described as follows: (1) inflammation; (2) activation of allogeneic or self dendritic cells (DCs) and migration to draining lymph nodes; (3) T cell activation by DCs, resulting in expansion of anti-donor T cells; and (4) migration of T cells to the graft where they mediate cytotoxicity.[16,89] The development of the Edmonton Protocol dramatically improved clinical outcomes in islet transplantation by using sirolimus, low-dose tacrolimus, and induction anti-interleukin-2 receptor antibody. While improvements have been made in the immunosuppression protocol, these patients require lifelong immunosuppression, which can be problematic for a disease with a juvenile onset.[90] The side effects brought by the permanent use of such systemically administered agents include increased vulnerability to infection, cytotoxicity or even diabetogenicity to the transplanted islets/cells. This section describes emerging research counteracting immune recognition and immune destruction, either locally at the site of transplant and/or systemically, that could enhance engraftment of the allogeneic cell transplant.

1.4.1 Local Immune Modulation

The local modulation of immune responses is motivated by at least two observations: i) that the inflammatory response contributes to islet loss following hepatic infusion[91], and ii) immunoprivileged sites, such as those in the thymus, brain, and testis[37], can limit immune responses without the need for systemic immune suppression. The inflammatory response following cell transplantation reduces transplanted cell survival, often substantially, and is

mediated initially by innate immune cells, whereas adaptive immune cells mediate cell killing in many autoimmune and allogeneic cell transplantations.[16] The function of innate and adaptive immune cells can be modulated at immunoprivileged sites in the body, such as the anterior chamber of the eye and the testis. The immunoprivileged properties for both the ocular and testes microenvironments result from a combination of structural features and immunoregulatory and immunosuppressive molecules.[92] In the testes, the Sertoli cells provide a physical blood barrier to isolate germ cells from the immune system along with a host of immunoregulatory compounds that suppress pro-inflammatory responses from the immune cells to maintain the immunoprotected environment.[93] The eye and testes are generally not considered translationally relevant due to the large number of islets that would need to be delivered into these small organs. Nevertheless, the basic principles associated with these immunoprivileged microenvironments have served to inspire approaches to protect allogeneic cell transplants at extrahepatic sites.

The local release of cytokines from scaffolds has been employed to modulate the inflammatory reaction and enhance both cell survival and function. These drugs and cytokines stimulate the recruitment of suppressive immune cells or polarize immune cells toward an anti-inflammatory phenotype. Examples of immune regulatory molecules that have been investigated for protection of islets and β-cells include transforming growth factor-β (TGF-β), IL-10, IL-4, CCL2 (MCP1), CXCL12 (SDF1), IDO1, LTB4, IL-33, and PGE2.[13,16] An important consideration is the impact these factors have at the specific site of transplantation. While IL-33 exerted graft-protective effects in both murine skin transplant and cardiac transplant models,[94–96] IL-33's ability to protect islets has shown mixed results. IL-33 delivery with islet transplantation into the peritoneal fat IL-33 upregulated graft-protective T cells and prolonged graft survival; however, at this site, IL-33 also led to an increased expression of pro-inflammatory cytokines responsible for potential host

protective immunity.[35] TGF-β1 delivered from porous scaffolds downregulated TNF-□, IL-12, and MCP-1 population by at least 40% compared to control scaffolds.[97] The transplanted allogenic islets on the TGF-β1 releasing scaffolds had an enhanced engraftment and functioned significantly longer than those transplanted on empty scaffold controls.

Chemokine delivery influences the function of numerous cell types within the environment. CXCL12 delivery protected transplanted islets and stem cell-derived β-cells, through either protection or enhanced function for allogeneic and xenogeneic sources.[98] CXCL12 directs monocyte differentiation towards a macrophage phenotype with immunosuppressive functions.[99] Additionally, dendritic cells derived following CXCL12 modulation induce activity from antigen-specific regulatory T cells.[99,100] CXCL12 can directly signal to β-cells for survival and regeneration[101], and increased the homing of autologous MSCs and HSCs that can substantially reduce the infiltration of inflammatory cells, and improve blood vessel formation.[102,103]

Chemokines invoked by tumor cells that protect tumors from immune destruction have also been employed for local immunomodulation. CCL22 is released by tumor cells to recruit regulatory T cells (Tregs) to the site through a chemokine receptor (CCR4) expressed on Tregs. Tregs possess potential capabilities of mitigating autoimmunity in T1DM and inducing local and systemic tolerogenic effects. This tolerogenic effect is achieved by suppressing T-cell and dendritic-cell (DC) maturation and activation by secreting immunosuppressive factors, such as IL-10 and TGF-β1.[104,105] Donor-specific tolerance to fully mismatched islet allografts has been achieved by locally recruiting Tregs in CCL22-expressing allografts (Figure 1.4A).[106] Allogeneic islets transduced to express CCL22 conferred prolonged protection and preservation of transplanted murine β-cells from rejection and maintained normoglycemia in 75% of recipients over 80 days, with elevated number of Tregs in the islet grafts and an absence of anti-donor

antibodies or lymphocyte proliferation after exposure to donor splenocytes. In a different transplant model, CCL22 releasing microparticles delivered locally at a graft prolonged complete MHC mismatched allograft survival over 200 days.[107] Effector T cells were significantly reduced in skin and draining lymph nodes of treated transplant recipients, with a resulting tissue architecture similar to muscle and skin biopsies from normal tissue.

Natural immunoprivileged sites often employ constitutive expression of FasL to control the entry of lymphoid cells expressing Fas. A synthetic FasL was developed by Yolcu and Shirwan, which consisted of an engineered streptavidin-FasL (SA-FasL) that could be attached to biotinylated pancreatic islet grafts. This FasL modification of islets in combination with a transient course of rapamycin treatment (0.2 mg/kg daily for 15 days) resulted in indefinite survival and function of allogeneic cells in the absence of chronic immunosuppression.[108] In another study, graft recipients were also systemically immunomodulated by intraperitoneal injection of donor SA-FasL– engineered splenocytes on days 1, 3, and 5 after the transplantation of the S-FasL attached islets. This regimen resulted in the survival of all allogeneic islet grafts for the 250-day observation period since SA-FasL protein preferentially induced apoptosis in autoreactive T-effector cells while sparing $CD4^{+}CD25^{+}FoxP3^{+}$ T-regulatory cells.[109] This result has recently been translated to biomaterials used for islet transplantation, with the materials being biotinylated and functionalized with FasL rather than the islets. PEG hydrogel beads modified with FasL that were co-transplanted with islets, or FasL modified porous PLG scaffolds supported allogeneic islet transplantation and function for over 200 days (Figure 1.4B).[110,111] Decreased numbers of both CD4+ and CD8+ T-effector cells and increased number of T-regulatory cells were observed in the tissues of mice receiving SA-FasL-engineered microgels. These islets were transplanted into either the kidney capsule or the epididymal fat pad with transient administration of rapamycin. These

islets had sustained survival and normalized blood glucose levels in chemically diabetic graft recipients. Long-term graft recipients generated a normal immune response to donor alloantigens, implicating localized tolerance to the graft.

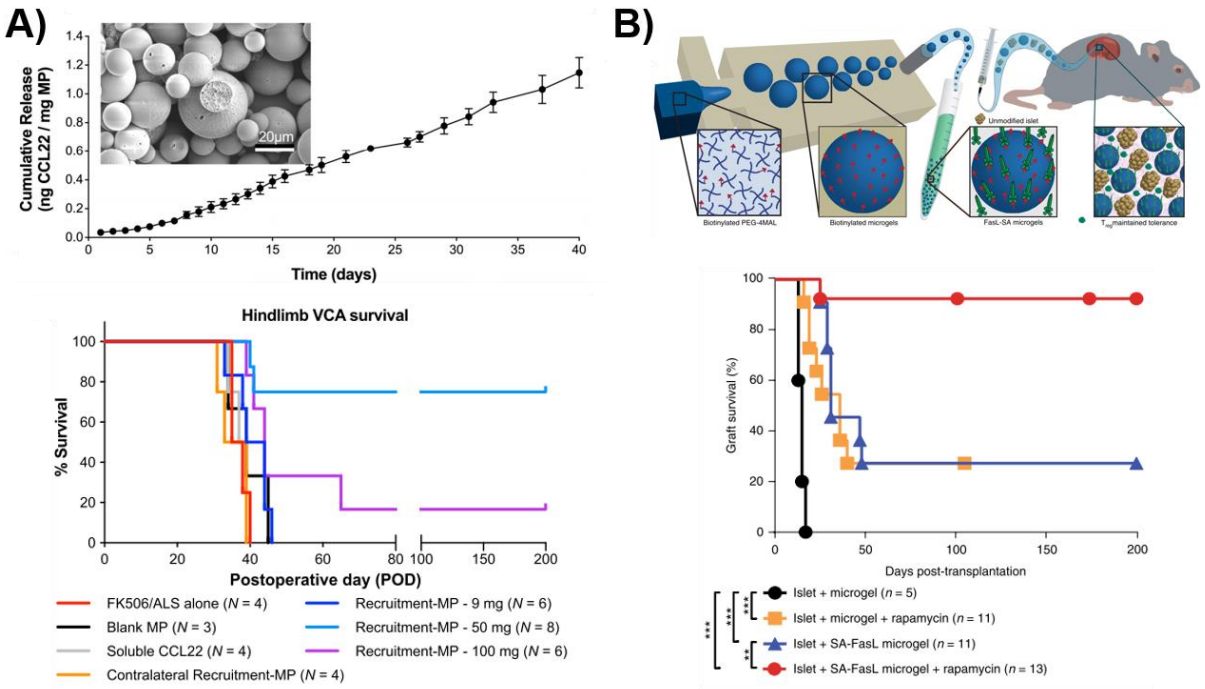

Figure 1.4. Immunomodulation strategies: CCL22 released microparticles in VCA model and Fas-L attached microgels in T1D model. A) Characterization and 40-day release profile of recruitment-MP. Treatment with Recruitment-MP (50 mg) prolongs allograft survival indefinitely. Hindlimb allograft survival curve showing indefinite survival (>200 days) in six of eight rats treated with Recruitment-MP (50 mg). Adapted from Fisher et. al[107] 2020 with permission from Creative Commons Attribution-Non Commercial license. B) Flow-focusing microfluidics were used to generate biotinylated microgels from biotin-functionalized PEG-4MAL macromers. Islet graft survival: SA-FasL-presenting or control microgels and unmodified BALB/c islets were co-transplanted under the kidney capsule of chemically diabetic C57BL/6 recipients. Adapted from Headen et. al 2018[111] originally published in Nature Materials, with permission from author Dr. Lonnie D. Shea.

An alternative to delivery or presenting factors within the environment is to directly transplant cells to modulate the immune response. Biomaterial scaffolds can serve to support the co-localization of the cell types. Perhaps the most widely studied cell type for co-transplantation involves mesenchymal stem cells (MSCs).[112,113] MSCs, derived from the infrapatellar fat pad, have been co-transplanted with islets in order to capitalize on the immunomodulatory[114] and proangiogenic[115] properties of MSCs. MSC treatment significantly enhanced islet engraftment and function, resulting in stabilized blood glucose levels, reduced exogenous insulin requirement, and

increased numbers of regulatory T-cells in peripheral blood.[116] An alternative to recruiting Tregs is the direct transplantation of Tregs with islets. Tregs co-transplanted with islets on a microporous PLG scaffold provided long-term graft protection from an autoimmune response.[117] The seeded Tregs were replaced by recipient-derived Tregs over time, suggesting that host-derived Tregs induce tolerance to islet grafts on the PLG scaffold. Normoglycemia was restored, and co-transplanted Tregs extended graft survival indefinitely in several instances. Interestingly, in this study, local co-delivery of Tregs with an initial islet transplant also led to protection against autoimmune destruction of a second islet transplant (without Tregs), indicating systemic tolerance to islet antigens.

1.4.2 Antigen-Specific Immune Modulation

Antigen-specific immune modulation strategies represent a significant opportunity for all cell and solid organ transplantation, which currently employ systemic immunosuppression to prevent rejection of the graft yet is associated with increased risk of infection and neoplasia for the patient. Modulation of systemic immune responses may be an alternative or complementary strategy to local immune modulation. These systemic therapies may be administered prior to cell transplantation, and/or as needed following transplantation. These strategies aim to either i) delete autoreactive or alloreactive T cells, ii) induce anergy in the naïve cells, and/or iii) induce the formation of regulatory T cells that can modulate the graft reactive T cells. Immunosuppressive drugs, such as those used in the Edmonton Protocol, function by suppressing the function of one or more cell types in an antigen-independent manner. Localized delivery of these immunosuppressive factors can reduce the required dose and avoid some of the systemic side-effects of the compounds.[118] This localized delivery strategy can prolong graft survival; however, the graft contains a finite quantity of drug and seems unlikely to support indefinite graft survival

and function. Numerous strategies are being investigated, such as pharmaceuticals, nanoparticles, or engineered cells, to limit allograft rejection and have been reviewed elsewhere[119–121], and we describe a few representative examples applied to islet transplantation.

Antigen-specific immune modulation offers the opportunity to prevent rejection of the graft while leaving the remainder of the immune system intact. One approach toward this goal has been the use of donor splenocytes, which have donor antigen and upon treatment with 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (ECDI) and subsequent intravenous infusion has had success in inducing tolerance in a variety of autoimmune and allotransplant models.[122–125] Treated splenocytes, which induce apoptosis in the donor splenocytes, are infused with one dose prior to transplantation and one dose post-transplantation. Infusions of ethylene carbodiimide (ECDI)-treated donor splenic antigen-presenting cells result in indefinite survival of allogeneic islet grafts in the absence of immunosuppression (Figure 1.5A).[124] The site of islet transplantation does influence the effectiveness of tolerance induction, with the kidney capsule and epididymal fat pad having high levels of success, and with one report indicating lower levels of tolerance induction in the liver.[125] Recently, this approach of ECDI treated splenocytes has been translated to studies in non-human primates using ECDI treated apoptotic donor leukocytes. The regimen promoted stable islet allograft tolerance (>356 days) in monkeys by suppressing effector cell expansion and expanding regulatory networks.[126]

The capability of ECDI coupled splenocytes to treat autoimmune disease and prevent transplant rejection has been extended to synthetic nanoparticles, which have the potential for greater consistency in GMP manufacturing that would facilitate translation. In autoimmune models of diabetes, antigen-loaded nanoparticles have been able to prevent islet destruction with nanoparticles administered prior to and after disease initiation.[127] Additionally, this approach has

been extended to allogeneic cell transplantation by coupling of cell lysates to the nanoparticles and subsequent infusion prior to and after allogeneic cell transplantation.[109] The particles in combination with a 3-day course of low-dose rapamycin were able to induce long term graft function in the absence of sustained immunosuppression(Figure 1.5B).[128] The particles were effective at limiting T cell activation induced by indirect antigen presentation; however, the particles were not able to influence T cell activation by direct antigen presentation.[129] This inability to affect T cell responses resulting from direct antigen presentation likely reflects that antigen coupling to particles or cells does not appear to modulate activation induced by major antigen mismatch.[129]

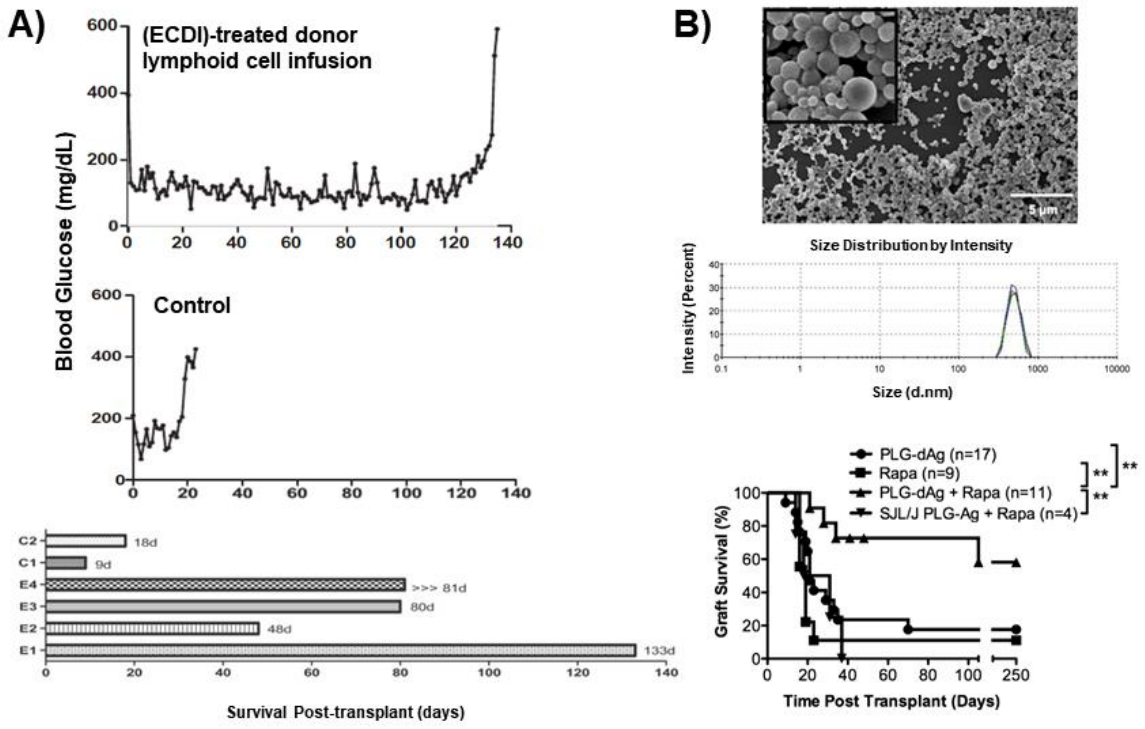

Figure 1.5. Immunomodulation strategies: ECDI-treated donor lymphoid cell infusion and ECDI-attached PLG nanoparticles in T1D model. A) Glycemic control posttransplant: four ECDI-DLI experimental group and two control group diabetic NHPs each promptly achieved normoglycemia post-allogeneic islet transplant. Adapted from Lei et. al 2015[134] with permission from John Wiley and Sons. B) STEM image and size of PLG particles prior to lysate coupling with ECDI. A short course of low dose rapamycin with PLG-dAg demonstrated significantly greater islet allograft survival (n = 11) compared with mice treated with rapamycin alone (n = 9) **p < 0.01. Adapted with permission from Bryant et. al 2014[128] with permission from Elsevier.

1.5 Final Thoughts

Substantial advances have been made in recent years for the differentiation of hPSC to β-cells. These advances have substantially raised the prospect of a cell-based therapy for T1D and highlight the critical need for cell manufacturing and delivery systems that can provide adequate numbers of cells that can efficiently engraft and function for long times without the need for sustained immunosuppression. Advances in CRISPR technology may also allow for the engineering of cells that have improved capacity for insulin secretion, or the ability to evade the immune system .[130] These opportunities in cellular engineering will benefit from technologies that can provide control over the cellular microenvironment, both during cell manufacturing and following transplantation. These biomaterial platforms provide the tools to sequester key signaling factors, facilitate the organization of cells into structures, support cell-cell and cell-matrix interactions, recruit or deliver accessory cells to aid maturation or function, limit inflammatory responses, and evade the adaptive immune responses. Establishing a supportive environment at the time of manufacture or transplantation can create a stable niche that can support cell function for extended periods of time. Collectively, the capabilities of hPSC-derived β-cells have advanced, and engineering the microenvironment can help to translate these capabilities.

1.6 Structure of Thesis

In **Chapter 2**, I present a study that shows the impact of local delivery of the trophic factor Exendin-4 on the maturation and function of maturing hPSC-derived β cell progenitors. The hPSC-derived β cell progenitors were produced using the protocol published from the Timothy J. Kieffer's lab, Alireza Rezania et al 2012, and subsequently transplanted into diabetic mice using either blank PLG scaffolds, or scaffolds with an Exendin-4 releasing inner layer. Kidney capsule injection was used as a control. The mice that received cells loaded on scaffolds with Exendin-4

had higher levels of circulating C-peptide, and explanted tissue revealed higher proportions of monohormonal insulin cells.

Our lab later fostered a collaboration with Dr. Douglas Melton's lab at Harvard, in which they shipped us hPSC-derived β cells produced from their 6-stage protocol. In **Chapter 3**, I present results from these studies, in which the clinically translatable sites of the peritoneal fat pad and the subcutaneous space were tested for their effect on the maturation and function of hPSC-derived β cells transplanted within collagen-IV coated PLG scaffolds. Additionally, I present findings from testing the impact of blood glucose levels on maturation of the hPSC-derived β cells. One of the most valuable insights from this study was that we observed similar functional dynamics (blood glucose levels, body weight changes, C-peptide levels, glucose tolerance test results, and duration to normoglycemia) between the kidney capsule, a site typically used to test hPSC-derived β cells, and the clinically translatable site of the peritoneal fat pad. Chapter 3 corresponds to a published peer-reviewed paper. The format of this chapter has been adjusted to match the rest of the thesis with minimal changes to the content. **Chapter 4** presents results from studies designed to enhance our understanding of how scaffolds can be used as a tool for in vitro manufacturing. The hPSC-derived β cell progenitors were produced using protocols published by Jeffrey R. Millman's lab, Leonardo Velazco-Cruz et al. 2019 and Nathaniel J. Hogrebe et al. 2020. We tested the developmental stage in which progenitors are seeded into the scaffold niche, and two differentiation strategies prior to seeding: monolayer and suspension culture. The timing of scaffold seeding was found to be important, as seeding too early hindered maturation within scaffolds, while pre-endocrine seeding promoted the continued maturation of the cells through the endocrine progenitor (stage 5) and maturating beta cell (stage 6) stages. Lastly, the conclusions of this thesis and future directions are presented in **Chapter 5**.

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Chapter 2. Microporous Polymer Scaffolds for the Transplantation of Embryonic Stem Cell Derived Pancreatic Progenitors to a Clinically Translatable Site for the Treatment of Type I Diabetes

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Contributions. Dr. Tadas Kasputis, 1st author, led execution of the studies and completion of the manuscript for publication. Daniel Clough, 2nd author, was involved with all aspects of the studies, assisted with transplantation, performed mouse glucose monitoring, c-peptide collection, tissue staining, writing and data analysis. Dr. Fallon Noto initiated these studies, taught Dr. Tadas Kasputis and Daniel Clough methods required to execute the studies. Kevin Rychel led image quantification of tissue sections. Dr. Briana Dye performed gene expression analysis. Dr. Lonnie Shea lead study design and oversaw all aspects of the studies.

2.1 Introduction

Type I diabetes (T1D), which affects an estimated 1.5 million Americans, is caused by autoimmune destruction of the pancreatic β-cells that currently results in the need for lifelong insulin therapy.[1–3] Rigorous glucose monitoring combined with insulin administration can manage symptoms in T1D patients; however, secondary microvascular and macrovascular complications

eventually afflict most T1D patients.[1–3] Cellular therapy holds promise for permanent control of glucose within the physiologically required range and would allow for endogenous insulin production.[4,5] Human allogeneic islet transplantation as a β-cell replacement strategy reached a major milestone in 2000 when investigators achieved diabetes reversal in seven out of seven recipients by hepatic infusion of islets from multiple donor pancreata combined with cortico-steroid-free immunosuppression.[3] Since then, improvements in islet transplantation have led to 56% of patients transplanted becoming insulin dependent after 3 years,[6] with limited long-term function being attributed to immunosuppression protocols, and the local milieu within the liver contributes to limited islet engraftment.[7–9]

Alternative cell sources for transplantation are being sought as the supply of donor islets is limited.[6,10–14] Pluripotent stem cell derived β-cell therapy is emerging for T1D treatment as the recent ability to direct stem cell differentiation of renewable pluripotent stem cells toward a β-cell lineage was developed.[15–21] Several milestones have been achieved with the development of culture systems that enable pluripotent stem cells (PSCs) to form definitive endoderm[22] with subsequent development through pancreatic endoderm to endocrine cells capable of synthesizing pancreatic hormones.[23] While cells have been differentiated in vitro to various stages of immature β-cells, transplantation in vivo is necessary to complete cell maturation.[4,16,18,21,24,25]

Alternative transplantation sites may be necessary for the transplantation of in vitro derived pancreatic progenitors. Previous reports have primarily employed transplantation of PSC derived pancreatic progenitors into the kidney capsule.[15–18,20,21] Biomaterial scaffold systems employed for transplanting the progenitors have, until recently,[26] been less efficient relative to transplantation of pancreatic progenitors to the kidney capsule or have involved components such as Matrigel that are not translational for clinical applications.[4,27,28] A recent report indicated that encapsulation of

immature β-cells within an alginate hydrogel and transplanted to the intra- peritoneal space of diabetic mice can restore euglycemia within 14 days following transplantation and provides long-term glucose control.[29] Importantly, these cells following transplantation must continue to mature in the presence of signals from the local host microenvironment in vivo.

Herein, we investigated the use of microporous scaffolds for extrahepatic, extrarenal transplantation of pancreatic progenitors with localized delivery of trophic factors to enhance maturation. We previously reported on scaffolds for extrahepatic transplantation[30,31] of murine, human, and porcine islets,[31,32] which has led to engraftment and long-term function. Furthermore, the scaffolds were fabricated for the sustained, localized release of exendin-4, a glucagon-like peptide-1 (GLP- 1) receptor agonist that stimulates glucose dependent insulin secretion, protects islets against apoptosis, and improves outcome in animal islet transplantation models.[33–35] Exendin-4 has also been previously reported to enhance the differentiation of mouse embryonic stem cells to insulin producing β-cells, primarily by upregulating Neurod1 and Glut2 gene expression.[36,37] Taken together, we hypothesize that using PSC-derived pancreatic progenitors along with a cell delivery platform, capable of localized and sustained delivery of trophic factors, will enhance in vivo cell maturation toward monohormonal insulin producing cells at a clinically translatable site.

2.2 Methods and Materials

2.2.1 Microporous PLG Scaffolds

Microporous scaffolds were fabricated as previously described.[30–32,38–41] Briefly, microporous scaffolds were fabricated by compression molding PLG microspheres (75:25 mol ratio D,L-lactide to glycolide) and 250−425 μm salt crystals in a 1:30 ratio of PLG microspheres to salt. The mixture was humidified in an incubator for 7 min and then thoroughly mixed again.

Nonlayered scaffolds were compression molded with 77.5 mg of polymer−salt mixture; layered scaffolds were compression molded with an inner layer containing exendin-4 loaded PLG microspheres sandwiched between two 38.75 mg layers.[40] Both nonlayered and layered scaffolds were compression molded into cylinders 5 mm in diameter by 2 mm in height using a 5 mm KBr die (International Crystal Laboratories, Garfield, NJ) at 1500 psi for 30 s. Molded constructs were gas foamed in 800 psi carbon dioxide for 16 h in a pressure vessel. The vessel was depressurized at a controlled rate for 30 min. On the day of transplantation, scaffolds were leached in water for 1.5 h, changing the water once after 1 h. Scaffolds were sterilized by submersion in 70% ethanol for 30 s and multiple rinses with phosphate buffer solution. Scaffolds were coated with a 1 mg/mL solution of collagen IV for 20 min prior to cell seeding.

2.2.2 In Vitro Differentiation of PSCs into Pancreatic Progenitor Cells

Pluripotent H1 PSCs (WiCEll, Madison, WI, United States), which are on the list of FDA approved PSC lines, and previous reports have demonstrated their capacity for developing into insulin producing cells,[16,17,19,42–44] were cultured in MTeSR1 media (Stem Cell Technologies, Vancouver, BC, Canada) on tissue culture treated plates (Corning Life Sciences, Tewksbury, MA, United States) over a layer of Geltrex murine tumor basement membrane extract (Life Technologies/Thermo-Fisher, Waltham, MA, United States). Pluri- potent cell clusters were lifted into a single cell suspension using Accutase (Stem Cell Technologies) and plated on Geltrex coated 6-well tissue culture treated plates (Corning Life Sciences) at a concentration of 1.5×10^6 cells per well of a 6-well plate. The pluripotent cells were differentiated according to a previously established 15 day differentiation protocol,[16] in which fresh growth factors are added fresh daily to direct differentiation through the first four stages of embryonic development: (1) definitive endoderm (3 days), (2) primitive gut tube (3 days), (3) posterior foregut (4 days), and finally (4)

pancreatic endoderm and endocrine precursors (5 days). At the end of stage 4, differentiated cells were lifted from the plates using Accutase and seeded on sterilized, collagen IV coated scaffolds at a concentration of 3×10^6 cells/scaffold.

2.2.3 qRT-PCR

Gene expression of pancreatic markers was assessed to track progress through stages of differentiation. First, cells were pelleted and flash frozen using liquid nitrogen. RNA was isolated (Qiagen; Hilden, Germany), and qRT-PCR was conducted (Qiagen; Hilden, Germany) against key pancreatic differentiation markers using the primers found in Table 2.1. Gene expression was calculated based on fold change in comparison to house keeper gene GAPDH, followed by normalization to marker expression in pluripotent ESCs.

Table 2.1. Primers of Pancreatic Differentiation Markers Used for qRT-PCR

Gene	Primer	Sequence (5' to 3')
KIR2	Forward	AAGGAGATGACCAGCCTCAG
KIR2	Reverse	GCCCACGAAAGTTATGAGGA
SUR1	Forward	AAGGAGATGACCAGCCTCAG
SUR1	Reverse	GCCCACGAAAGTTATGAGGA
PDX1	Forward	CCTTTCCCATGGATGAAGTC
PDX1	Reverse	CGTCCGCTTGTTCTCCTC
NKX6.1	Forward	GGGGATGACAGAGAGTCAGG
NKX6.1	Reverse	CGAGTCCTGCTTCTTCTTGG
MAFA	Forward	GAGAGCGAGAAGTGCCAACT
MAFA	Reverse	TTCTCCTTGTACAGGTCCCG
Insulin	Forward	TTCTACACACCCAAGACCCG
Insulin	Reverse	CAATGCCACGCTTCTGC
Glucagon	Forward	TGCTCTCTCTTCACCTGCTCT
Glucagon	Reverse	AGCTGCCTTGTACCAGCATT

2.2.4 Streptozotocin Induced Diabetic Mouse Model

Male NSG mice (Jackson Laboratories, Bar Harbor, ME, United States) between 8 and 12 weeks of age received an intraperitoneal injection of 130 mg/ kg streptozotocin 4−7 days prior to transplant. (Sigma-Aldrich, St. Louis, MO). Each day leading up to surgery, blood glucose levels were measured using the tail vein prick technique (Accu-chek/Roche, Basel, Switzerland), and body weight was recorded. The criteria to include a mouse in the study was the following: glucose

readings above 350 mg/ dL on 2 consecutive days leading up to transplant surgeries, and no more than a 20% reduction in weight since streptozotocin injection. A sustained release insulin pellet (LinBit, LinShin Canada, Inc., Scarborough, ON, Canada) containing USP grade bovine insulin was inserted subcutaneously at time of surgery to assist in mouse health during engraftment of transplanted cells, and every four weeks until the end point of the study. Following surgery, glucose and body weight for each mouse were measured three times per week. All studies involving mice were approved by the University of Michigan Animal Care and Use Committee.

2.2.5 Transplantation of Pancreatic Progenitors to Streptozotocin-Induced Diabetic NSG Mice

The day before scaffold seeding, 100 mg of fibrinogen (EMD Millipore, Billerica, MA, United States) was dissolved in 2 mL of tris-buffered saline (TBS) at 37 °C for 2 h. The solution was added to a 10,000 MW cutoff dialyzing cassette and dialyzed overnight in 1 L of TBS. The dialyzed fibrinogen solution was estimated to be approximately 20 mg/mL, and this solution was diluted to 4 mg/mL in TBS. On the day of surgery, stage 4 pancreatic progenitors were lifted from tissue culture plates using Accutase, and 3×10^6 cells were resuspended in 6 µL thrombin mix (50 U/ml thrombin, 50 mM CaCl2, in TBS). Six µL of 4 mg/mL fibrin solution was added to the mix, and the cell suspension was promptly added to semidried scaffolds. Mice were anesthetized with 2% isoflurane until loss of consciousness was confirmed by pinch reflex test. The abdominal area was shaved and sterilized with betadine and ethanol. An approximate 5 mm incision was made in the peritoneal wall, in the middle of the abdominal region, and the epididymal fat pads (EFP) were located and unwrapped outside of the abdominal wall. Scaffolds were wrapped within the epididymal fat pad, then placed back into the peritoneal cavity.[45] Kidney capsule transplantation was adapted from a previously published report.[16] Five million cells were pelleted and suspended

in a fibrin thrombin mix. The mix was injected into the kidney capsule. Mice were given a dose of 0.005 mg/gram body weight Rimadyl carprofen (Zoetis, Florham Park, NJ, United States) and allowed free access to food and water postoperatively.

2.2.6 Serum Collection and Human C-Peptide Analysis

Once every four weeks, blood serum was collected to quantify circulating human C-peptide levels. Mice were fasted 5 h before blood collection. Thirty minutes prior to blood collection, mice received an intraperitoneal injection of 2 g/kg glucose in the form of 20% glucose. Approximately 200 µL of blood was collected from the saphenous vein during the period between 30 and 60 min post glucose stimulation. Serum was collected from whole blood by 20 min of coagulation followed by centrifuging at 2000g for 20 min. Isolated serum was analyzed for human C-peptide levels using the manufacturer's provided ELISA protocol (AbCam, Cambridge, MA, United States).

2.2.7 Immunostaining

Immunostaining of in vitro cell differentiation was performed at stages 0 (pluripotent), 1 (definitive endoderm), 2 (posterior foregut), and 4 (pancreatic progenitors). Cells differentiated in 6-well plates were fixed using 4% paraformaldehyde (Electron Microscopy Sciences; Hatfield, PA, United States), permeabilized using 0.5% Triton in tris-buffered saline, blocked using normal donkey serum, and stained for key differentiation markers at each stage (Oct3/ 4 and Sox17 for stages 0 and 1, FoxA2 and HNF4-alpha for stage 2; C- peptide, Nkx6.1, insulin, and glucagon for stage 4) and cell nuclei were counterstained with DAPI. For immunohistochemical assessment of in vivo cell maturation, scaffolds were removed at 4 and 8 weeks post transplant. Epididymal fat pads were isolated, and a majority of the fat sounding the scaffold was removed with forceps. Scaffolds were cryopreserved in isopentane cooled on dry ice and then embedded within OCT

embedding medium and cryosectioned. Sections were stained for C-peptide, human mitochondria (HuMit), insulin, and glucagon, and cell nuclei were counterstained with DAPI. Digital images were acquired with a MicroFire digital camera (Optronics, Goleta, CA) connected to an Olympus BX-41 fluorescence microscope (Olympus, Center Valley, PA, United States). Image quantification was conducted using MATLAB software using an object-based colocalization analysis. DAPI+ cells were identified and quantified by applying Otsu's thresholding method, the watershed transform, and individual cluster thresholding. Then, each cell's colocalization with immunofluorescent markers was quantified.

2.2.8 Statistics

All statistical analyses were conducted using Prism graphing and data analysis software (GraphPad Software, Inc., La Jolla, CA, United States). Values were reported as the mean ± SEM.

2.3 Results

2.3.1 In Vitro Differentiation of Pancreatic Progenitors and Seeding on Scaffolds

PSCs were cultured and differentiated in vitro to pancreatic progenitors following a previously established protocol by Rezania et al.[16,17] Pluripotent cells were stained and imaged for Sox17 (green) and Oct3/4 (red) 24 h after plating, prior to the induction of differentiation to the definitive endoderm lineage, indicating that a population of Oct3/4-expressing undifferentiated pluripotent cells was obtained (Figure 2.1A). Three days after induction of differentiation to the definitive endoderm lineage, staining for Oct3/ 4 and Sox17 indicates the presence of a Sox17-expressing cell population (Figure 2.1B), demonstrating the differentiation of pluripotent stem cells to definitive endoderm cells. Furthermore, upon differentiating cells to the primitive gut tube lineage (stage 2), cellular staining for FoxA2 and Hnf4 was prevalent. Successful differentiation

into pancreatic progenitors was verified by immunostaining for insulin and glucagon, as well as Pdx1 and Nkx2.2 expression (Figures 2.1D and E). Additionally, qRT-PCR was used to quantify the expression of key factors involved in the differentiation from PSCs to pancreatic progenitors throughout in vitro differentiation (Figure 2.2). The qRT-PCR results indicate the progressive increase in expression of Ngn3 and NeuroD, which are markers for β-cell maturity, as well as coexpression of Pdx1 and Nkx6.1, which are markers of β-cell fate. Furthermore, expression of Sur1, an ATP channel protein, and Kir2, a calcium channel- closer, increased at stages 3 and 4, corresponding to the expression of insulin and glucagon.

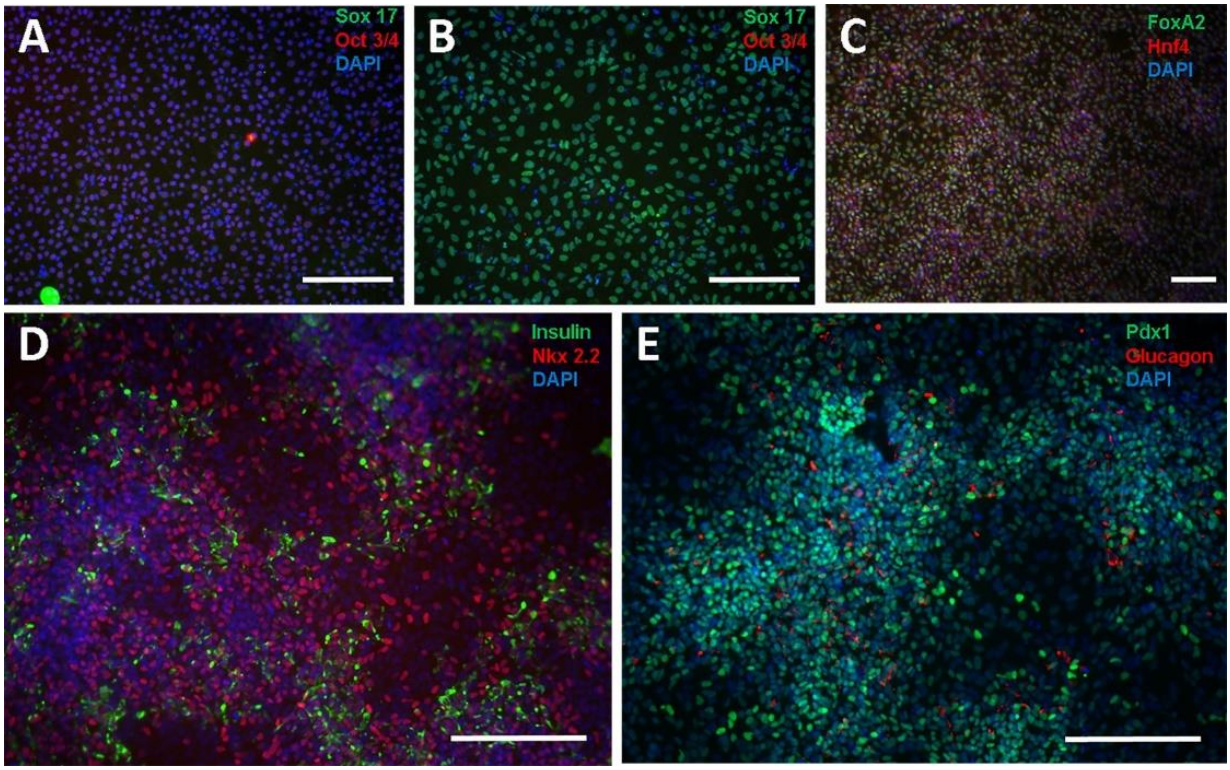

Figure 2.1. In vitro differentiation of PSCs to pancreatic progenitor cells. H1 pluripotent PSCs were seeded to Matrigel-coated 6-well plates at a density of 1.5 million cells per well, and pluripotent cells were imaged for sox17 (green) and oct3/4 (red) prior to beginning the differentiation (A). Definitive endoderm cells were imaged for sox17 (green) and oct3/4 (red) following stage 1 of the differentiation (B). Primitive gut tube cells were imaged for FoxA2 (green) and Hnf4 (red) (C), and pancreatic progenitor cells were stained for insulin (green) and Nkx2.2 (red) (D) as well as Pdx1 (green) and glucagon (red) (E) following the fourth stage of differentiation. All cells were counterstained with DAPI (blue). Scale bars for all images = 200 µm.

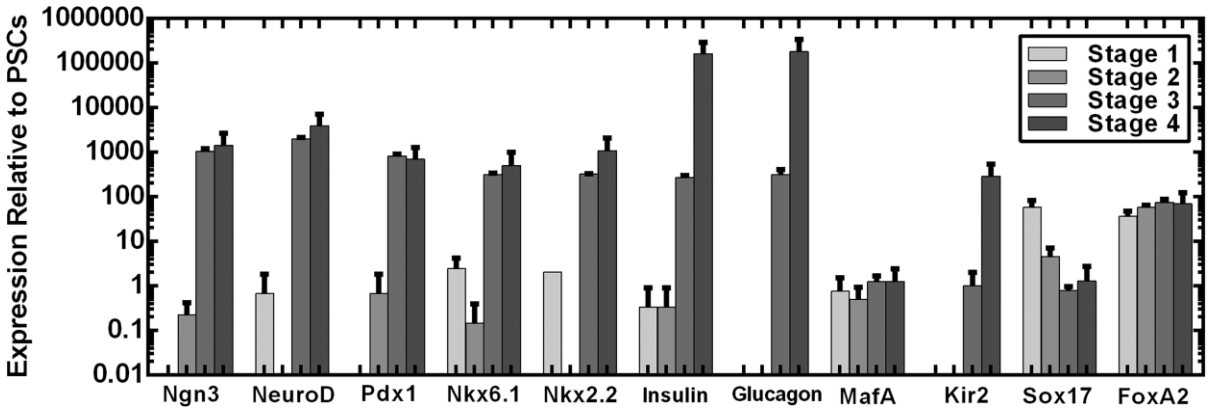

Figure 2.2. qRT-PCR of in vitro differentiation of human PSC derived pancreatic progenitors.

2.3.2 Transplantation of PSC-Derived Pancreatic Progenitors to Epididymal Fat Pad

Following in vitro differentiation, the pancreatic progenitors were seeded onto collagen IV-coated microporous PLG scaffolds (Figure 2.3A), and pancreatic progenitor live/dead staining at 1 h after seeding indicated good cell viability (Figure 2.3B) and a homogeneous distribution of cells throughout the pores of the PLG scaffold (Figure 2.3B).

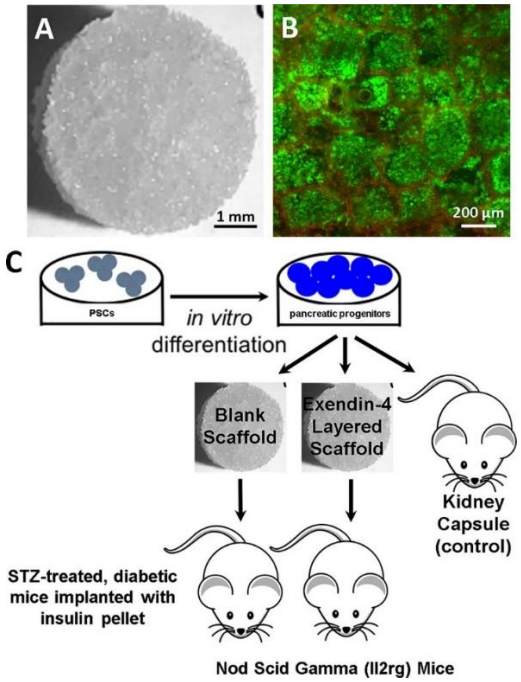

Figure 2.3. Microporous PLG scaffold morphology (A) and live/dead stain of seeded pancreatic progenitors (B). Schematic of experimental workflow (C). PSCs were differentiated in vitro to pancreatic progenitors and then seeded to either microporous PLG scaffolds without exendin-4 or to scaffolds containing an encapsulated exendin-4 microparticles within an inner layer disk. Scaffolds were subsequently transplanted to the epididymal fat pads of STZ-treated, diabetic NSG mice.

Microporous PLG scaffolds seeded with pancreatic progenitors were transplanted into the epididymal fat pads of NSG mice (Figure 2.3C)[46] with transplantation under the kidney capsule serving as a control. The function of these transplanted cells was assessed through blood glucose and human C-peptide measurements in serum (Figures 2.4A−C). Blood glucose levels for the kidney capsule transplant cohort stabilized (between approximately 100−250 mg/dL) upon administration of insulin pellets at week 4. In contrast, mice receiving pancreatic progenitors seeded to PLG scaffolds had high glucose levels (approximately 400−600 mg/dL) that were statistically different from mice transplanted with pancreatic progenitors under the kidney capsule, despite the presence of insulin pellets in both cohorts (Figure 2.4A).

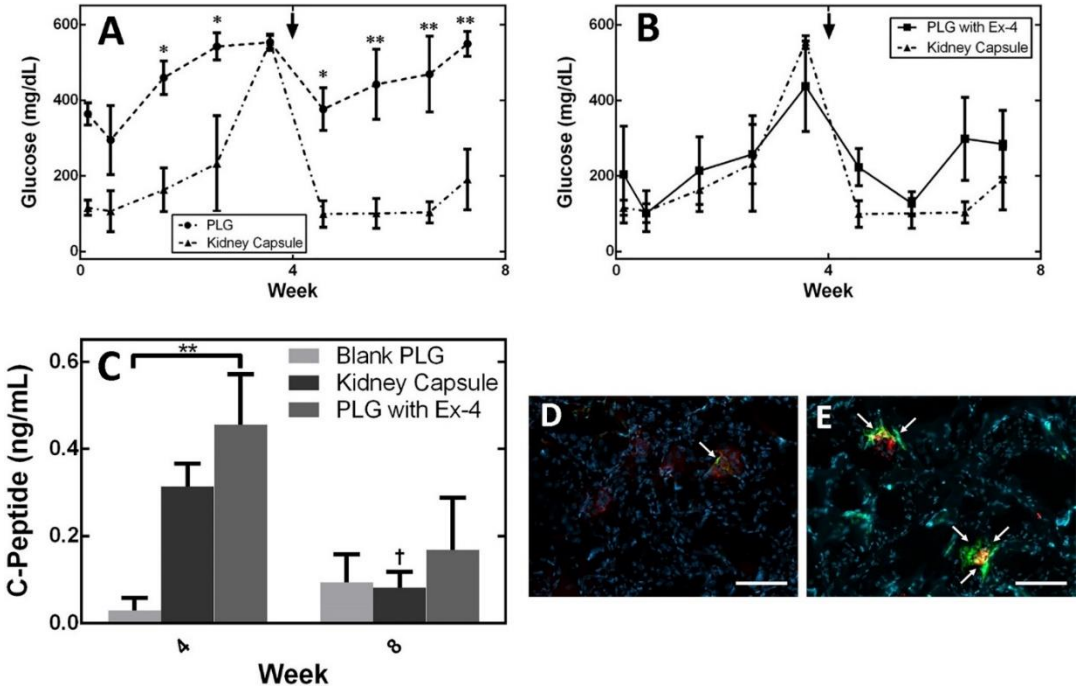

Figure 2.4. Blood glucose measurements of mice transplanted with pancreatic progenitor cells, seeded to blank PLG scaffolds or transplantation of cells under the kidney capsule (A, arrow indicates administration of insulin pellet). Blood glucose measurements of mice transplanted with pancreatic progenitors seeded to PLG scaffolds containing exendin-4 or transplantation of cells under the kidney capsule (B, arrow indicates administration of insulin pellet). Human C-peptide in serum for a cohort of mice receiving microporous nonlayered PLG scaffolds containing PSC-derived pancreatic progenitors (n = 4), a cohort of mice receiving layered PLG scaffolds containing exendin-4 (n-4), and a cohort of mice transplanted with PSC- derived pancreatic progenitors under the kidney capsule (n = 4) (C). Histology of a blank scaffold (D) and exendin-4 layered scaffold (E) removed at week 4, stained for c-peptide (green), human mitochondria (red), and counterstained with DAPI (blue). All values reported as the average ± standard error; * indicates $p < 0.05$, ** indicates $p < 0.01$, † indicates $p < 0.05$ difference between week 4 and week 8 among a cohort of mice. Arrows on images (D and E) indicate regions of C-peptide/human mitochondria coexpression. All scale bars for images D and E = 100 μm.

The concentration of human C- peptide in serum, which was 10.6-fold greater (n.s. alpha = 0.05) for cells transplanted into the kidney capsule compared to cells transplanted on the PLG scaffolds into the peritoneal fat at 4 weeks. At week 8, C-peptide production for cells within the kidney capsule decreased and is not significantly different (n.s. alpha = 0.05) relative to the cells transplanted on PLG within the epididymal fat pads. Cellular colocalization of both human mitochondria and C-peptide at week 4 (Figure 2.4D) indicates that human cells are present within the scaffold but producing very small amounts of C-peptide, which reflects the measurement of C-peptide at week 4 (Figure 2.4C). Image analysis of insulin and glucagon producing cells indicates no significant differences between insulin, glucagon, and polyhormonal cells (insulin and glucagon producing cells) at week 4 and glucagon producing cells were significantly greater ($p < 0.01$) than insulin producing and polyhormonal cells at week 8 (Figure 2.5).

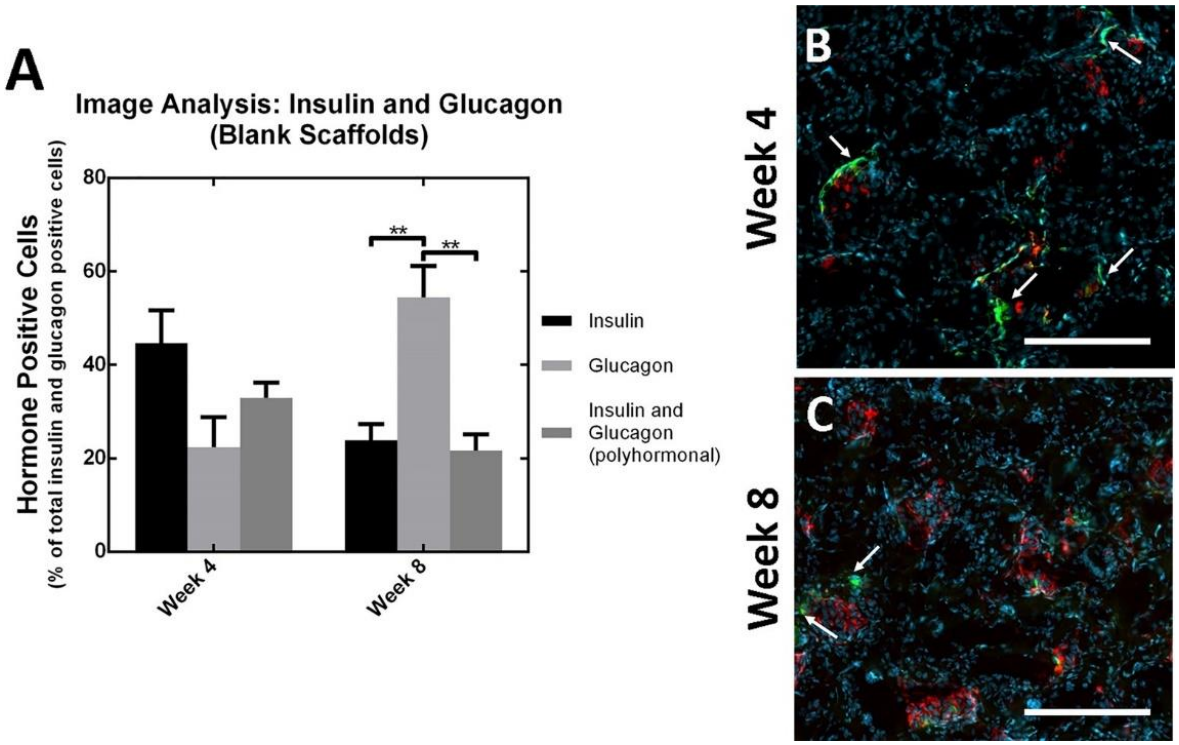

Figure 2.5. Image quantification of monohormonal insulin producing cells and polyhormonal insulin/glucagon producing cells for blank scaffolds at weeks 4 and 8 (A). Histology of blank layered scaffolds (B and C) removed at weeks 4 and 8, stained for insulin (green), glucagon (red), and counterstained with DAPI (blue). All values reported as the average ± standard error; ** indicates $p < 0.01$. Arrows indicate regions of monohormonal insulin production. Scale bars = 200 μm.

2.3.3 Exendin-4 Delivery from Scaffold Enhances Pancreatic Progenitor Function

We subsequently investigated the localized delivery of trophic factors from scaffolds as a means to enhance maturation and function of the transplanted pancreatic progenitors. Blood glucose levels of mice transplanted with scaffolds containing exendin-4 were statistically similar to blood glucose levels of mice transplanted with pancreatic progenitors under the kidney capsule (n.s. alpha = 0.05, Figure 2.4B). Average measurements of serum C-peptide for cells transplanted on exendin-4 containing scaffolds to the fat pad were 15.7-fold greater relative to cells transplanted on blank PLG scaffolds to the fat pad ($p < 0.01$) and 1.5-fold greater relative to cell transplantation to the kidney capsule (n.s. alpha = 0.05) (Figure 2.4C). Cellular colocalization of both human mitochondria and C-peptide at week 4 (Figure 2.4E) indicates that human cells are present within the scaffold and producing C-peptide. Furthermore, image analysis indicates that the presence of insulin producing cells was significantly greater than glucagon producing and polyhormonal cells at weeks 4 ($p < 0.0001$) and 8 ($p < 0.01$) (Figure 2.6).

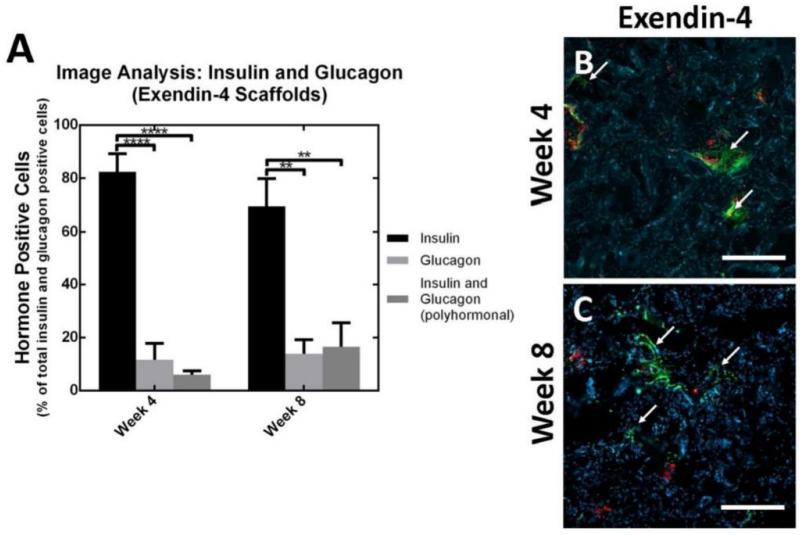

Figure 2.6. Image quantification of monohormonal insulin producing cells and polyhormonal insulin/glucagon producing cells for exendin-4 layered scaffolds at weeks 4 and 8 (A). Histology of exendin-4 layered scaffolds (B and C) removed at weeks 4 and 8, stained for insulin (green), glucagon (red), and counterstained with DAPI (blue). All values reported as the average ± standard error; ** indicates $p < 0.01$; **** indicates $p < 0.0001$. Arrows indicate regions of monohormonal insulin production. Scale bars = 200 μm

2.4 Discussion

Allogeneic islet transplantation for the treatment of T1D is a therapy in which donor islets are infused intrahepatically, which has led to the transient reversal of diabetes. However, allogenic transplantation has several therapeutic limitations, which include a shortage of donor islets, long-term immunosuppression, and high risk of tissue rejection. All of these limitations have led to the investigation of embryonic or induced pluripotent stem cells as an unlimited source of functional β-cells. Successful differentiation into pancreatic progenitors was verified by immunostaining for insulin and glucagon producing cells as well as Pdx1- and Nkx2.2-expressing cells (Figure 2.1D, E) and indicate the differentiation of PSCs into pancreatic progenitors that have the potential to mature into β-cells, as shown by the presence of both Pdx1 and Nkx2.2 as well as the presence of insulin producing cells. The qRT-PCR results suggest differentiation into pancreatic progenitors, as evidenced by significant upregulation in expression of markers for pancreatic tissue between stages 0 and 4 of differentiation and analogous to values in a previously reported study by Rezania et al.[16] More recently, protocols have been developed for the differentiation of PSCs to stage 6 or 7 immature β-cells in vitro.[18,21,25,29] For both pancreatic progenitors or later stage immature β-cells, further maturation is necessary following transplantation,[25] with instructive cues being provided by the in vivo environment. Biomaterial platforms provide the opportunity to facilitate the organization of cells into structures or provide cues that can enhance the in vivo maturation and ultimately function.

Microporous scaffolds were employed for transplantation of the cells at a clinically translatable site, which provides for integration with the host tissue similar to kidney capsule transplants, yet distinct from the isolation of cells from the host obtained with encapsulation systems. Transplantation of progenitor cells into the kidney capsule has been the primary method

by which these cells are able to survive, mature, and ultimately normalize blood glucose levels.[16,18,21] For translational purposes, studies have been investigating extrarenal sites as well as extrahepatic sites to avoid the instant blood mediated inflammatory reaction[47] and may enable retrieval of the cells should it become necessary. Herein, we employed the epididymal fat pad, which has similar properties as the omentum that has emerged as a leading candidate for clinical trials. The peritoneal fat is a relatively large tissue that could accommodate a volume of transplanted cells or, more likely, devices such as cell pouches and scaffolds that contain the transplanted cells.[47,48] The integration of the host tissue with the transplanted cells offers the potential to vascularize the cells, which provides the opportunity to sense blood glucose and distribute insulin. However, our results indicated that the cells did not produce C-peptide at levels comparable to the kidney capsule, which suggests that the host tissue interactions in the peritoneal fat did not readily support the maturation and function of the transplanted cells. Additionally, blood glucose levels measured between the two cohorts of mice suggested that transplantation of pancreatic progenitors into epididymal fat site was less effective at supporting β-cell function relative to transplantation under the kidney capsule.

Modulation of the local environment has the potential to augment the endogenous properties of the transplant microenvironment to enhance differentiation of maturing pancreatic progenitors toward the mature β-cell fate.[4,27,28] In a previous investigation using microporous PLG scaffolds for human islet transplantation to diabetic murine models,[30–32,38–40] the trophic factor exendin-4 was delivered as a means to enhance the glucose stimulated insulin secretion from the transplanted islets. The localized release of exendin-4 was applied herein to influence pancreatic progenitor survival and differentiation following transplantation. Exendin-4 has also been previously reported to enhance the differentiation of mouse embryonic stem cells to insulin

producing β-cells, primarily by upregulating Neurod1 and Glut2 gene expression.[36,37]

We report that modifying this post-transplant microenvironment by sustained, localized delivery of exendin-4 was found to significantly enhance the survival and differentiation of the pancreatic progenitors when transplanted to a clinically translatable site: the peritoneal fat (epididymal fat pads). The studies herein employed scaffolds coated with collagen IV as a means to support and enhance the survival and maturation of pluripotent cells because previous reports have demonstrated that ECM proteins such as collagen IV and laminin are key components of the pancreatic basement membrane that are necessary for maintaining islet morphology as well as enhancing β-cell proliferation and insulin gene expression.[49,50] This investigation demonstrated that exendin-4 release to transplanted pancreatic progenitors significantly increased C-peptide production relative to blank control scaffolds (0.6 ± 0.1 ng/mL C-peptide in mice with exendin-4 and 0.3 ± 0.1 ng/mL in mice containing blank control scaffolds; $p < 0.05$). Also, the percentage of insulin producing cells in scaffolds releasing exendin-4 was four- to fivefold greater than that in glucagon producing and polyhormonal cells and significantly greater than the percentage of insulin producing cells in scaffolds without exendin-4, where insulin producing cell percentage was not significantly greater than glucagon producing and polyhormonal cells. These results support the potential of exendin-4 to enhance the in vivo maturation of the progenitor cells toward mature β-cells.

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Chapter 3. Normoglycemia Achieved in Diabetic Mice Following Islet Organoid Maturation in the Peritoneal Fat

Contributions. Daniel Clough led the design and execution of all aspects of the studies. Richard Youngblood ran the gene expression analysis. Dr. Tadas Kasputis assisted with transplantation of mice. Kevin Rychel, Georgios Mentzelopoulos, and Sam Steinberg assisted with blood glucose and weight monitoring. Rohit Maramraju cryo-sectioned tissue and provided tissue staining guidance. Cells were differentiated and shipped by Elise Engquist, Dr. Ramona Pop, Dr. Douglas Melton. Dr. Lonnie Shea lead study design and oversaw all aspects of the studies.

3.1. Introduction

Type 1 diabetes (T1D) is a chronic metabolic disorder characterized by hyperglycemia due to autoimmune destruction of insulin-producing β-cells. Individuals living with T1D must frequently monitor their blood glucose and inject exogenous insulin. Even with the aid of advanced insulin pumps and real time feedback systems, blood glucose levels still deviate outside of the range maintained by native islets. These deviations outside of normoglycemia put the individual at risk for vascular complications and life-threatening hypoglycemic events.[1–3] Cell replacement therapies have demonstrated the capacity to more tightly control blood glucose levels, while preventing severe hypoglycemia and maintenance of appropriate HbA1c. The Edmonton Protocol, first reported in 2000, describes infusing donor islets into the portal vein in conjunction with immune suppression. Gradual improvements over the years have led to islet transplantation

becoming an established standard of care for recurrent severe hypoglycemia, though it is not widely practiced due to the limitations of sustained immunosuppression.

An additional challenge with extending islet transplantation more broadly is the availability of islets, which has motivated the manufacturing of stem cell derived β cells. *In vitro* protocols have been developed to guide human pluripotent stem cell (hPSC) differentiation through the stages of pancreatic islet organogenesis towards immature insulin producing β cells.[4] A few reports have demonstrated the capacity of the transplanted stem cell derived β cells to restore normoglycemia [5–7], and this restoration of normoglycemia does not occur instantaneously as the β cells undergo further maturation in vivo. Due in part to the harsh environment of the portal vein, extrahepatic sites are being investigated for their potential to support therapeutic cells. A majority of the studies published to demonstrate production of hPSC-derived β cells utilized the kidney capsule as the site to study graft function.[5–13] This site has been a gold standard for testing β cell's ability to restore normoglycemia in mice; however, the kidney capsule is not considered clinically translatable for humans.[14,15] Biomaterial scaffolds are being developed to support the engraftment and survival of hPSC-derived β cells at extrahepatic sites that may be translationally relevant. Biomaterial encapsulation approaches present a physical barrier between the host immune system and therapeutic cells, which has a critical drawback of limiting access to host blood vessels, and limits the exchange of oxygen, nutrients, waste byproducts, and the capacity to rapidly distribute the secreted insulin. Non-encapsulating scaffolds allow for integration of therapeutic cells within the host tissue, but this approach will require the use of local or systemic immunomodulators to allow for graft tolerance. The subcutaneous site is a relatively easy surgical site access and can accommodate the large volume of cells that would be required for maintenance of normoglycemia[15], yet the vascularity of this site is low relative to other options and may require

prevascularization to support sufficient graft survival.[16] The peritoneal or omental pouch similarly has the capacity to support large numbers of transplanted cells. Islets transplanted into the peritoneal fat pad of diabetic mice have been achieved using porous scaffolds composed of either poly (lactide-co-glycolide) (PLG) or PDMS. Engraftment and function of these transplanted cells has been observed for hundreds of days.[17]

In this study, we investigated the transplantation of hPSC-derived β cells at clinically relevant sites on ECM coated microporous PLG scaffolds, which allow integration of transplanted cells with the host and determined the ability to support normoglycemia. The transplantation sites we tested included the epididymal fat pad, the murine surrogate for the omental pouch, the subcutaneous space, and the kidney capsule as a control. Additionally, we investigated maturation of the hPSC-derived β cells by transplanting these cells into diabetic mice compared to transplantation into non-diabetic mice with diabetic induction following the in vivo maturation period. Following transplantation, the blood glucose levels, body weight, levels of human C-peptide levels in circulation, and responses to glucose challenges were measured. The results from these studies provided insights into maturation and function of hPSC-derived β cells following transplantation, which in turn inform the development long term β cell replacement therapies for patients.

3.2. Methods

3.2.1 Fabricating Microporous PLG Scaffolds

Microporous scaffolds were fabricated as previously described. Briefly, PLG microporous scaffolds were fabricated by compression molding PLG microspheres (75:25 mole ratio D,L-lactide to glycolide) and 500-600 micron-sized salt crystals in a 1:30 ratio of PLG microspheres to salt. The mixture was humidified in an incubator for 7 min and then thoroughly mixed again.

Scaffolds were compression molded with 77.5 mg of polymer–salt mixture into cylinders 5 mm in diameter by 2 mm in height using a 5 mm KBr die (International Crystal Laboratories, Garfield, NJ) at 1500 psi for 45s. Molded constructs were gas foamed in 800 psi carbon dioxide for 16 h in a pressure vessel. The vessel was depressurized at a controlled rate for 30 min. On the day of cell seeding, scaffolds were leached in water for 1.5 h, changing the water once after 1 h. Scaffolds were sterilized by submersion in 70% ethanol for 30 seconds and multiple rinses with phosphate buffer solution. Scaffolds were coated with a 1 mg/mL solution of collagen IV for 20 min prior to cell seeding.

3.3. hPSC-Derived β Cells

Pluripotent stem cells were differentiated to immature beta cells (hPSC-derived beta cells) via a 6 stage protocol by the Melton group.[8] Cells were shipped from Dr. Douglas Melton's lab, Harvard University, Cambridge, MA to Dr. Lonnie Shea's lab, University of Michigan, Ann Arbor, Michigan.

3.3.1 qRT-PCR Analysis

For gene expression analysis, cell containing scaffolds were analyzed prior to transplant, and following removal of the graft. Cell-laden scaffolds were mechanically homogenized in a tube containing Trizol® reagent (Life Technologies), and RNA was isolated according to the manufacturer's instructions. RNA concentration was determined using a NanoDrop spectrophotometer. The iScript™ Reverse Transcription Supermix was used to transcribe RNA into cDNA. SYBR Green dye was used to detect fluorescence. The amplification profile was assessed using a LightCycler® 480 (Roche,

Germany). Gene expression was quantified using the ΔΔCt method and fold change was calculated using the formula $2^{-\Delta\Delta Ct}$. Values for the genes of interest were normalized to the housekeeping

gene (GAPDH) followed by normalization to marker expression in pluripotent hPSCs. Primers used for qPCR analysis are listed in **Table 3.1**.

Table 3.1. Primers of pancreatic differentiation markers used for qRT-PCR

gene	primer	sequence (5' to 3')
PDX1	forward	CCTTTCCCATGGATGAAGTC
PDX1	reverse	CGTCCGCTTGTTCTCCTC
NKX6.1	forward	GGGGATGACAGAGAGTCAGG
NKX6.1	reverse	CGAGTCCTGCTTCTTCTTGG
MAFA	forward	GAGAGCGAGAAGTGCCAACT
MAFA	reverse	TTCTCCTTGTACAGGTCCCG
MAFB	forward	CATAGAGAACGTGGCAGCAA
MAFB	reverse	ATGCCCGGAACTTTTTCTTT
INS	forward	TTCTACACACCCAAGACCCG
INS	reverse	CAATGCCACGCTTCTGC
GLUC	forward	TGCTCTCTCTTCACCTGCTCT
GLUC	reverse	AGCTGCCTTGTACCAGCATT
ECAD	forward	TTGACGCCGAGAGCTACAC
ECAD	reverse	GACCGGTGCAATCTTCAAA
PCSK1	forward	CTCTGGCTGCTGGCATCT
PCSK1	reverse	CGGGTCATACTCAGAGGTCC
PCSK2	forward	TGCAAAGGCCAAGAGAAGAC
PCSK2	reverse	TTTCGGTCAAATCCTTCCTG
G6PC2	forward	TGGTATGTCATGGTAACCGC
G6PC2	reverse	CACTCCAAAGAAATGACCAGG
ABCC8	forward	AAGGAGATGACCAGCCTCAG
ABCC8	reverse	GCCCACGAAAGTTATGAGGA
SLC30A8	forward	CCAAGATGCATGCTTTCACA
SLC30A8	reverse	CTGACTCCAGCTCCTCTGGT
GAPDH	forward	AAGGTGAAGGTCGGAGTCAA
GAPDH	reverse	AATGAAGGGGTCATTGATGG

3.3.2 Streptozotocin Induced Diabetic Mouse Model

Male NSG mice (Jackson Laboratories, Bar Harbor, ME, United States) between 8 and 12 weeks of age were induced diabetic 7 days prior to transplantation. Mice were fasted for 4-6 hours, and then received an intraperitoneal injection of 140 mg/ kg streptozotocin. (Sigma-Aldrich, St. Louis, MO). Each day leading up to surgery, blood glucose levels were measured using the tail vein prick technique (Accu-chek/Roche, Basel, Switzerland), and body weight was recorded. The criteria to include a mouse in the study was the following: glucose readings above 350 mg/ dL for 2 consecutive days leading up to transplant surgeries, and no more than a 20% reduction in weight since streptozotocin injection. Following surgery, glucose and body weight for each mouse were

measured three times per week. All studies involving mice were approved by the University of Michigan Animal Care and Use Committee.

3.3.3 Transplantation of hPSC-Derived β Cells

On the day of surgery, scaffolds were leached in water for 1.5 h, changing the water once after 1 h. Scaffolds were sterilized by submersion in 70% ethanol for 30 seconds and multiple rinses with phosphate buffer solution. hPSC-derived β cell clusters were seeded onto both sides of collagen IV coated PLG scaffolds prior to implantation into the peritoneal at pad or the subcutaneous space. Mice received either a dose of 2000 clusters, equating to a dose concentration of 2,564 clusters/cm^2, or 4000 clusters, equating to a dose of 5,128 clusters/cm^2.

To test the effect of hyperglycemia on hPSC-derived β maturation, one cohort of mice was induced diabetic with streptozotocin prior to transplantation, and another cohort of mice were left normoglycemic until receiving a treatment of streptozotocin 12 weeks after transplantation. These two cohorts were transplanted with hPSC-derived β cells on PLG scaffolds into the peritoneal fat pad at a concentration of 2,564 clusters/cm^2.

Mice were injected with 0.005 mg/gram body weight Rimadyl carprofen (Zoetis, Florham Park, NJ, United States). Mice were anesthetized with 2% isoflurane until loss of consciousness was confirmed by pinch reflex test. The abdominal area was shaved and sterilized with betadine and ethanol. An approximate 5 mm incision was made in the peritoneal wall, in the middle of the abdominal region, and the epididymal fat pads were located and unwrapped outside of the abdominal wall. Scaffolds were wrapped within the epididymal fat pad, then placed back into the peritoneal cavity. Kidney capsule transplantation was adapted from a previously published report.[8] 1000 clusters were pelleted and injected into the kidney capsule. Mice were given a second dose of carprofen 24 hours following surgery.

3.3.4 Intraperitoneal Glucose Tolerance Test and Human C-peptide Analysis

Mice were fasted for 4 hours to control for variability caused by spontaneous feeding. Following the fast, an injection of a 2g/kg of D-glucose in Ringer's Buffer solution was administered into the intraperitoneal cavity. Blood was collected from the saphenous vein between 30-40 minutes post-injection, and processed to isolate the serum for human C-peptide analysis (Alpco Ltd, 80CPTHUE01.1). An intraperitoneal glucose tolerance test (IPGTT) was performed at day 126 (week 18). Blood glucose was measured with a glucometer following a prick of the lateral tail vein, before injection of 2g/kg of D-glucose in saline solution t=0, and at 15 min, 30min, 45 min, 60 min, and 120 min post-injection.

3.3.5 Immunohistological Staining

Immunostaining of in vivo cell differentiation was performed on explanted tissue samples. Explanted tissue samples were cryopreserved in isopentane cooled on dry ice and then embedded within OCT embedding medium and cryo-sectioned. Sectioned tissue samples were fixed using 4% paraformaldehyde (Electron Microscopy Sciences; Hatfield, PA, United States), permeabilized using 0.5% Triton in tris-buffered saline, blocked using normal donkey serum, and stained insulin, human mitochondria, and cell nuclei were counterstained with DAPI. Digital images were acquired with a MicroFire digital camera (Optronics, Goleta, CA) connected to an Olympus BX-41 fluorescence microscope (Olympus, Center Valley, PA, United States). Image quantification was conducted using FIJI Image J open-source software using an object-based colocalization analysis.

3.3.6 Statistics

Statistical analysis was performed in PRISM (Graphpad Software, Inc.). Results were presented as mean ± standard error of the mean (SEM). Statistical significance was indicated on

plots by showing P values using the following notation: *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001. IPGTT and C-peptide figures were analyzed via 2-Way repeated measures ANOVA with Bonferroni multiple comparisons correction to compare two group's means at each timepoint.

PCR plots were analyzed by 1-Way ANOVA for each gene, followed by multiple comparisons with Tukey correction. Errors bars shown as standard error of the mean (SEM).

3.4. Results

Initial studies investigated the transplantation of the stem cell derived beta cells on PLG microporous scaffolds into the epididymal fat pad, with transplantation into the kidney capsule serving as a control. The kidney capsule transplantation delivered 1000 clusters, whereas transplantation on the scaffolds involved two scaffolds delivered each with 2000 clusters. For an individual mouse to be included in the diabetes reversal group, it needed to first show three consecutive readings below 250mg/dL. Graft failure was declared if a mouse who had reversed later showed two consecutive readings above 250mg/dL graft failure. Glucose remained elevated for approximately 3 months after transplantation, with normoglycemia achieved at day 151 for mice within the kidney capsule cohort and day 143 for mice within the fat pad cohort. Survival explant was performed at day 160 for the fat pad cohort, and the increase in blood glucose indicated the graft was responsible for the observed glucose control (Fig 3.1A). The weight declined during the initial two weeks following diabetes induction, yet the weight increased gradually and surpassed the weights measured prior to treatment with streptozotocin (Fig 3.1B). At week 18, the intraperitoneal glucose tolerance test for the kidney capsule and scaffold transplantation demonstrated similar blood glucose dynamics, with a return of glucose levels to normoglycemia by the 2-hour mark (Fig 3.1C). Finally, we assessed serum human C-peptide levels within

circulation, which indicated similar levels for transplantation to both the kidney capsule and the epididymal fat pad (Fig 3.1D). Collectively, these studies demonstrate that transplantation into the epididymal fat pad using microporous scaffolds could induce normoglycemia and allow for high insulin distribution, though the efficiency of the fat pad transplantation is lower than the kidney capsule as greater numbers of clusters were delivered to the fat pad.

hPSC-derived β cells were next transplanted into the subcutaneous space at a density of 2000 clusters on each of two scaffolds. Glucose was observed to rise following treatment with streptozotocin, and the mice remained hyperglycemic throughout the entire study (Fig 3.2A). Body weight was observed to drop during the initial period following treatment with streptozotocin, and gradually increase, although the weight never returned to pre-streptozotocin levels (Fig 3.2B). At week 18, the IPGGT showed a lack of glucose control as shown by the failure to achieve a normoglycemic reading after 2-hours (Fig 3.2C). Serum C-peptide levels started at 54 pM at week 2, and gradually increased to 308 pM by week 18 (Fig 3.2D). These amounts were significantly lower than the amounts observed from the peritoneal fat pad and kidney capsule.

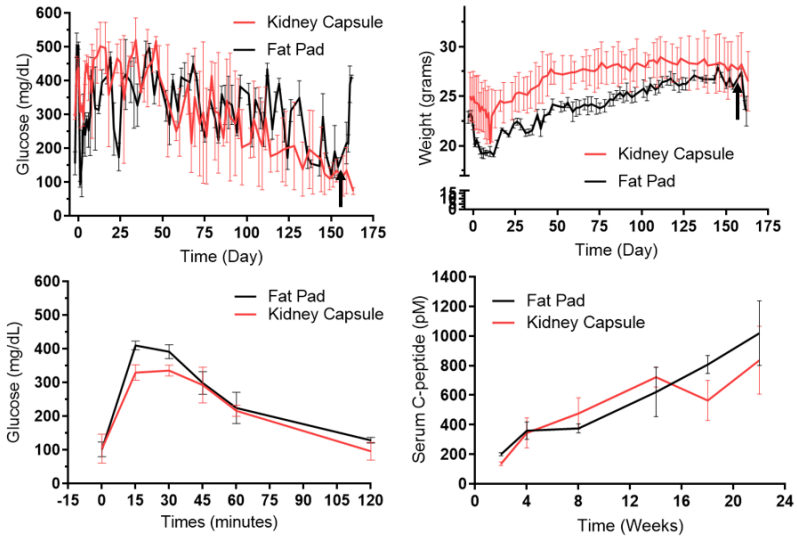

Figure 3.1. Comparison of In Vivo graft function between fat pad and kidney capsule: A) blood glucose, B) weight, C) intraperitoneal glucose tolerance test, and D) serum human C-peptide. No significant differences in C-peptide or IPGTT, as determined by comparing the cohort's means across timepoints via 2-Way repeated measures ANOVA with Bonferroni multiple comparisons.

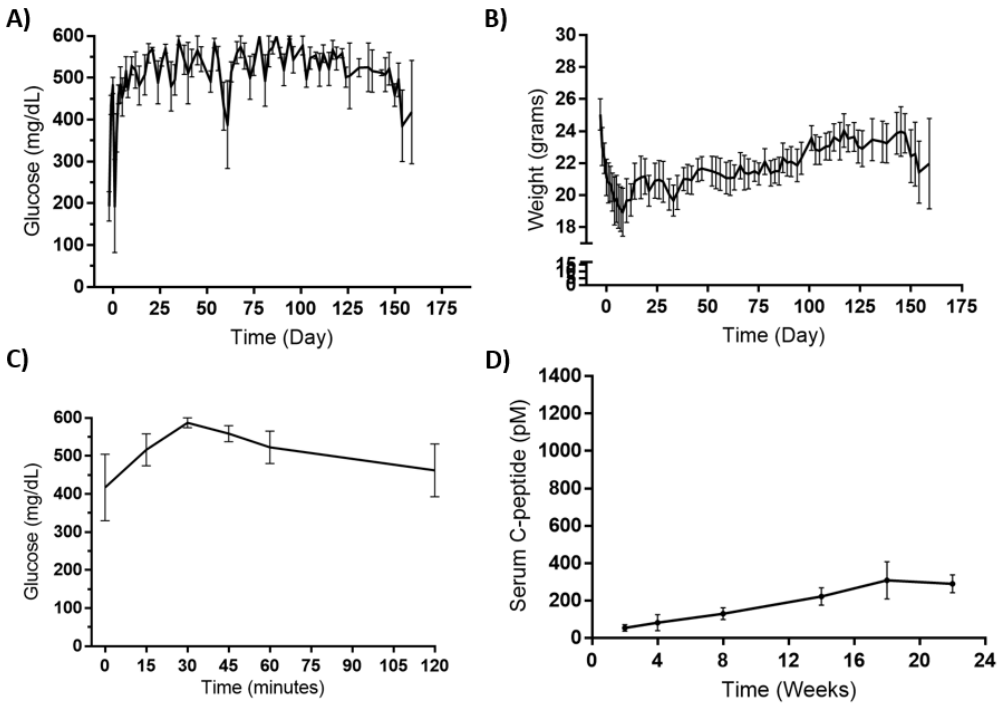

Figure 3.2. Graft function from subcutaneous implantation: A) blood glucose, B) weight, C) intraperitoneal glucose tolerance test, and D) serum human C-peptide.

We next investigated the maturation of stem cell derived beta cells following transplantation by analyzing gene expression by the beta cells before transplantation and following retrieval at day 160. mRNA was harvested from cell lysate and PCR was employed to characterize expression of a pancreatic panel (Fig 3.3). The panel included genes for transcription factors: pancreatic and duodenal homeobox 1 (PDX1), NK6 homeobox 1 (NKX6.1), v-maf avian musculoaponeurotic fibrosarcoma oncogene homolog A and B (MAFA, MAFB); islet hormones: Insulin, Glucagon; genes involved in glucose processing and insulin section: Proprotein Convertase Subtilisin/Kexin Types 1 and 2 (PCSK1, PCSK2), islet-specific glucose-6-phosphatase catalytic subunit-related protein (G6PC2), the sulfonylurea receptor (ABCC8), cation efflux transporter ZNT8 (SLC30A8); and cell surface adhesion protein epithelial cadherin (ECAD). PDX1 expression significantly increased following maturation within the kidney capsule, and the kidney capsule expression for PDX1 was higher than the expression from the fat pad and the subcutaneous site. Insulin

expression was significantly increased after maturation in all three sites. Interestingly, the subcutaneous site had the highest expression for the pancreatic hormones insulin and glucagon, despite not providing control over blood glucose. In addition, the subcutaneous site showed enhanced expression for PCSK2, G6PC2, and SLC30A8 following in vivo maturation. MAFA was found to have higher expression following maturation in the kidney capsule. No significant differences were detected for NKX6.1, MAFB, PCSK1, ECAD, or ABCC8.

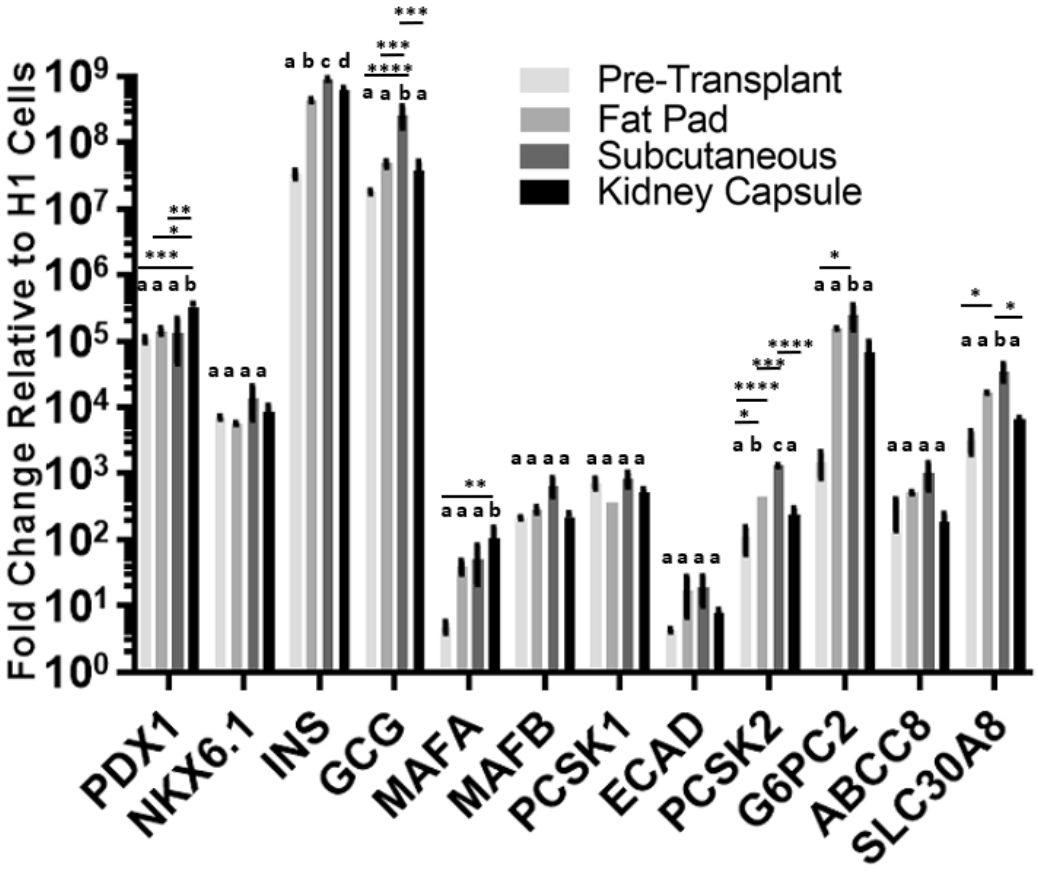

Figure 3.3. Changes in hPSC-derived β cell gene expression following 160 day maturation period in fat pad, subcutaneous site, and kidney capsule. Gene expression was analyzed via PCR. Values were normalized to the house keeping gene GAPDH and shown relative to pluripotent H1 expression. **P*<0.05, ***P*<0.01, ****P*<0.001, *****P*<0.0001.

We subsequently investigated whether the diabetes status of the host was a potential factor that influence the function of the transplanted cells for transplantation into the peritoneal fat pad. Mice were either treated with streptozotocin to induce diabetes or left untreated, and each cohort

received two scaffolds loaded with 1000 clusters/scaffold transplanted into the peritoneal fat pad. In the cohort that was diabetic at the time of transplantation, blood glucose levels were initially hyperglycemic, and gradually reduced until achieving normoglycemia at approximately day 200. The mice that were not diabetic at the time of transplantation were treated with streptozotocin on day 84, and glucose numbers were observed to briefly fluctuate, and then followed a similar trend as the cohort that were diabetic at the time of transplantation (Fig 3.5A). For the cohort that was diabetic at transplant, body weight was observed to rise and surpass pre-streptozotocin weight measurements (Fig 3.5B). The non-diabetic cohort had a similar body weight by the end of the study. The assessment of function of the transplanted cells by IPGTT at week 18 indicated a similar response for both cohorts (Fig 3.5C). The return to normoglycemic glucose levels following glucose challenge demonstrated robust glucose control from the fat pad grafts with a return to normoglycemia within 2 hours. Analysis of serum showed that human C-peptide levels for both cohorts increased through week 14 and declined by approximately 25% afterwards (Fig 3.5D). Interestingly, the cohort that were diabetic at the time of transplantation had significantly more C-peptide at weeks 10 and 14, yet these differences were not significant at weeks 18 and 22.

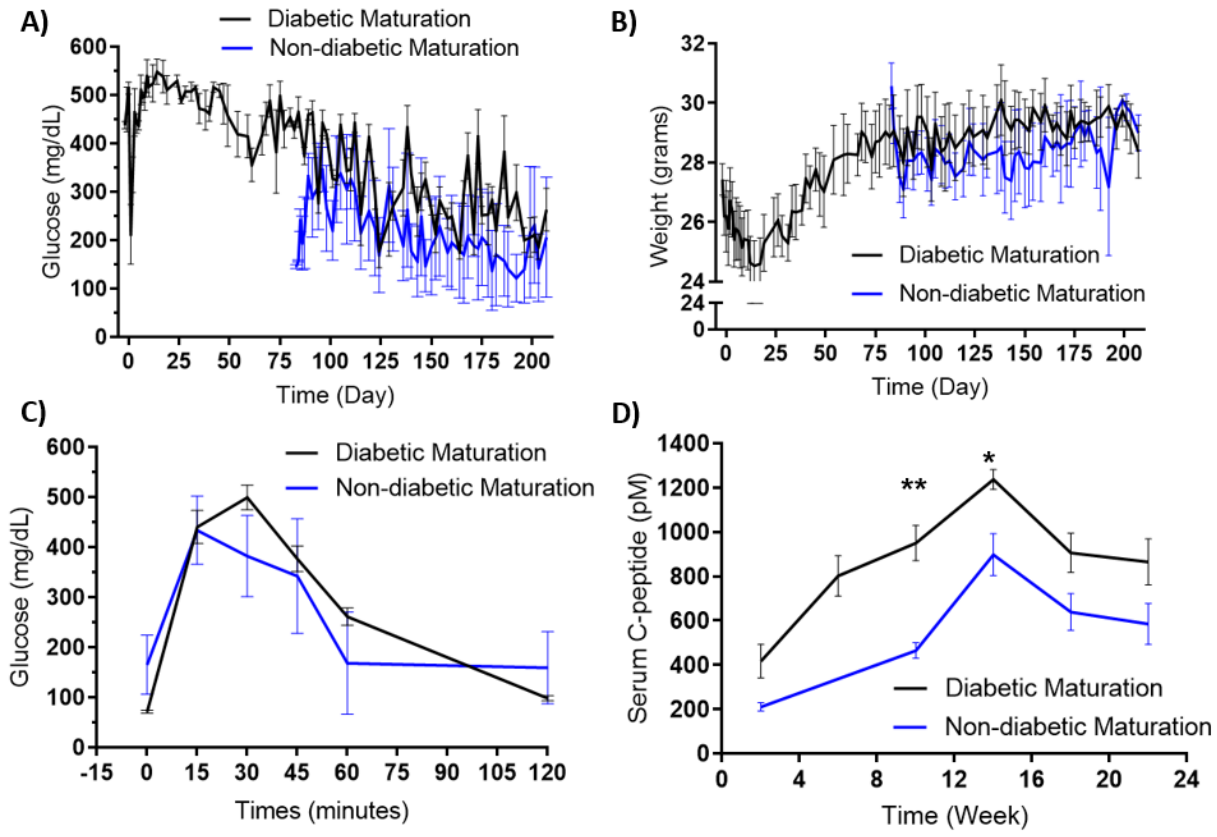

Figure 3.4. Comparison of graft function from the fat pad in grafts exposed to diabetic or non-diabetic environments: A) blood glucose, B) weight, C) intraperitoneal glucose tolerance test, and D) serum human C-peptide. *P<0.05, **P<0.01, Two-Way repeated measures ANOVA with Bonferroni test multiple comparisons was used to compare the cohort's means at each timepoint.

Gene expression profiling was performed on cells prior to transplantation and following graft retrieval to assess changes in maturation of the cells. In the diabetic cohort, significant differences in expression were observed pre- and post- transplantation for PDX1, insulin, and glucagon. The cohort with non-diabetic maturation, significant changes in expression were detected for PDX1, NKX6.1, INS, MAFA, ECAD, and G6PC2. Interestingly, the cohort that was not diabetic at transplantation had significantly higher expression for NKX6.1, insulin, glucagon, and G6PC2 compared to the cohort that was diabetic at transplantation, suggesting that the environment associated with diabetes may impact beta cell maturation. We did not detect significant changes in expression for MAFB, PCSK1, PCSK2, ABCC8, or SLC30A8.

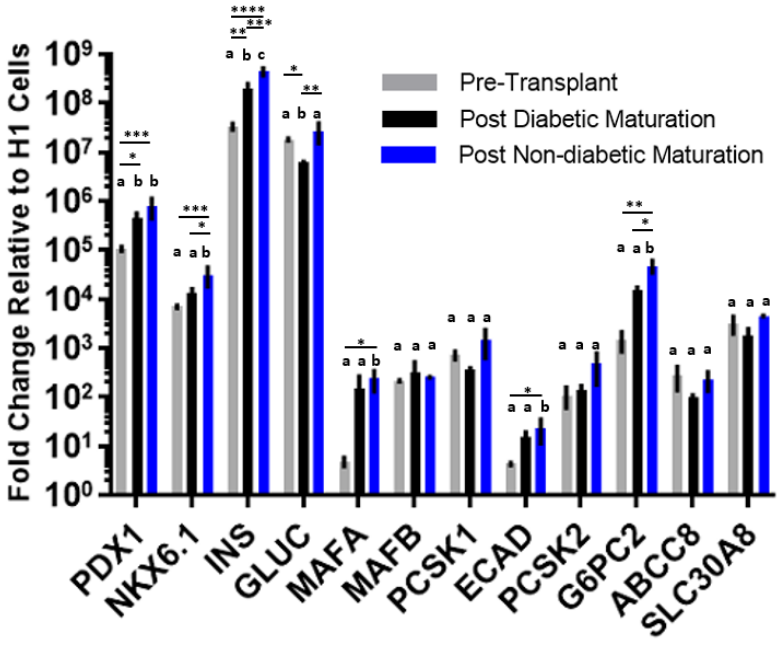

Figure 3.5. Changes in hPSC-derived β cell gene expression following 210 days of In Vivo maturation, with 12-week period of diabetic or non-diabetic environment exposure: Gene expression was analyzed via PCR. Values were normalized to the house keeping gene GAPDH and shown relative to pluripotent H1 expression. **P*<0.05, ***P*<0.01.

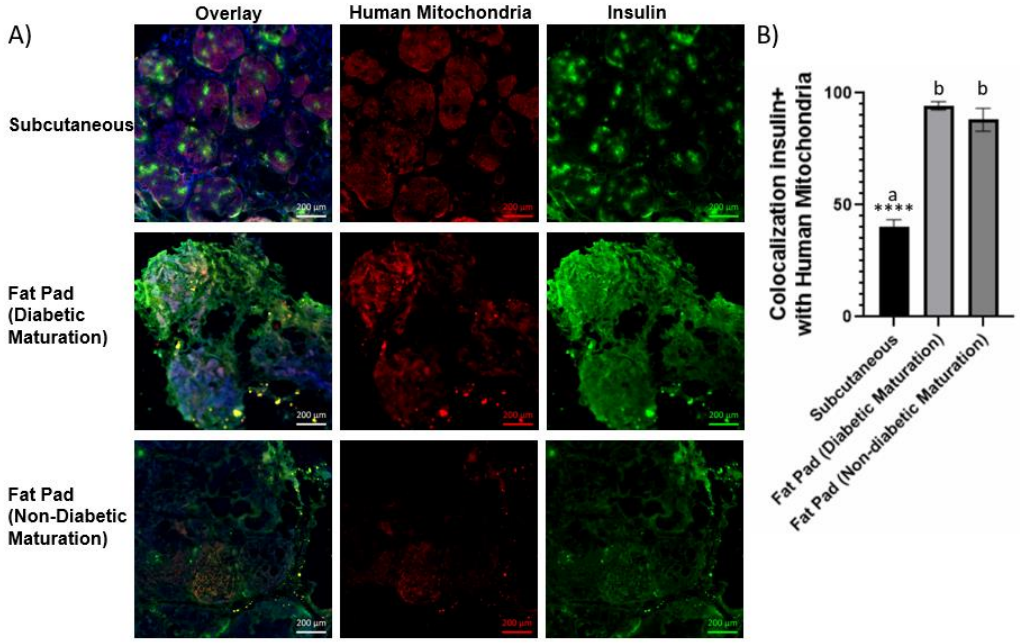

Figure 3.6. Immunofluorescent staining of surviving cells: A) Explanted tissue from cohorts that received subcutaneous, fat pad (diabetic maturation), or fat pad (non-diabetic maturation) scaffolds, was sectioned and stained for insulin (green), human-mitochondria (red). B) The images were analyzed for colocalization of human mitochondria and insulin. The Fat Pad cohorts (diabetic and non-diabetic maturation) both showed significantly higher percentage of insulin positive human mitochondria area compared to subcutaneous stained tissue. The diabetic and non-diabetic maturation cohorts did not show a significant difference in the percentage of insulin positive human mitochondria area.

3.5. Discussion

hPSC-derived β cells were transplanted on microporous PLG scaffolds into clinically relevant sites to study their function and maturation within extrahepatic sites. These studies utilized porous, non-encapsulating scaffolds that allow for integration of the therapeutic cells into the host tissue rather than a more traditional encapsulation strategy.[18,19] This integration of therapeutic cells into the host tissue is hypothesized to allow for rapid sensing of glucose and distribution of insulin. Embedding the scaffold within the fat pad reduces the leakage of clusters into the peritoneum that has been shown in studies delivering free clusters to the site[6]. Importantly, we report that hPSC-derived β cells could be supported within the extrahepatic site of the peritoneal fat pads using microporous scaffolds, and achieved normoglycemia, following a period of maturation in vivo.

Transplanted cells induced normoglycemia by week 20 for transplantation on the scaffolds, which was similar to the timing observed with kidney capsule transplant, a site that is commonly used for islet transplantation and had been employed to test hPSC-derived β cell function.[5,7,8,11,13] The duration between transplantation and normoglycemia was longer than what has been demonstrated in recent differentiation protocols[7,10]. A contributing factor may have been the shipment of the cells, which may have impacted cell viability and motivated the comparison of transplantation between peritoneal fat and kidney capsule. Blood glucose levels were initially hyperglycemic and gradually decreased to normoglycemia over 20 weeks. Body weight gradually increased over 10 weeks. At week 18, IPGTT for cells transplanted to the kidney capsule or peritoneal fat had similar response to glucose challenge. Additionally, circulating C-peptide was 807 pM at week 18, similar to what was observed in Rezania el al 2014[6].

Subcutaneously transplanted grafts did not yield glucose control or significant insulin secretion into the blood stream as indicated by low serum C-peptide levels. Our results are consistent with

other studies that have compared transplantation between the epididymal fat pad and subcutaneous space. Islets were transplanted on pro-angiogenic fibrin matrices into either the epididymal fat pad or the subcutaneous space, the epididymal fat pad cohort demonstrated reduced duration to diabetes reversal.[20] Cell survival at the subcutaneous space was lower relative to that observed with the peritoneal fat. The subcutaneous space may require strategies to enhance vascularization to improve graft survival.

This study indicates that these immature hPSC-derived β cells continue to mature post transplantation. At week 4, blood glucose levels were elevated and C-peptide levels were 360 pM, both of which had significantly improved by week 18. Multiple changes in gene expression were observed following the in vivo engraftment period. The genes MAFA, NKX6.1, PDX1, PCSK1, PCSK2, SLC30A8, and G6PC2 have been shown to be differentially overexpressed in adult beta cells compared to hPSC-derived insulin producing cells.[4] Interestingly, the kidney capsule cohort showed higher MAFA expression than the others, suggesting the kidney capsule microenvironment may have valuable maturation cues for beta cells. MAFB and ABCC8 were not differentially expressed between in vivo matured beta or hPSC-derived beta cells, consistent with previous reports comparing hPSC-derived beta cells and adult islets[4]. Interestingly, the subcutaneous transplantation exhibited the highest gene expression for insulin, glucagon, PCSK2, G6PC2, and SLC30A8, yet had the worst functional outcome that our histological analysis indicated an overall lower survival rate for the subcutaneous site.

Our results indicate that the process of in vivo maturation was not significantly impacted by the diabetes status of the recipient. The grafts exhibited similar functional outcomes following maturation in the diabetic and normoglycemic environments as shown by the blood glucose, weight changes, IPGTT, and serum C-peptide. The majority of animal models evaluating hPSC-

derived beta cell transplantation use a hyperglycemic model, in which newly transplanted cells are exposed to high blood glucose levels until diabetes can be reversed. The potential impact blood glucose levels can have on post-transplantation maturation motivated us to examine how hPSC-derived beta cells mature and function within the peritoneal fat pad in the presence of a hyperglycemic or normoglycemic environment. While function was comparable between the two environments, gene expression for NKX6.1, insulin, glucagon, and G6PC2 was significantly greater in the cohort whose grafts matured in a non-diabetic environment. A recent study examined the impact that blood glucose levels may have on beta cell phenotype. They found that increased blood glucose levels following partial pancreatectomy had a measurable impact on beta cell regulation, including downregulation of important beta cell transcription factors, downregulation of genes associated with insulin granule trafficking, and changes to genes transcribed by mitochondrial DNA.[21] This result supports that persistent hyperglycemia can have a detrimental impact on beta phenotype. In contrast, a recent study highlighted how fluctuating glucose levels can promote immature beta cells to mature towards a more glucose responsive adult stage.[22] Fetal beta cells have amino acid induced insulin secretion, whereas the postnatal nutrient environment regulates mTOC1, which in turn triggers beta cells to shift to more glucose induced insulin secretion. The non-diabetic environment, which has more physiologic fluctuations in glucose levels between meals, may have beneficially impacted beta cell maturation when compared to grafts exposed to consistently high glucose for 12 weeks. These insights into maturation highlight that blood glucose control may be an important consideration for translation of beta cell replacement therapy for patients with T1D.

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Chapter 4. Enhanced pancreatic endocrine induction of pancreatic progenitors following culture within microporous scaffolds

Contributions. Daniel Clough led these studies, and cultured suspension culture and suspension seeded scaffolds. Richard Youngblood originally initiated these studies using suspension seeded scaffolds. Elizabeth Bealer and Kelly Crumley led aspects of the study that involved culture of planar cultured cells and scaffolds seeded with these cells. Jessica King led the adoption of planar culture methods in Shea Lab. Feiran Li performed flow cytometry analysis that was crucial to characterizing progress through differentiation stages. Dr. Lonnie Shea led study design and oversaw all aspects of the studies.

4.1 Introduction

Type 1 diabetes is caused by the autoimmune destruction of insulin-producing β-cells, resulting in a chronic metabolic disorder typically treated with exogenous insulin. Even with the aid of advanced insulin pumps and real time feedback systems, blood glucose levels still deviate outside of the range maintained by native islets, which places the individual at risk for vascular complications and life-threatening hypoglycemic events. Cell replacement therapies have demonstrated the capacity to tightly control blood glucose levels. The wide adoption of cell replacement therapy is hindered by limited availability of donor islets, and the lack of effective methods to support the long-term function of these cells within a clinically accessible site.

The limited supply of donor islets has motivated research into methods for differentiating pancreatic β-cells from renewable pluripotent stem cells. Many of the current protocols

differentiate human pluripotent stem cells (hPSCs) and subsequent developmental stages above a feeder layer in monolayer, or in suspension within a bioreactor. [1–5] The two protocols utilized within this study involved differentiating pluripotent cells through six-stages: pluripotent stage 0 (S0), definitive endoderm (DE) S1, primitive gut tube (PGT) S2, early pancreatic progenitor (PP1) S3, late pancreatic progenitor (PP2) S4, endocrine progenitor (EN) S5, and finally hPSC-derived β-cells (SC-β) S6. Typically, these protocols require the disruption of the cell niche during key differentiation stages or pre-transplantation handling. Biomaterial scaffolds maintain the integrity of cell-to-cell and cell-to-matrix connections by providing both a space for cell niche development as well as a vehicle for transplantation into the body. In developing cell replacement strategies for T1D, the manufacturing of the cell product is critical to its ultimate function following transplantation. Cells are cultured to the final in vitro stage either in suspension [6], or as a monolayer [5]. During these cultures, the cells spontaneously form into clusters, which can be impacted by factors such as the shear rate in a suspension culture.[7]

Biomaterial platforms allow for controlled assembly of cells into tissues.[8] Factors secreted by cells are retained within the tissue niche, and this may support their long-term function. The scaffold culture's supportive niche can be employed at different stages along the differentiation process to drive the cells to become progressively more committed to the final beta cell fate. Differentiating cells receive many signals during maturation, including but not limited to: paracrine factors, cell-to-cell communication, and cell-to-extracellular matrix connections. Cell to cell connections, and cell to ECM connections, are extremely important during stem cell development [9,10], and as a result, the method by which these cells are cultured plays an important role in development.

In this study, we investigated the 3D culture of hPSC-derived progenitors on porous biomaterial scaffolds at multiple stages of differentiation between Stage 0 and 6. We previously reported the feasibility of culturing hPSC-derived pancreatic progenitors (Stage 4) within the pores of three dimensional poly(lactide-co-glycolide) (PLG) scaffolds when seeded as single cells.[11] Herein, we investigate the relative differentiation when seeding the hPSCs from planar culture[5] or as clusters generated from suspension bioreactors[4]. Furthermore, we investigated the maturation within scaffolds following the introduction of progenitors at different developmental stages. Maturation was characterized via gene expression analysis, glucose stimulated insulin secretion assay, and nondestructive microscopy utilizing a sfGFP-C-peptide cell line that reports C-peptide production and secretion.

4.2 Methods

4.2.1 Microporous scaffold fabrication

Poly(lactide-co-glycolide) (PLG) microporous scaffolds were fabricated as previously described.[12] Briefly, PLG microporous scaffolds were fabricated by compression molding PLG microspheres (75:25 mole ratio D,L-lactide to glycolide) and 500 to 600 μm micron-sized salt crystals in a 1:30 ratio of PLG microspheres to salt. The mixture was humidified in an incubator for 7 min and then thoroughly mixed again. Scaffolds were compression molded with 77.5 mg of polymer–salt mixture into cylinders 5 mm in diameter by 2 mm in height using a 5 mm KBr die (International Crystal Laboratories, Garfield, NJ) at 1500 psi for 45s. Molded constructs were gas foamed in 800 psi carbon dioxide for 16 h in a pressure vessel. The vessel was depressurized at a controlled rate for 30 min. On the day of cell seeding, scaffolds were leached in water for 1.5 h, changing the water once after 1 h. Scaffolds were disinfected by submersion in 70% ethanol for 30 seconds and rinsed multiple times with phosphate buffer solution (PBS).

4.2.2 Designing sfGFP-Cpep Fluorescent Insulin Reporter hPSC line

Briefly, HEK293FT cells were co-transfected using lentiviral packaging vectors (pMDL-GagPol, pRSV-Rev, pIVS-VSV-G, with a CpepSfGFP construct [13,14] using Lipofectamine 2000 (Life Technologies, Grand Island, NY,) for 48 h. The CpepSfGFP construct (i.e., with the Superfolder-GFP cDNA [15] ligated into the XhoI site of the human C-peptide coding sequence[14]) driven by the upstream 2.2-kb rat Ins1 promoter included a phosphoglycerol kinase (PGK)-promoter-mCherry selection marker. Using PEG-it (System Biosciences, Mountain View, CA), supernatant was concentrated for 24 h. Then it was precipitated using ultracentrifugation, resuspended in PBS and stored at −80 °C until use. Through viral transfection and FACS sorting, clones can be identified in which the vector was correctly integrated into the cell line. The sfGFP-Cpep cell line was then expanded into a subclonal population and characterized.

4.2.3 Differentiation of hPSCs and Scaffold Seeding

Differentiations were either performed as suspension culture[4] and planar culture[5] as previously described. hPSC differentiations were performed in 30-mL spinner flasks on a stirrer plate set at 60 RPM in a humidified incubator set at 5% CO2 and 37 °C. hPSCs were differentiated to SC-β cells using the protocol previously described[6]. Briefly, undifferentiated cells were single-cell dispersed and seeded at 6 × 105 cells/mL in a 30-mL spinner flask. Cells were cultured for 72 hr in mTeSR1 and then cultured in the differentiation media for 6 stages. To initiate scaffold culture differentiations, Stg5 pancreatic progenitor clusters were seeded as clusters on scaffolds at 125 million cells/cm3 then further differentiated according to the protocol[11]. Prior to cluster seeding, scaffolds were washed in cell media solution then briefly dried on sterile gauze to improve the absorption of the cell solution into the scaffold. Clusters were distributed across both faces of

the scaffold and then incubated for 10min to allow cell solution to be further absorbed into the scaffold before differentiation media was added.

4.2.4 qRT-PCR Analysis

Gene expression analysis on fixed, sorted cells was obtained by first centrifugation of the cell pellet at 60 g for 1min at 4°C. The supernatant was discarded. Total RNA was isolated from the pellet using the RecoverAll Total Nucleic Acid Isolation kit (Ambion), starting at the protease digestion stage of manufacturer recommended protocol. The following modification to the isolation procedure was made: instead of incubating cells in digestion buffer for 15 minutes at 50°C and 15 minutes at 80°C, we carried out the incubation for 1 hour at 50°C. RNA concentration was determined using a NanoDrop spectrophotometer. The iScript™ Reverse Transcription Supermix was used to transcribe RNA into cDNA. Universal RT microRNA PCR assays were performed using SYBR Green MasterMix Universal RT (Exiqon), according to the manufacturer's instructions. The amplification profile was assessed using a LightCycler® 480 (Roche, Germany). Gene expression was quantified using the ΔΔCt method and fold change was calculated using the formula 2-ΔΔCt. Values for the genes of interest were normalized to the housekeeping gene (GAPDH) followed by normalization to marker expression in pluripotent hPSCs. Primers used for qPCR analysis are listed in Table 2.

4.2.5 Statistics

All statistical analyses were conducted using Prism graphing and data analysis software (GraphPad Software, Inc., La Jolla, CA, United States). Statistical differences were determined using a two-tailed Student t test or one-way ANOVA with Dunnett test for multiple comparisons. Values were reported as the mean ± standard error of mean (SEM). n indicates the total number of biological replicates.

4.3 Results

4.3.1 Pre-Pancreatic Progenitor Scaffold Culture

We initially investigated the seeding and culture of suspension clusters at the earliest stages of development. Pluripotent clusters, stage 0, were cultured in suspension bioreactors. These pluripotent clusters were seeded into scaffolds for the first day of Stage 1, definitive endoderm initiation. Following the completion of stage 1, cell lysate was collected for gene expression analysis. The gene panel for this stage consisted of the pluripotency marker: OCT4, and definitive endoderm markers SOX17, FOXA2, cerberus (CER), Chemokine [C-X-C motif] receptor 4 (CXCR4), and goosecoid (GSC). No significant differences were detected for OCT4, SOX17, FOXA2, or GSC, however, CER and CXCR4 showed significantly higher expression for suspension culture relative to scaffold culture (Figure 4.1B). Following completion of stage 4 culture, the pancreatic progenitor stage, gene expression analysis was performed for the transcription factor of pancreatic commitment PDX1, the maturing pancreatic transcription factor NKX6.1, the beta cell hormone insulin (INS), the alpha cell hormone glucagon (GLUC), the endocrine precursor marker transcription factor NEUROD1, bHLH transcription factor Neurogenin3 (NGN3), the gene encoding proprotein convertase-1 involved in pro-insulin processing (PCSK1), the mature maturation marker for beta cells MAFA, and the cell surface adhesion protein E-cadherin (ECAD). Gene expression analysis showed glucagon, PDX1, NKX6.1, MAFA, ECAD, NEUROD1, and NGN3 had reduced expression in scaffold culture relative to suspension culture (Figure 4.1C), which led to studies investigating other stages of culture.

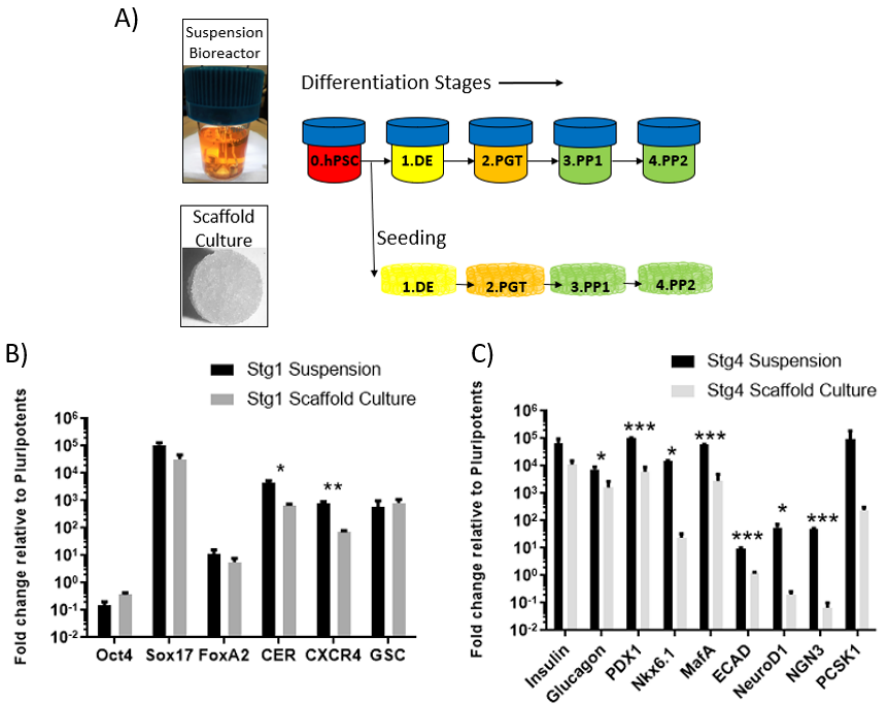

Figure 4.1. Early Stage (S0) Cluster Seeding On Scaffolds: (A)Schematic of seeding timing and analysis. (B) Clusters were cultured in suspension, or seeded onto scaffolds prior to stage 1, and cultured to completion of stage 1 definitive endoderm, (C) Clusters were cultured in suspension, or seeded onto scaffolds prior to stage 1, and cultured to completion of stage 4 pancreatic progenitor. Statistical significance was determined based on unpaired T-test for each gene pair *P<0.05, **P<0.01, ***P<0.001.

4.3.2 SC-derived β-Cell Scaffold Culture

We next investigated the seeding of cells from suspension culture and planar culture at the beginning of stage 6 of the differentiation protocol, which corresponds to maturing stem-cell-derived β-cells. Suspension clusters were cultured from pluripotency (stage 0) through endocrine progenitor (stage 5) and seeded into scaffolds at stage 6 day 1. Scaffolds were cultured through stage 6 day 10, and insulin and NKX6.1 were shown to be differentially expressed in suspension relative to scaffold culture (Figure 4.2B).

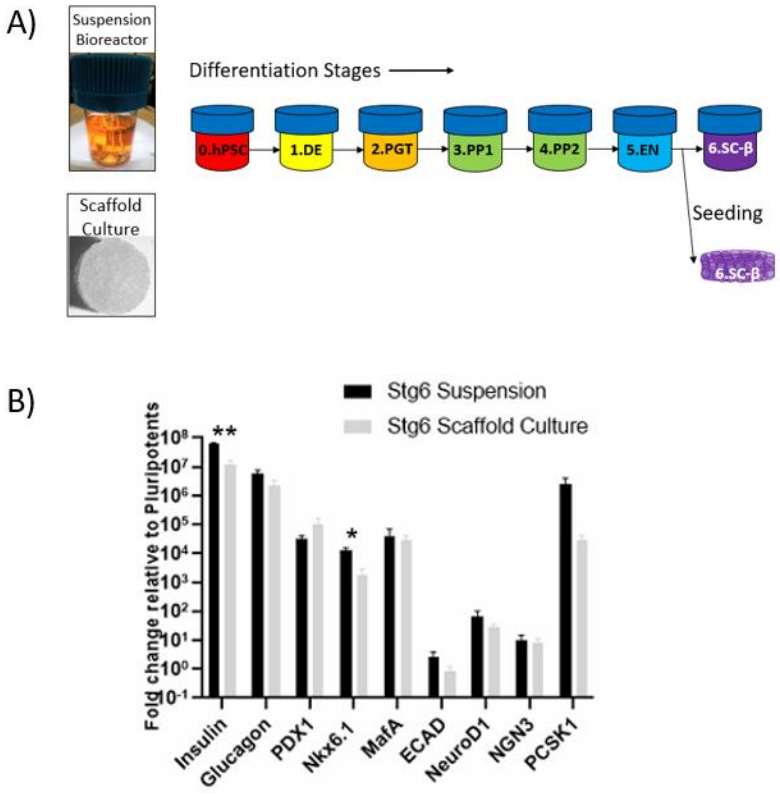

Figure 4.2. Late Stage (Stage 6 Day 1) Cluster Seeding on Scaffolds: (A)Schematic of seeding timing and analysis. Clusters were cultured in suspension, or seeded onto scaffolds prior to stage 6, and cultured to completion of stage 6. (B) Gene expression analysis of end of stage 6 cells cultured in suspension or on scaffolds. Statistical significance was determined based on unpaired T-test for each gene pair *P<0.05, **P<0.01.

4.3.3 Endocrine Progenitor Scaffold Culture

Next, we tested an earlier seeding by introducing pancreatic progenitors into the scaffold prior to the start of the endocrine initiation, stage 5 day 1. Gene expression analysis showed insulin, glucagon, NKX6.1, NEUROD1, NGN3, and PCSK1 had enhanced expression from scaffold cultures relative to suspension culture. (Figure 4.3B).

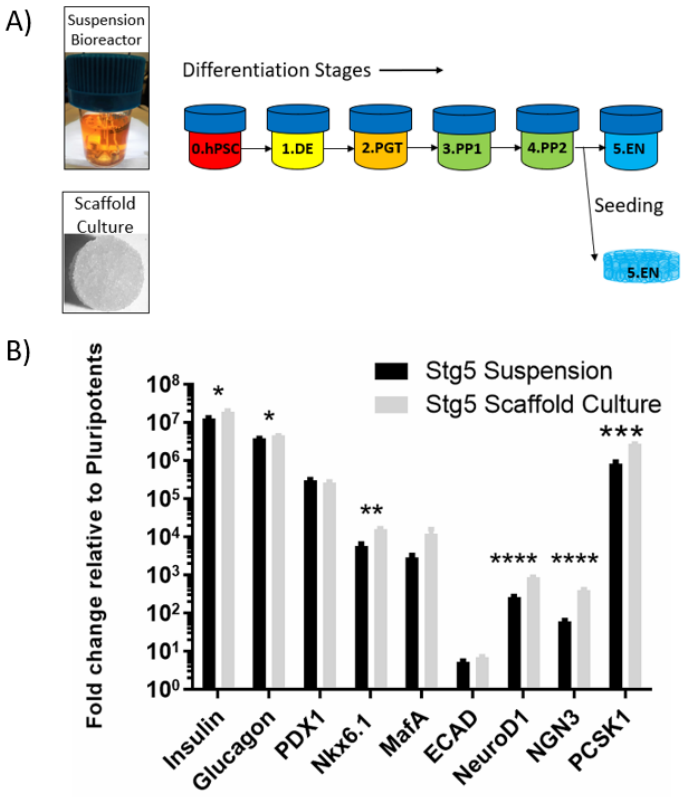

Figure 4.3. Pre-endocrine (Stage 5 Day 1) cluster seeding on scaffolds: (A)Schematic of seeding timing and analysis. Clusters were cultured in suspension, or seeded onto scaffolds prior to stage 5, and cultured to completion of stage 5. (B) Gene expression analysis at Stage 5 Day 7 for cells cultured in suspension or on scaffolds. Statistical significance was determined based on unpaired T-test for each gene pair *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

The non-destructive sfGFP-C-peptide reporter was utilized to track insulin production during suspension and scaffold culture. Signal was first detected during stage 5, and increased in intensity through stage 5 and stage 6 in both suspension and scaffold culture (Figure 4.4A). Following the completion of stage 6 day 10, scaffolds underwent the glucose stimulation insulin secretion assay and the sfGFP signal was quantified. It was observed sfGFP signal dropped during the period following exposure to 20 mM glucose. (Figure 4.4B).

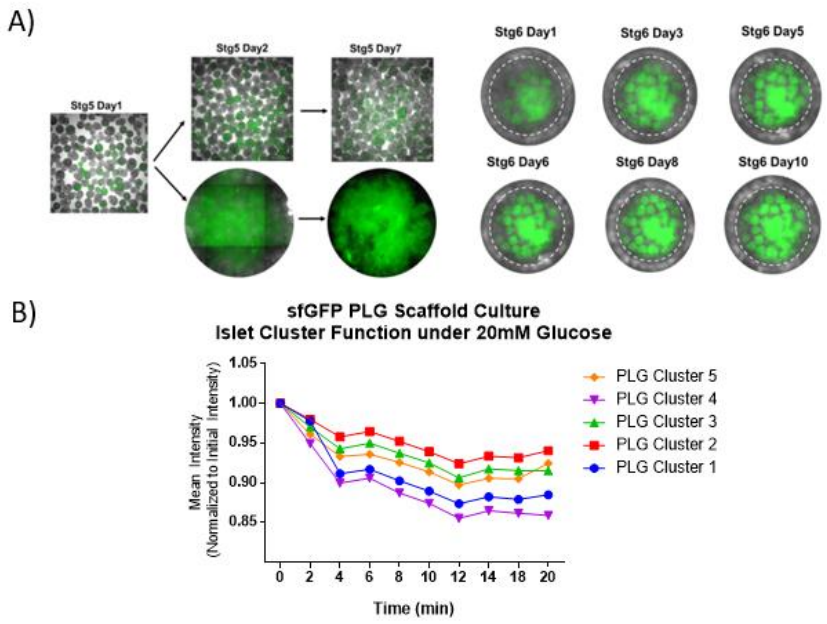

Figure 4.4. sfGFP-C-peptide reporter activity during culture and glucose stimulated insulin secretion: (A) sfGFP-C-peptide reporter signal for suspension clusters and pre-endocrine (Stage 5 Day 1) cluster seeded scaffolds during stage 5 and stage 6. (B) Florescent signal from C-peptide reporter within scaffolds measured during GSIS assay.

4.3.4 Planar Seeded SC-derived β-Cell and Endocrine Progenitor Scaffold Culture

Next, pluripotent cells were cultured according to the planar culture differentiation protocol, and were lifted and seeded into scaffolds at Stage 5 Day 1 and Stage 6 Day 1, and cultured until stage 6 day 10. Significantly enhanced expression for NGN3 was detected for scaffolds seeded at stage 5 Day 1. No other significant differences were detected. (Figure 4.5B).

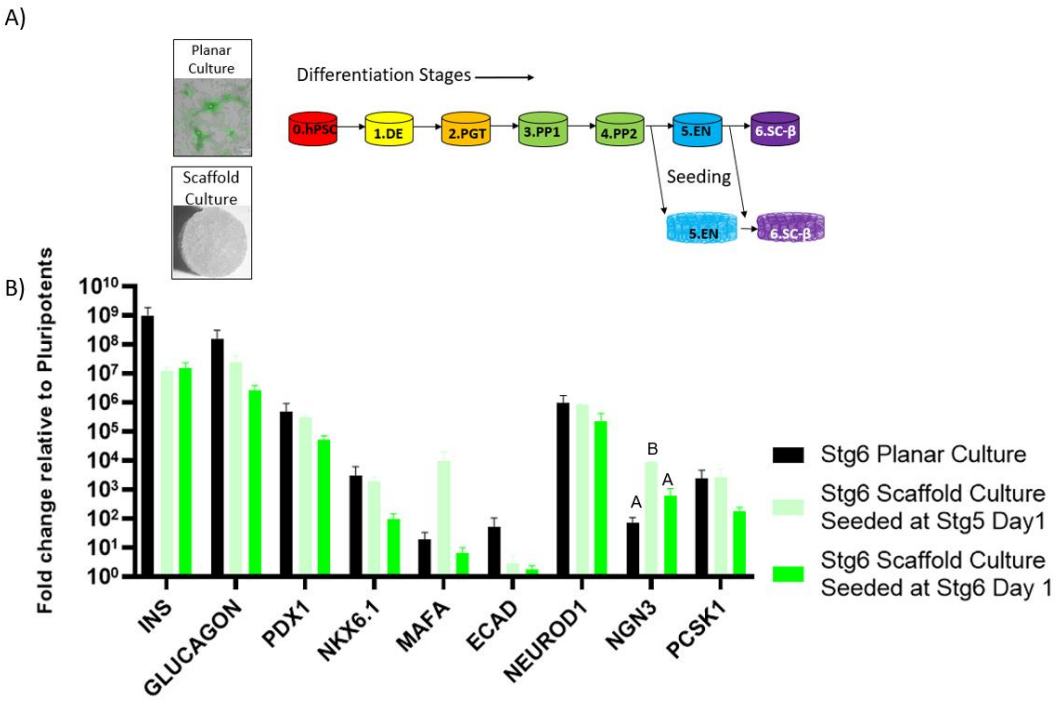

Figure 4.5. Planar culture seeding on scaffolds at Stage 5 Day 1 and Stage 6 Day 1: (A)Schematic of seeding timing and analysis. Clusters were cultured in suspension, or seeded onto scaffolds prior to stage 6, and cultured to completion of stage 6. (B) Gene expression analysis of end of stage 6 cells cultured in suspension or on scaffolds. Statistical significance was determined based 1-Way ANOVA with Tukey Multiple Comparisons. Significance detected in expression for NGN3.

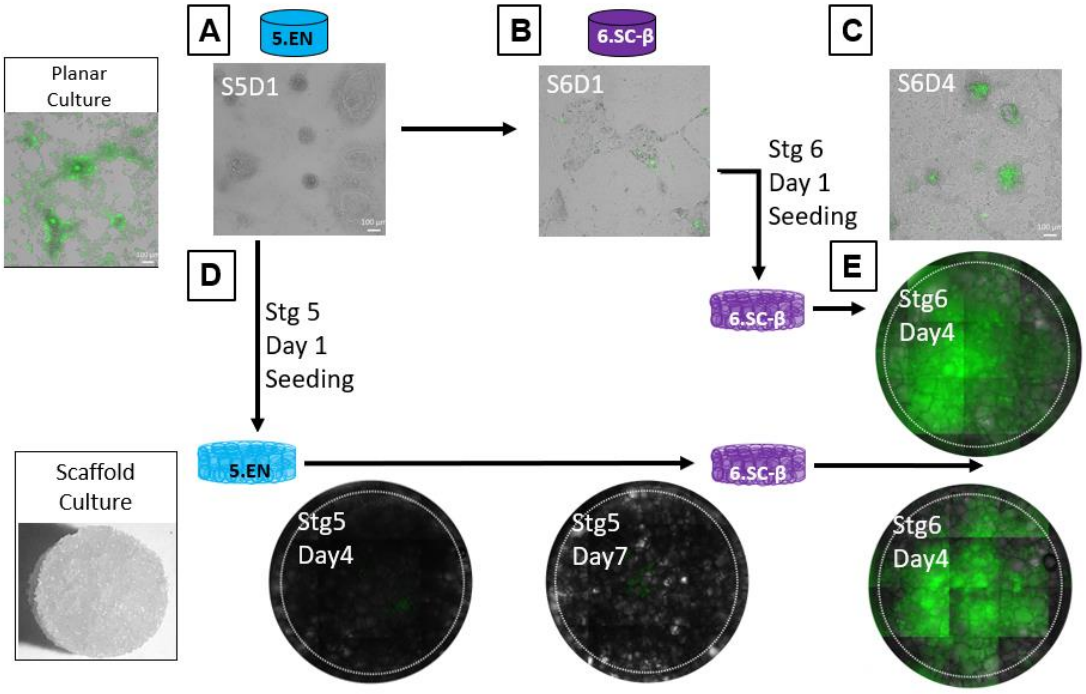

Figure 4.6. sfGFP-C-peptide reporter activity during planar culture, and scaffolds seeded at Stage 5 Day 1 and Stage 6 Day 1: (A) Stage 5 Day 1 Planar Culture, (B) Stage 6 Day 1 planar culture, (C) Stage 6 Day 4 Planar Culture, (D) Scaffold culture, seeded with Stage 5 Day 1 planar culture, at Stage 6 Day 4 and Stage 6 Day 7, (E) Scaffold Culture, either seeded with Stage 5 Day 1 or Stage 6 Day 1, at Stage 6 Day 4.

sfGFP-C-peptide reporter activity was first detected within the planar culture at the beginning of stage 6 (Figure 4.6B). Scaffold cultures were seeded with planar cultured cells at stage 5 Day1 and Stage 6 Day 1, and continued to the end of stage 6 Day 10. By stage 6 day 4, significant sfGFP signal was detected from both planar culture and scaffolds (Figures 4.6 C and E). These results indicate the cells were producing insulin both within the planar culture and within the scaffolds, and that this insulin production could be tracked in real time during the differentiation.

4.4 Discussion

The culture of hPSC-derived progenitors on the scaffolds supports and can enhance maturation of SC derived β-cells depending on the stage of seeding. When clusters were seeded into the scaffold prior to the initiation of definitive endoderm (DE), scaffold cultures showed reduced expression for the DE markers at the end of stage 1, as well as diminished PP markers at the end of stage 4. The early stages in the differentiation protocol, definitive endoderm (stage 1), primitive gut tube (stage 2), and pancreatic progenitor 1 (stage 3) are accompanied by rapid proliferation and changes in cluster morphology.

Clusters that were seeded following pancreatic endocrine development (post-stage 6, pre-stage 6) showed similar gene expression to suspension culture, but insulin and NKX6.1 expression was observed to be higher in suspension. However, when planar cultured cells were seeded at Stage 6 Day 1, no significant gene expression differences were observed between scaffold culture and planar controls. These results indicate the that progress through stem cell derived β-cell stage was promoted within the planar culture seeded scaffolds, but slightly diminished in suspension seeded scaffolds.

The seeding of PP2 stage 4 cells for stage 5 culture pancreatic endocrine culture enhanced maturation relative to planar or suspension culture controls. Gene expression analysis showed insulin, glucagon, NKX6.1, NEUROD1, NGN3, and PCSK1 had enhanced expression from suspension seeded scaffold cultures. sfGFP-C-peptide reporter activity was measured during suspension and scaffold culture. Signal was first detected during stage 5, and increased in intensity through stage 5 and stage 6 in both scaffold culture and suspension conditions. Scaffolds were exposed to 2mM and 20mM glucose during a glucose stimulated secretion (GSIS) assay and sfGFP signal was quantified. The sfGFP-C-peptide is secreted from the cells during insulin secretion, and we observed sfGFP signal dropped following exposure of the cells to elevated glucose levels, showing that the scaffold cultured cells were glucose responsive. When planar cultured cells were seeded into scaffolds prior to stage 5, significantly enhanced expression for NGN3 was detected relative to planar controls. NGN3 is required for commitment to the endocrine fate[16], and this enhanced expression for NGN3 was only detected in scaffolds seeded prior to endocrine stage. sfGFP-C-peptide reporter activity was first detected within the planar culture at the beginning of stage 6. By stage 6 day 4, significant sfGFP signal was detected from both planar culture and scaffolds.

The promising results from this study highlight the potential of biomaterial scaffolds to promote the continued maturation of cells within a 3D PLG scaffold. Importantly, maturation markers were enhanced in both suspension and planar culture seeded scaffolds when seeded prior to stage 5. The nondestructive sfGFP-C-peptide reporter identified real time maturation of cells within the scaffolds, and directly visualized the glucose responsive functionality of the cells during GSIS. These scaffolds provide a structure by which integrity of cell-to-cell and cell-to-matrix connections are not disrupted during niche development. One of the biggest advantages of this

culture system is that these microporous scaffolds are platforms for transplantation. The scaffolds promote 3D tissue formation during the final endocrine and SC-β cell stages, and eliminate disruption of the islet niche prior to delivery into the patient.

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Chapter 5. Conclusions and Future Directions

5.1 Conclusions

The studies presented in this thesis explored strategies for human pluripotent stem cell (hPSC)-derived β cell differentiation, transplantation, and maturation within microporous PLG scaffolds. A majority of islet cell replacement in the clinic has utilized hepatic portal vein infusion, yet this site is harsh on islets and leads to graft failure in the long term. Many of the latest differentiation protocols for production of pancreatic β cells study *in vivo* function using the kidney capsule as the site of transplantation, but the kidney capsule is not widely considered a clinically translatable site. Microporous scaffolds provide the support needed to deliver and contain the therapeutic cells subcutaneously or within the peritoneal fat, both considered good candidates for eventual clinical translation. However, few studies have investigated these sites for hPSC-β cell engraftment. The studies presented in this thesis show that the scaffolds can promote the survival and maturation of hPSC-derived β cells within these sites, with different degrees of overall function. Following *in vitro* production of pancreatic or β cell progenitors, maturation continues *in vivo*. We have shown that the scaffolds are able to provide maturation cues from within the engraftment site. Additionally, these microporous scaffolds have proven useful in *in vitro* manufacturing of the differentiating cells. Scaffold culture, which doubles as a transplantation platform, allows for 3D tissue formation with minimal disruption of the niche prior to transplantation. These studies highlighted exciting ways scaffolds could be employed to manufacture cell products which continue to mature within a clinically promising site. These are

important steps towards the ambitious goal of a widely utilized stem cell-based cell replacement strategy for type I diabetes.

We first tested the ability of microporous scaffolds to promote the *in vivo* maturation of human PSC derived pancreatic progenitors within the clinically translatable site of the epidydimal fat pad, a murine surrogate for the peritoneal fat pad. Initially, transplantation of the progenitor cells into the peritoneal fat did not support maturation and function at levels observed in the kidney capsule. We hypothesized that sustained release of Exendin-4 into the microenvironment would enhance the function of the transplanted cells in the peritoneal fat. The localized, sustained delivery of Exendin-4 did indeed promote the *in vivo* development of PSC derived pancreatic progenitors into mature insulin producing β-cells. This result was demonstrated by levels of C-peptide comparable to those of cells transplanted under the kidney capsule, improved glycemic control compared to that of cells transplanted on scaffolds without exendin-4, and significantly greater percentages of insulin producing cells compared to those in glucagon and polyhormonal cells four and eight weeks following transplantation. These studies provide strong evidence that modification of the microenvironment with a locally delivered trophic factor can enhance the function of transplanted progenitor cells. This proof of concept opens the door to other methods of continuing to influence cell behavior *in vivo*.

The optimal transplantation site and strategy by which to deliver cells is a major question facing T1D cell replacement therapy researchers. We transplanted hPSC-derived β cells into the clinically relevant sites of the peritoneal fat and the subcutaneous space, with the kidney capsule serving as a control. Hyperglycemia was ameliorated in the cohorts of mice that received scaffolds seeded with hPSC--derived β cells transplanted into the peritoneal fat pad, following a period of *in vivo* maturation. The function of these cells was demonstrated by the reduction in blood glucose

levels, healthy increase in weight, therapeutic levels of circulating human insulin, and healthy responses to glucose challenge tests. The function from the clinically translatable peritoneal fat was observed to be comparable to the kidney capsule, although the fat pad required more cells to achieve this functional level. The kidney capsule has been the most frequently used transplantation site for hPSC-derived beta cell function murine models, so it was exciting to see robust survival and function from the peritoneal fat with the support of PLG scaffolds. While the subcutaneous site did not result in normoglycemia within this study, the enhanced maturation supports further studies that can enhance cell survival, such as through enhancing vascularization. Lastly, no differences were observed in graft function between the cohorts whose grafts matured in a diabetic or non-diabetic environment, yet differences in gene expression were present. These studies highlight that stem cell derived beta cells continue to mature following transplantation, and produce strong glucose stimulated insulin secretion from the peritoneal fat. This motivates future work to investigate tissue engineering approaches that support and direct maturation *in vivo*.

Lastly, an important insight from the scaffold culture studies was that the stage in which scaffolds were seeded had an impact on the maturation of the cells. When clusters were seeded prior to definitive endoderm, for culture through definitive endoderm to pancreatic progenitor, gene expression showed diminished maturation within the scaffolds. Two protocols, planar and suspension, were used to test seeding at stage 5 day 1 and stage 6 day 1. The nondestructive sfGFP-c-peptide reporter proved useful in observing maturation of cells on the scaffolds. These results indicate the cells were producing insulin within the planar and suspension, as well as within planar and suspension seeded scaffolds. Maturation markers were enhanced in both suspension and planar culture seeded scaffolds, and insulin production and secretion were tracked in real time during differentiation and glucose stimulation insulin secretion (GSIS). We observed that pre-endocrine

seeding (Stage 5 day 1) promoted the continued maturation of the cells through the endocrine progenitor (stage 5), and enhanced gene expression for insulin, glucagon, NKX6.1, NeuroD1, NGN3 and PCSK1 in the suspension seeded scaffolds, and enhanced NGN3 gene expression in the planar seeded scaffolds. This pre-endocrine (stage 5) seeding in planar culture provided superior maturation relative to pre-SC-β (stage 6) seeding, indicating that the timing of scaffold culture initiation could be an important variable in the design of cell manufacturing processes.

In conclusion, as our understanding of the developmental biology of beta cells progresses, we can expect to see improvements in hPSC-derived β-cells glucose responsive insulin secretion and metabolic maturity. While these advances are being made, continuing to advance methods for the manufacturing of these cells can allow for large scale and consistent production of cell products for T1D recipients. Additionally, our ability to deliver these cells to an accessible site within the body, support and protect these cells through the engraftment period, and promote further maturation will depend on our understanding of how to appropriately engineer the local microenvironment.

5.2 Future Directions

While the results presented in this thesis contain important insights into considerations for the manufacturing and post-transplant support of hPSC-derived beta cells, there is much more work to be done before therapy will be widely adopted in the clinic. As discussed in Chapter 2, the ultimate cell replacement therapy for T1D will likely require advancements in: manufacturing of hPSC-derived beta cells, strategies towards enhancing the maturation and function of therapeutic β-cells, the survival of transplanted insulin-producing cells amid revascularization and inflammation, and the application of immune engineering methods to allow transplanted cells to avoid autoimmune or allogeneic host immune responses.

5.2.1 Proposed Cell Culture Opportunities

The studies presented in this thesis present steps towards understanding beta cell manufacturing, but there are many more variables to test to further understand and optimize beta cell culture *in vitro*. In this study, planar cultured cells were dispersed prior to seeding in the pores of the scaffold. Testing dispersal methods, such as different dissociation reagents and pipetting methods could improve results due to better maintained cell-to-cell connections. Scaffolds were seeded at stage 5 day 1 and stage 6 day 1, but other timepoints are worth investigating such as stage 4 day 1. This stage is important for directing cells along the pancreatic islet lineage, and it would be interesting to see if scaffolds provide a useful structure for niche formation during this critical timepoint.

In the past scaffold seeding studies, cells were seeded within scaffolds and cultured on an static culture on top of an air-liquid interface (air-liq) membrane.[1] Future studies can be designed to investigate alternative methods to culturing scaffolds within differentiation media. Two conditions to consider in addition to the static air-liq: i) static submerged culture- scaffolds submerged into differentiation media using a device to prevent the scaffolds from floating or sinking (media surrounds each surface), ii) dynamic submerged culture - scaffolds submerged into differentiation media on a shaker plate, with a device that prevents floating or sinking. Following transfer of 2D cultured cells to 3D scaffolds, the cell density increases by a large degree, and this could have an impact on diffusion of nutrients, waste products, and growth factors. Dynamic culture may help to circulate the media around the cells, potentially enhancing the differentiation.

When conducting the stem cell differentiation protocol, we have observed batch-to-batch variability in the proportion of beta cells and the degree to which the cells demonstrate glucose-responsive insulin secretion. These features of the batch are an important predictor of functional

success *in vivo*. The co-expression of NKX6.1 and C-peptide, specifically percentages flow cytometry co-expression (C-peptide+/NKX6.1+) above 30%, is considered favorable for transplantation success. With the current differentiation protocols, batch to batch variability can create the challenge of producing consistently high-quality cells for studying transplantation outcomes. One approach to overcoming this hurdle is to purify the population for β cells. There are at least two potential approaches to accomplishing purification: i) reaggregation of cells, and ii) live cell sorting on beta cell specific markers. Regarding reaggregation, the Millman lab has shown some preliminary evidence that when a mixed population of pancreatic cell types: acinar cells, ductal cells, and endocrine islet cells are dispersed and reaggregated in suspension, the endocrine cells have enhanced affinity for one another.[2] This process results in the reduction in the proportion of exocrine pancreatic cell types, hence increasing the proportion of endocrine cells. Regarding live cell sorting, we showed in Chapter 5 the utility of a sfGFP-C-peptide reporter in identifying insulin producing cells, and this reporter could be used for live cell sorting. Another candidate strategy for cell sorting is sorting based on CD49a, a β cell surface marker, and CD26, an α cell surface marker.[3] Questions remain about the benefits and harms of transplanting a purified β cell population as opposed to a mixture of pancreatic cell types.[4] Glucagon producing α-cells, as well as other islet cells, provide β cells with regulatory signaling.[5] It is estimated that roughly $1\text{-}5*10^8$ β cells may be for a human transplant recipient, which means a substantial load of cells must be delivered and supported within a confined volume. Since the volume provides a design constraint, further studies are needed to elucidate if a pure β cell population is ideal, or if a mixture of pancreatic cell types might provide enhanced therapeutic outcomes due to islet organoid synergy. One study that could bring some insight into this question could involve transplanting several cohorts of mice with known proportions of β and α cells. For example, upon completion

of Stage 6 SC-β, half of the cells are sorted on the β-cell specific marker (ex: sfGFP-c-peptide). During sorting, a baseline percentage of β cells and α cells could be established through the staining controls, which will not be utilized for transplantation. Following sorting a portion of the pure β cells could be mixed with the unsorted cells at a 1:1 ratio to produce a population with a percentage directly between sorted and unsorted. For example, if it is determined there are initially 30% β cells, the three groups of cells would be i) 30% β cells, ii) 65% β cells (1:1 mix of sorted and unsorted), and 100% β cells (sorted). The pre-transplantation characterization of these three conditions can later be compared against the *in vivo* function of these cohorts, as well as the degrees of maturation. Following explanatation, grafts can be analyzed via bulk population gene analysis (PCR), single cell sequencing, and IF staining. If there were significant differences in functional performance, the single cell level of information can allow us to see if the presence of different numbers of alpha cells influenced β cell subpopulations.

5.2.2 Considerations for the In Vivo Diabetic Model

The design of the mouse model for testing our cell products will likely have an impact on the outcomes and insights derived from our studies. Aspects of the model within our control include: the method for making mice diabetic, the choice of pluripotent cell line, and the immune profile of the host species or mouse strain.

Throughout the studies presented in this thesis, we utilized a single dose of 140mg/kg dose of streptozotocin (STZ) to make our mice diabetic. While this protocol has been successfully utilized for islet transplantation in mice, the very high level of hyperglycemia may present too high of a bar for hPSC-derived beta cells with maturing insulin secretion capabilities. There is some evidence that a mild STZ protocol, of 45 mg/kg daily for 5-days, eases the mice into their diabetic state which can improve their health pre- and post- transplantation. In the clinical setting, patients

are likely to be weaning off exogenous insulin while the transplanted graft takes over total glucose control. In the mouse models utilized in this thesis and the literature, the transplanted cells are exposed to extreme hyperglycemia (ex: consistently >500mg/dL). This degree of hyperglycemia may not be a real world parallel. A patient weaning off exogenous insulin is unlikely to have glucose similar to the untreated high-dose STZ mouse models. A lower initial level of blood glucose will reduce the strain on newly transplanted cells to bring glucose down to the healthy homeostatic setpoint. Therefore, this mild STZ protocol may be beneficial for studying the function of our cell products, both from a clinical translation perspective as well as decreased barrier to the graft reversing hyperglycemia.

The studies throughout this thesis utilized immunodeficient mice, which allowed us to study the engraftment and maturation hPSC-derived beta cells without the added challenge of protecting the cells against this xenogeneic response. Clinical application of cell replacement therapy will require an effective protection of transplanted cells from an allogeneic and/or autoimmune response. One approach to protecting cells from the immune response is physical encapsulation, but a major drawback of this strategy is the inadequate nutrient and oxygen exchange with the host.[4,6] Non-encapsulating strategies allow for better integration into the host tissue, but these strategies do require local and/or systemic immune modulation to protect the cells.[7] Scaffolds functionalized with streptavidin-Fas-Ligand (SA-FasL) have been shown to almost entirely prevent the allogenic response following islet transplantation into C57BL/6 mice.[8] There is evidence that this SA-FasL can induce apoptosis in T effector cells and induce expansion of T regulatory cells. This strategy is a promising direction for eventually protecting SC-β cells, but it will be challenging to test this strategy in pre-clinical animal models due to the xenogeneic immune response.

The choice of human pluripotent cell line may have an impact on the immune response in rodent models and will likely have an impact on the extent of immune modulation required for patients in the clinic. It is likely the pluripotent cell source for clinical β cell production will either be banks of HLA compatible hPSCs or iPSCs derived from the future recipients. Many of the published studies in the field utilize the H1 or HUES8 lines. A recently published report demonstrated the capability to produce functioning β cells from induced pluripotent cells derived from patients with T1D, indicating allogenic sources of iPSCs are being considered.[9] There is some movement in the field to produce pluripotent lines engineered to elicit a reduced immune response.[10] Testing the manufacturing and immunomodulatory approach with several cell lines can reveal if the methods are robust across cell lines. An additional important consideration is that immune protection strategies that worked in murine models may likely have different degrees of efficacy in primate or human recipients, so it is important to test approaches in other animal models in parallel with murine studies.

5.2.3 Promotion of Survival During Early Engraftment

One major goal of cell replacement therapy is to maximize the number of transplanted cells that survive the engraftment period. Following transplantation, cells must rely on diffusion of oxygen and nutrients until a new blood vessel network can develop to support the cells long term. Additionally, even in immune deficient mice, it is expected that there is some degree of foreign body reaction during the days following transplantation. A large proportion of the graft's cell death is expected to take place during the first several days following transplantation. Scaffold modifications made to promote revascularization are likely to facilitate engraftment into the host.

Two main approaches could be taken to promote revascularization: i) SC-β cells co-seeded with supportive cell types such as endothelial cells and fibroblasts, and ii) local release of

angiogenic factors from the scaffold into the local microenvironment. For the co-seeded scaffolds, endothelial cells and fibroblasts could either be added immediately prior to transplantation, or as a co-culture *in vitro*. While the co-culture strategy may provide some challenges regarding media formulations that support all cell types, this direction may provide additional benefits regarding β cell maturation. Vascular endothelium grows in coordination with the developing pancreas and has been shown to provide signals that induce insulin expression in pre-pancreatic endoderm.[11,12] Additionally, transplantation of the hPSC-derived β cells on scaffolds modified to release pro-angiogenic factors could likely expedite access to blood vessels and therefore promote survival. Several angiogenic factors could be tested individually or in combination, including: VEGF, FGF, PDGF, Angiopoietin 1, and TGF-β.[13,14] VEGF is known to stimulate outgrowth and assembly of endothelial cells, while PDGF requires supportive cell types such as pericytes and smooth muscle cells. Angiopoietin-1 (Ang1) and TGF-β1 are known to stabilize developing blood vessels. The microporous PLG scaffolds have been previously utilized to promote angiogenesis for islet transplantation through the local release of VEGF and PDGF, with PDGF release delayed relative to VEGF.[14] This sequential release was shown to enhance vessel maturation. Utilizing the scaffolds to first release VEGF, followed by PDGF, and finally stabilizing signals of Ang1 and TGF- β1 may provide engrafting SC-β's the opportunity for increased survival. Explanted tissue can be characterized for blood vessel network maturity through cryo-sectioning and immuno-fluorescent (IF) staining for CD31 and α-SMA. A direct measurement of surviving cell number can be estimated from automated counting of IF labeled human cells across a large sample of the sections. Finally, this characterization of cell survival can be related back to the functional dynamics to help us understand what proportion of surviving cells is require for therapeutic outcomes.

5.2.4 Promotion and Characterization of Long-Term Maturation

The microporous scaffolds can be modified to locally released trophic factors within the graft's niche following transplantation. This thesis presented a study which involved transplanting hPSC-derived pancreatic progenitors on PLG scaffolds modified to provide sustained release of Exendin-4, and this method proved beneficial in terms of β cell maturation and insulin secretion. An additional preliminary study with Exendin-4 releasing scaffolds was used to deliver stage 6 SC-β cells (results shown in Appendix), which showed some initial promising signs of enhanced function. While this study was limited by the supply of cells being shipped from collaborators, continuing to test Exendin-4 is strongly advised and will likely have interesting results when combined with the SC-β derived from planar culture. In addition to Exendin-4, there are other potentially useful candidates for scaffold modified sustained release: Nerve growth factor (NGF), Prolatin, somatotropin, hepatocyte growth factor, insulin-like growth factor 1 (IGF-1), betacellulin, and Activin-A are some of the candidates identified in the literature that promote and support β cells. Cells should be characterized prior to transplantation (gene expression, protein expression/flow cytometry, GSIS, and single cell sequencing), and this characterization pipeline should be repeated following explantation with the addition of immune-fluorescent staining. While the bulk population PCR can provide some insights into overall graft maturation, single cell information gained from flow cytometry, single cell sequencing, and IF staining. This single cell level of information can give us insights into the heterogeneity of the β cell population and the state of the engraftment niche, including the profile of host cells (ex: vascular and immune cells).

The insights gained through these proposed directions, if pursued, would likely lead to greater understanding of how to best culture the cells *in vitro*, minimize cell loss during engraftment, and promote the long-term maturation and function. The design considerations examined in this thesis,

as well as the work that will continue to build upon these results, will bring us closer to realizing a stem cell derived beta cell replacement therapy that can improve the lives of millions of people around the world.

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