Generation and Functional Characterization of Human Pluripotent Stem Cell Lines That Report on INSULIN and GLUCAGON Expression

Generation and functional characterization of human pluripotent stem cell lines that report on *INSULIN* and *GLUCAGON* expression

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Abstract

Diabetes is characterized by high levels of blood glucose due to malfunction (Type II) or destruction (Type I) of the INSULIN producing beta-cells (β-) in the pancreatic Islets of Langerhans. INSULIN action in muscle and fat allows for rapid blood clearing after a meal. The INSULIN producing β-cells are one of many endocrine cell types present in the Islets. Most notably, the product of alpha-cells (α-), GLUCAGON, has been shown to work as a secreted repressor of INSULIN action. Since Type I diabetic patients can benefit from transplants of cadaveric Islets, novel cell replacement therapies have focus on generating stem cell derived β-cells from human embryonic stem cells. Previous research has shown the feedback cycle of α- and β-cells is important for adequate glucose control and there have been issues with obtaining adequate numbers of Islets for cell replacement therapy, making stem cell derived pancreatic cells an attractive option for treatment. This study focused on a way to enhance the function of stem cell derived pancreatic β-cells through the addition of pancreatic α-cells for the treatment of Type I diabetes. This idea hinged on the creation of transgenic human embryonic stem cell lines that targeted the mCherry red fluorescent protein to the *INSULIN* and *GLUCAGON* genomic loci. These cells lines would then be differentiated individually toward the β- or α-cell lineage and sorted by mCherry fluorescence so that they could be reaggregated together in Islet-like organoids (miniature 3D *in-vitro* organ) and tested for changes in glucose response. The goal of the study was to test if the generation of α- and

β-Islet organoids would enhance glucose stimulated INSULIN secretion (GSIS) in stem cell-derived β-cells.

Dedication

I'd like to dedicate this work to my parents and friends for tolerating me through the existential crisis that was my thesis, and to the Melton Lab: past, present and future. Thank you for all your suggestions, comments, and encouragement.

Acknowledgements

I'd like Dr. Douglas Melton for allowing me the opportunity to conduct my research in his lab, for the support and encouragement he provided me during my time working for him, and for his unfathomable patience in waiting for me to generate reporter lines. I'd also like to thank Dr. José Rivera-Feliciano, my lab guru, who volunteered to be my thesis director and has provided an immeasurable amount of wisdom and guidance which I have used to complete this work. I'd also like to thank Ronny Helman for allowing me to use his aggregation protocol and for providing help in troubleshooting. In addition, I'd like to thank Jeff Davis for sharing his GSIS protocol, for allowing me to use the 1016-IRN reporter he generated, and for answering every single question I asked him when I couldn't find José.

I am also grateful to Dr. Ramona Pop for her help in keeping me on track with my experiments and for help with running FACS on my reporter samples, and to Jenny Kenty and Deanne Watson for their insights and suggestions for my ELISA experiments. I am indebted to the help I received from Dr. Laurence Daheron, Dr. Li Li, Caroline Becker, Sarah Fahmy, and Haihan Zhang of the Harvard iPS core who provided much assistance in my efforts to target *GLUCAGON* and *INSULIN*, including the TIDE analysis for the *GLUCAGON* guides. I also want to thank Dr. Torsten Meissner, who was among the first to encourage my desire to explore working with small molecules to enhance the efficiency of HDR.

I would also like to express my gratitude to Edwin Rosado-Olivieri who allowed me to use his unpublished 1016 data, and for helping me analyze my ELISA results. Adrian Veres was extremely helpful in the early stages of reporter generation and designed the original *INSULIN* gRNA7 sequence I eventually put into eCas9. Dr. Quinn Peterson also has my gratitude for developing the SC-α protocol I used and for aiding my initial efforts to target *GLUCAGON*. In addition, I'd like to thank my friend, Steph Tsai, for helping me stay sane during this process and for providing commiseration as she is currently working on her Ph.D. thesis. And finally, to Rebeca Ramos-Zayas who pushed me to break into the stem cell biology field, allowing me to end up at the Melton lab.

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Chapter I.

Introduction

The dysfunction of INSULIN-secreting β-cells in the pancreas is a hallmark trait in both Type I and Type II diabetes. This in turn results in an inability to regulate blood glucose levels which can be lethal if left untreated. The most common form of treatment remains the daily administration of exogenous INSULIN, while a more permanent solution involves the transplantation of cadaveric Islets. Unfortunately, there is a shortage of available Islets, prompting researchers to turn towards producing stem cell-derived β-cells instead. The goal of this work was to investigate glucose responsiveness in β- and α-cell organoids to provide an alternative to cadaveric Islets for cell replacement therapy in Type I Diabetes.

Physiology of Glucose Regulation

Glucose homeostasis is essential for normal physiology. After a meal the food is processed, and complex sugars are metabolized into glucose which is then absorbed by the intestine. Glucose is then distributed and used throughout the body as an energy source and when levels are abundant it is taken up by the liver for storage in the form of glycogen (Jiang *et al.,* 2003). When glucose levels are low glycogen can be converted into glucose and then secreted back into the bloodstream.

Critical to this process is the pancreas. The Islets of Langerhans are regions of the pancreas containing multiple subsets of endocrine cells including alpha (α-), beta (β-), delta, epsilon, and gamma cells each secreting a separate hormone essential for glucose metabolism (Kelly *et al.,* 2011). The hormone INSULIN is secreted from the pancreatic β-cells when glucose levels are too high, inducing the cellular uptake of glucose and the synthesis of glycogen. GLUCAGON is a hormone secreted by the pancreatic α-cells when blood glucose levels are too low and causes the liver to convert stored glycogen into glucose which will then be released into the bloodstream to raise the circulating levels of glucose. Both INSULIN and GLUCAGON are part of a feedback cycle to keep blood glucose levels stable in the body (Habener *et al.,* 2012).

In the structure of the human islet, β-cells make up about 64% of the islet cells while α-cells make up almost the remaining volume (Rodriguez-Diaz *et al.*, 2011). In addition, the cytoarchitecture of an islet reveals that almost 70-80% of β-cells face α-cells and maintain a strong association even upon dispersion. Structurally, it appears that α-cells are optimally placed to influence β-cells. While there is a reciprocal relationship between these two cell types in the maintenance of glucose metabolism, it has also been demonstrated that Acetylcholine secretion by α-cells sensitizes the β-cell response to increases in glucose concentration (Briant *et al.,* 2016). This action is mediated by paracrine interactions which occur via the interstitial space between endocrine cells. Thus, we hypothesize the presence of other paracrine signals from α-cells that would be important for the maintenance of β-cell function.

Diabetes

Diabetes is a disease which affects more than 300 million people worldwide. This disease results in abnormally high levels of glucose in the blood which is essential for normal physiology. In Type I diabetes, formerly known as juvenile diabetes, pancreatic β-cells are subject to destruction by an autoimmune attack which is currently not understood, while Type II diabetes results from INSULIN resistance as a symptom of obesity and metabolic syndrome (World Health Organization, 2012). Type I diabetes accounts for 5-10% of the total population of diabetic patients and there is no cure. Currently, diabetes management is controlled through the administration of exogenous INSULIN in response to elevated glucose levels, however constant glucose monitoring is needed as the injections fail to reach the same level of control as endogenous INSULIN secreted by β-cells. These long-term complications can result in episodes of hypoglycemia and/or ketoacidosis, both of which can be life-threatening.

Several more permanent options have been considered for the treatment of Type I diabetes. Whole pancreas transplants and cadaveric islet transplants have been used in the past as potential treatment options. The Edmonton Protocol (Shapiro *et al.*, 2000) was the first effective cell replacement therapy in which cadaveric Islets were isolated and infused into the portal vein within the livers of diabetic patients coupled with immunosuppression. Several patients experienced normal glucose tolerance and half of the patients were independent from exogenous INSULIN injection for more than five years. However, even successful islet transplants with this approach have potential complications. The first is the scarcity and quality of viable islets that can be harvested from cadavers and the second is the need for immunosuppressant treatment to fend off

autoimmune attacks from the body. While cell replacement therapy remains the most attractive option for the treatment of Type I diabetes, it is essential that an unlimited supply of pancreatic β-cells be obtained.

Pluripotent Stem Cells

Pluripotent stem cells (PSC) are defined by their ability to both self-renew and differentiate. Self-renewal is the ability of the stem cell to divide and generate at least one daughter cell that is equivalent to the parent cell while differentiation describes the ability to create different cell types. Human embryonic stem cells have been praised for their disease modeling and treatment potential given that they are generally chromosomally normal and can generate any cell type in the body both *in vitro* and *in vivo*. These cells are derived from the inner cell mass of a blastocyst and many stable cell lines have been generated from donated human blastocysts (Davis *et al.,* 2009).

Given the controversial process of producing these embryonic stem cells a breakthrough by Yamanaka *et al.* provided a way to circumvent this issue by reprogramming adult human fibroblast cells into PSCs also known as iPSCs, or induced pluripotent stem cells (Takahashi & Yamanaka, 2006). This was accomplished by viral introduction of Oct3/4, Sox2, c-Myc, and Klf4 factors into fibroblasts. With this technology, unlimited amounts of pluripotent stem cells can be generated and used for a multitude of disease research. In addition, this technique paved the way for patient specific cells to be reprogrammed into genetically identical PSCs which can be

differentiated into cells for cell replacement therapy without the need for immunosuppressants (Maehr *et al.,* 2009).

Pancreas Development

During embryogenesis, the pancreas forms from the endodermal germ layer. Pancreas development involves the generation of two distinct tissue types: exocrine, which includes acinar cells, centro-acinar cells, and ducts, and endocrine, which includes the multiple cell types of the Islets of Langerhans (Gittes, 2015). In humans, pancreas formation begins around gestational day 26 (G26d) with the dorsal bud formation followed by two central buds appearing around G30d. The dorsal pancreatic bud forms the head, neck, body, and tail while the ventral pancreatic bud forms the hooked uncinate process. The eventual fusion of these two buds forms the definitive pancreas. The cells in the pancreas undergo two different paths of differentiation. Multi-potent pancreatic progenitor cells can differentiate into either exocrine or endocrine cell types and are characterized by the co-expression of the *PDX1* and *NKX6.1* transcription factors (Jennings *et al.,* 2015).

Though it remains difficult to directly study human pancreas development, pancreatic differentiations of pluripotent stem cells have allowed researchers to examine expression profiles in progenitor cells and their progeny. In humans, the pancreatic progenitor cells under the expression of NEUROGENIN-3 and ISL1 and in the absence of the Notch receptor signaling form two lines of committed endocrine precursor cells (Jennings *et al.,* 2015). The first line forms alpha cells (α-) and gamma cells (γ-), which produce GLUCAGON and pancreatic polypeptides, respectively, through the expression

of *ARX* and absence of *PAX4*. The second line, influenced by *PAX4* and in the absence of *ARX*, produces INSULIN secreting beta cells (β-) with somatostatin secreting delta cells (δ-) expressing neither *ARX* nor *PAX4* (Gittes, 2015).

Initially, islet clusters contain about 3-4 fold fewer α-cells than β-cells around G9-13w due to *INSULIN*-expressing cells appearing first around G7.5W. However, this ratio about evens out to 1:1 by G14-16w and continues until after birth (Pan *et al.,* 2014). In addition, it appears that a majority of the α, β, and δ-cells in the fetal human pancreas are monohormonal in expression with a considerable percentage of *INSULIN* and *GLUCAGON* co-expressing (polyhormonal) cells appearing between G9w and G16w, declining significantly by G21w. Markers of mature β-cells include *INSULIN*, *MAFA*, and *NKX6.1* while markers of mature α-cells include *GLUCAGON* and *ARX* (Gittes, 2015).

Directed Differentiation of Pluripotent Stem Cells

Scientists have been able to mimic specific cell development pathways using compounds and small molecules to guide the pluripotent stem cell differentiation to the desired cell type (Kroon et al., 2008). Lineage-specific PSC differentiations attempt to recapitulate normal human development in vitro via the addition of exogenous growth factors or small molecules which mimic the key developmental transitions. Generating functional INSULIN-producing β-cells from PSCs has been a goal for many researchers in the diabetes field. Differentiation protocols for pancreatic α- (Rezania et al., 2011) and β-cells (Pagliuca et al., 2014) have been developed for stem cells and provide optimism for the treatment of diabetes via cell replacement therapy. Both protocols have been

successful in generating GLUCAGON and INSULIN-expressing cells, respectively. While stem cell derived β-cell transplants in vitro and in vivo have shown the ability to mimic the function of Islets, actual INSULIN secretion has yet to reach the level seen in normal Islets. As previously mentioned, there is evidence to suggest that incorporation of other pancreatic cells types like α-cells are important for precise control of β-cell function (Rodriguez-Diaz et al., 2011). To explore this idea, it is critical to identify, purify, and enrich for INSULIN and GLUCAGON-expressing cells.

Use of Reporter Cell Lines

One of the most useful molecular biology tools is the generation of stable reporter cell lines. These lines are generated by targeting a specific gene via homologous recombination of a DNA vector. The vector will contain homology arms which flank the 5' and 3' regions around the target site as well as a reporter protein, such as Green Fluorescent Protein, GFP, which is under the control of a specific promoter. For a successful reporter line, it is essential that the insertion of the vector will not cause a disruption in the coding region of the gene. The expression of the reporter protein is dependent on the expression of the targeted gene and will not turn on if the integration of the vector negatively impacts the gene function. For this reason, the 5' or 3' untranslated regions of a gene are usually targeted. These lines allow for marking of the target cells and are often used for in vivo modeling in mice (Kretzschmar & Watt, 2012).

Transgenic lines are especially useful for the visualization of a specific cell type of interest in a directed differentiation (Gupta *et al.,* 2018). For example, a β-cell

differentiation protocol would have use for an *INSULIN*-targeted reporter line to allow for the quantification of *INSULIN*-expressing cells produced during the protocol. Cells that express *INSULIN* would also express the reporter gene which would allow for the isolation of these populations of cells. Targeting of reporter constructs through homologous recombination can be a challenging task. Previously, constructs were transfected into cells and would simply rely on Homologous Directed Repair, HDR, for integration. Since the rate of HDR is so low the generation of double stranded DNA breaks in the target genome became a necessity so that the reporter construct would be able to integrate into the genomic locus at higher efficiency.

Emergence of the CRISPR System

Around 2013, the gene editing field exploded with the implementation of the CRISPR/Cas9 system. CRISPR is family of sequences generated by bacteria as a defense mechanism against foreign invaders. Specific sequences are obtained from viruses that have previously attacked the bacterium and RNA harboring those sequences guide the protein component to recognize and cut exogenous DNA. This induces a DNA double-strand break at the targeted genomic locus. Non-homologous end joining (NHEJ) repairs the damaged DNA, though this mechanism is error prone and often results in nucleotide deletions or insertions. This is turn causes a frameshift downstream of the cleavage site that can result in the formation of a premature STOP codon and the disruption of gene expression (Ran *et al.*, 2013). This system can be used in molecular biology by synthetically editing the target site of the gRNA which allows for the formation of double stranded DNA breaks at any desired genomic locus (Ding *et al.,* 2013).

Recently, Slaymaker et al. engineered a more specific version of the Cas9 endonuclease called enhanced Cas9, or eCas9 (Slaymaker *et al.*, 2016). Through the neutralization of positive charges on residues in the non-target (nt) groove of Cas9, they were able to drastically reduce off-target indel (insertion or deletion) formation while preserving on-target activity. Another form of CRISPR targeting has also been developed called the CRISPR/Cpf1 system (Zetsche *et al.*, 2015). This system was developed for targeting regions of genomic DNA which are particularly AT rich and incompatible with the NGG PAM sequence used by the CRISPR/Cas9 system.

These advancements have markedly improved the efficiency of gene editing and have been very successful in the generation of indels within a given gene, however HDR targeting has remained difficult. Since Non-homologous end joining, NHEJ, is the primary mechanism of DNA damage repair, it is essential to stimulate the HDR pathway instead (Mao *et al.,* 2008). To address this issue, the discovery and implementation of small molecule enhancers (Yu *et al.*, 2015 and Song *et al.*, 2016) in conjunction with the CRISPR system have managed to increase the rate of HDR by 2-3 fold, allowing for the successful integration of homologous vectors into the genome. The use of these improved gene editing tools should improve the success of engineering knock-in cell lines.

Here, we hypothesize that the generation of Islets-like organoids composed of α-cells, and β-cells can improve the level of INSULIN secretion so that it can more closely mimic that of human Islets. This study utilized CRISPR/Cas9 technology and pluripotent stem cells to generate fluorescent reporter cell lines which report on the *INSULIN* and *GLUCAGON* genes and can be differentiated towards an α- or β-cell fate. The success of this research can provide information on regulatory mechanisms of β-cell function which

can eventually be used to engineer stem cell derived islets. Advancements in cell replacement therapy can provide more efficient treatment for patients suffering from Type I diabetes.

Chapter II.

Materials and Methods

The following section outlines the materials and protocols used for the study. Briefly, advanced molecular biology techniques were used for the gRNA design and selection, small molecule optimization, and production of reporter constructs for targeting the *INSULIN* and *GLUCAGON* genomic loci. Next, pluripotent stem cells were cultured and electroporated with the constructs and gRNAs, and positively targeted clones were expanded and characterized by karyotype, immunostaining, and FACS analysis. Finally, the *INSULIN* and *GLUCAGON* reporter lines were differentiated towards an α- or β-cell fate, sorted by mCherry fluorescence, reaggregated into Islet-like organoids, and subjected to GSIS to assess their functionality. All experiments were carried out in a BSL-2 level lab.

gRNA Design and Construction

Guide RNA sequences for the *INSULIN* and *GLUCAGON* genomic regions were designed using the CRISPR tool on the Benchling website which uses the specifications outlined from the Zhang lab at MIT (Ran *et al.*, 2013). Guide sequences were chosen based on their off-target scores, and proximity to the stop codon of each gene. Guide sequences were ordered as forward and reverse primer pairs through IDT and ligated into

either eCas9 (Addgene 71814) or LbCpf1 (Addgene 84742) CRISPR plasmids using the protocol outlined on the MIT genome engineering website (crispr.mit.edu). Ligation reactions were then transformed into TOP10 competent bacterial cells and incubated overnight at 37°C on agar plates containing ampicillin. Positive bacterial colonies were picked and mini-prepped using the Qiagen mini-prep kit and sent for sequencing through the Dana Farber sequencing core through Harvard university. Correctly sequenced clones were midi-prepped (ZymoPURE Plasmid Midiprep Kit) and the plasmids were used for guide testing.

293T Transfection

HEK 293T cells were seeded into a 6 well plate (Corning) at a density of $2x10^6$ cells and left for 24hrs. Each well of 293T cells was then transfected with 5ug of a single gRNA using the Mirus 293 transfection kit and left for 72hrs. Cells were dispersed into single cells using TrypLE Express (Life Technologies) at 37°C and genomic DNA was extracted using the Zymo Research quick DNA prep kit. Primer pairs flanking a 700bp region within the target site of each gene were generated through IDT and used with the Phusion High-Fidelty PCR kit.

gRNA Analysis

PCR on the gDNA extracted from the 293T transfections was run with the primers flanking the 700bp regions around the *INSULIN* and *GLUCAGON* genomic

regions. PCR samples were run on a 1% agarose gel and gel purified with the Qiagen Gel Extraction Kit. For *INSULIN* a surveyor assay was done using the IDT kit and for *GLUCAGON* TIDE analysis (Brinkman *et al*., 2014) was done by the iPS Core at Harvard Stem Cell Institute using the software found on the https://tide.deskgen.com/ website.

Generation of Constructs

Targeting constructions were designed using the Benchling website. Primers flanking the stop codon region in the last exon of the *INSULIN* and *GLUCAGON* genes were generated through IDT. Homology arms flanking ~750bp upstream and downstream of the stop codon were generated using these primers and PCR products were gel purified using the Qiagen Quickgel Extraction kit. 5' and 3' homology arms were ligated with the NEB quick ligase into a previously generated backbone vector containing an mCherry fluorescent protein and a puromycin antibiotic selection marker. All constructs were sequenced through the Dana Farber sequencing core through Harvard University.

Cell Culture

Undifferentiated hPSC lines were maintained in mTeSR1 (StemCell Technologies) in 10cm plates (Corning) in a 37°C incubator, 5% CO_2, and 100% humidity. The human embryonic stem cell line HUES8 and hiPSC cell line iPS-1016 were utilized for all experiments shown. Cells were fed every day with 10-15mL of

mTeSR1 depending on confluency. Confluent cells were passaged as single cells using TrypLE Express at 37°C and the cell number was counted automatically by a Vi-Cell (Beckman Coulter). Cells were then seeded at a density of $10x10^6$ cells in mTeSR1+10μM Y27632 with on a 10cm plate coated with Matrigel.

Cell Electroporations

HUES8 and iPS1016 cells were dispersed into single cells using TrypLE Express at 37°C and the cell number was counted automatically by a Vi-Cell (Beckman Coulter). The Invitrogen 100μL nucleofector kit was used for electroporations and cells were resuspended in R buffer at a density of $2.5x10^6$ cells per 100μL. Cells were electroporated at 1100mV for 1 30ms pulse in 100μL aliquots with 25μg of targeting construct and 5μg of gRNA plasmid per $10x10^6$ cells. Cells were seeded at $10x10^6$ cells per Matrigel coated 10cm plate in mTeSR1 media with 10μM Y27632. For small molecule studies cells were incubated with either 7.5μM RS-1 (Xcessbio biosciences) or 5μM L755507 (Xcessbio biosciences) for 24hrs post electroporation.

Clone Screening

72hr post electroporation, cells were treated with puromycin at a concentration of 1mg/mL for 7 days to obtain single colonies. Colonies were picked under a microscope roughly 18-21 days post electroporation into a 96 well plate and dispersed into clumps. After 3 days colonies were dispersed with TrypLE Express at 37°C and seeded into both

a 96 well plate and a 48 well plate. gDNA from the 96 well plate was extracted using the Zymo Research Quick-DNA 96 Plus Kit. PCR assessing the presence of the 5' and 3' homology arms was done with the clone gDNA and clones positive for both were then subjected to an additional PCR to assess for heterozygosity. The PCR product was isolated and sequenced to ensure no mutations had been made to the unmodified allele. Heterozygous clones were expanded from 48 well plates to 12 well plates to 6 well plates and then to 10cm plates where cells were then sent for karyotype analysis through Cell Line Genetics.

α- and β-Cell Differentiations

For initiation of SC-β cell differentiation, HUES8 or hiPSC-1016 cells were seeded at 5×10^6 cells/ml in mTeSR1 media+10μM Y27632. The differentiation was started 24 hr later by changing media to Day media. The protocol was taken from Pagliuca et. al with the following additions: Y27632 through stages 3 and 4, ActivinA through stage 4, S3 media instead of CMRLS media for stage 6. The alpha cell protocol was adapted from Rezania et. al with unpublished observations from the Melton lab. Both protocols are included in Appendix II. In the final stage, cells were analyzed between 35-42 days of the protocol.

Immunohistochemistry

Differentiated cells in 6 well plates were fixed with 4% PFA for 30 min at room temperature, washed once in PBS, and incubated in blocking buffer (PBS+0.1% Triton X-100+5% donkey serum) at room temperature for 1 hr. Cells were then incubated in blocking buffer with C-peptide (1:100, rabbit, Cell Signaling Technology), GLUCAGON (1:500, mouse, Abcam), or mCherry (1:500, rat, Invitrogen) primary antibodies at 4°C overnight. Cells were washed twice in PBST and were then incubated in blocking buffer with secondary antibodies (Alexa Fluor® anti-mouse 488, anti-rat 594, anti-rabbit 647, 1:500) at room temperature for 2 hr. Cells were then washed twice with PBST and incubated with DAPI stain.

FACS Analysis

Differentiated cells were dispersed into single-cell suspension by incubation in TrypLE Express at 37°C, fixed with 4% PFA for 30 min at 4°C, washed once in PBS, and incubated in blocking buffer (PBS+0.1% Triton X-100+5% donkey serum) at room temperature for 1 hr. Cells were then resuspended in blocking buffer with C-peptide, GLUCAGON, or mCherry primary antibodies and incubated at 4°C overnight. Cells were washed twice in blocking buffer and were then incubated in blocking buffer with secondary antibodies at room temperature for 2 hr. Cells were then washed three times and analyzed using the LSR-II flow cytometer (BD Biosciences).

Reaggregation

Differentiated cells at were dispersed into single cells using TrypLE Express and resuspended in tissue culture grade PBS with 0.1% BSA and 10μM Y27632 on ice. For *INSULIN*-targeted lines 1:2000 dilution of TSQ was added. Cells were then sorted for mCherry fluorescence and TSQ (for *INSULIN* lines) using a MoFlo Astrios or XDP into tissue culture medium with 10μM Y27632 and kept on ice for the duration of the sort. After sorting cells were pelleted at 350rcf and resuspended at a concentration of 50,000 cells per mL of S3 medium with 10μM Y27632. Cells were then plated at 100μL per well in 96 well V-bottom plates and pelleted at 350rcf to allow for organoid formation. Plates were fed every 48 hours with 100μL of S3 medium with spinning before and after aspiration and feeding.

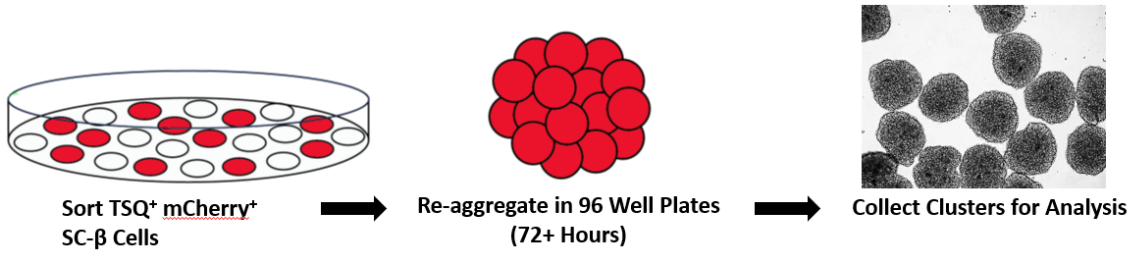

Figure 1. Schematic for the sorting and reaggregation of fluorescent reporter cells for GSIS analysis.

Cells are differentiated towards an α- or β-cell fate until ~21 days into stage 6 of the protocol and are then sorted by fluorescence to isolate pure populations of INSULIN- or GLUCAGON-expressing cells. These cells are then placed in a 96 well plate and allowed to reaggregate into organoids for analysis.

Islet Culture

5000 IEQ (Islet equivalent, measurement that corresponds to an estimate of the tissue volume of a perfectly spherical islet with a diameter of 150 μm) of Islets were

obtained from Prodo Laboratories and maintained in a low attachment 6 well plate (Corning) at ~2000 IEQ per well in 5mL CMRLS medium with 10% FBS and 2% penstrep in a 37°C incubator, 5% CO_2, and 100% humidity. Cells were fed every 48hrs. For plating for GSIS, islet clusters were dispersed into single cells with Accutase and resuspended at a concentration of 50,000 cells per mL of CMLRS medium with 10μM Y27632. Cells were then plated at 100μL per well in 96 well V-bottom plates and pelleted at 350rcf. Plates were fed every 48 hours with 100μL of S3 medium with spinning before and after aspiration and feeding.

Glucose Stimulated INSULIN Secretion (GSIS)

96 well plates of reaggregated sorted cells and islets were pelleted at 230rcf and washed in 150μL KRB (2.8mM glucose). Cells were then pelleted and incubated in 150μL 2.8mM KRB for 1.5 to 2 hr at 37°C. Cells were pelleted at 230rcf and washed with 150μL 2.8mM KRB. Cells were pelleted at 230rcf and incubated in 100μL of 2.8mM KRB (low glucose), 16.7mM KRB (high glucose), or KCl (low glucose + 30mM KCl, for depolarization) at 37°C for 1 hr. The top 70μL supernatant was removed and transferred to a new 96 well PCR plate and spun down at 230rcf. The top 50μL of this supernatant was then removed and transferred to an ultra-low attachment 96 well plate, parafilmed, and stored at -20°C until used for INSULIN ELISA. Supernatant from original 96 well plate was aspirated and clusters were lysed in 100μL RIPA buffer parafilmed, and stored at -20°C until used to assess for total INSULIN content. An ultrasensitive INSULIN ELISA kit (ALPCO) was used for assessing INSULIN secretion and content.

Chapter III.

Results

The focus of this study was to observe the effect of co-culturing pure populations of stem cell-derived α- and β-cells on GSIS performance. To do this, fluorescent reporter lines targeting the *INSULIN* and *GLUCAGON* loci were generated and differentiated towards either an α- or β-cell fate. All reporter lines were generated in human pluripotent stem cells. Prior to this, optimizations were made to the targeting of these lines through the testing of advanced CRISPR proteins and small molecule homologous recombination enhancers to improve efficiency in obtaining positive clones. Differentiated cells were sorted by fluorescence and reaggregated together in 5000 cell populations of β-cells, α-cells, or a 2:1 or 1:1 ratio of β-cells to α-cells. These aggregates were then subjected to GSIS to evaluate any changes in efficiency as compared to normal human Islets. These results were used to gain a better understanding of the reciprocal relationship between α- and β-cells during glucose homeostasis.

The Optimization of CRISPR Targeting

Guide RNA Design and Testing

The CRISPR/Cas9 system was used for the targeting of the *INSULIN* and *GLUCAGON* reporter lines. Given the difficulty of introducing a homologous template into a cell, extensive guide RNA testing was done to select guides with the highest

frequency of indel formation. These guide sequences were generated around the last exon of the *INSULIN* gene through the CRISPR tool feature on the Benchling website. Off-target scores were considered during guide selection to account for non-specific cutting in other genomic loci. Seven guide sequences were ordered as oligos and ligated into a plasmid containing the Cas9 protein to use for surveyor assay analysis. While normally surveyor assays are performed using HEK 293T cells, the embryonic stem cell line HUES8 was used instead given that it was the desired cell line for targeting. However, given the difficulty of editing stem cells, the bands produced by the surveyor assay were very faint suggesting that many of the guides were not efficient (Figure 2A).

Despite the faint bands, guides 1, 6, 7, 8, and 9 were selected and used for targeting *INSULIN*. While gRNAs 6, 8, and 9 showed slightly more intense bands on the surveyor gel, only 3, 5, and 2 colonies were obtained respectively on each plate after drug selection while gRNA7 produced 9 colonies. None were positively targeted. Despite this, gRNA7 (cgRNA—GCCGACAGGCATGGCCGCTTT) was chosen for targeting given the higher frequency of colony production and because the guide sequence falls entirely within the 3'UTR of the *INSULIN* gene while the other gRNAs fall within the coding region of the last exon. Guide RNA 7 was eventually ligated into the enhanced cas9 (eCas9) protein and was able to produce positively targeted clones (figure 3).

GLUCAGON targeting proved to be more difficult given that the genomic region around the 3' UTR is particularly AT-rich. A TALEN pair had been previously generated but was unsuccessful in producing any drug-resistant clones. In addition, Cas9 options were limited given the NGG PAM sequence and those that were generated produced exceptionally low indel frequencies (1-2%) when evaluated in 293T cells using TIDE. To

correct this issue, guides were made with the Cpf1 CRISPR protein which contains a TTTN PAM sequence that was more suited for the *GLUCAGON* locus. Six different guide sequences were tested in both the Lb and As strains of Cpf1 and gRNA5 (cgRNA—ATCTCTAGGAAATAACTATA) was selected for targeting since it had the one of the highest cutting efficiencies (13.4%) while also spanning across the stop codon of the last exon (Figure 2B). A slight variability between the two strains of Cpf1 was observed with Lb being slightly more efficient than As. Guide 5 was used for targeting and produced 60 drug-resistant clones, though none of them were positively targeted.

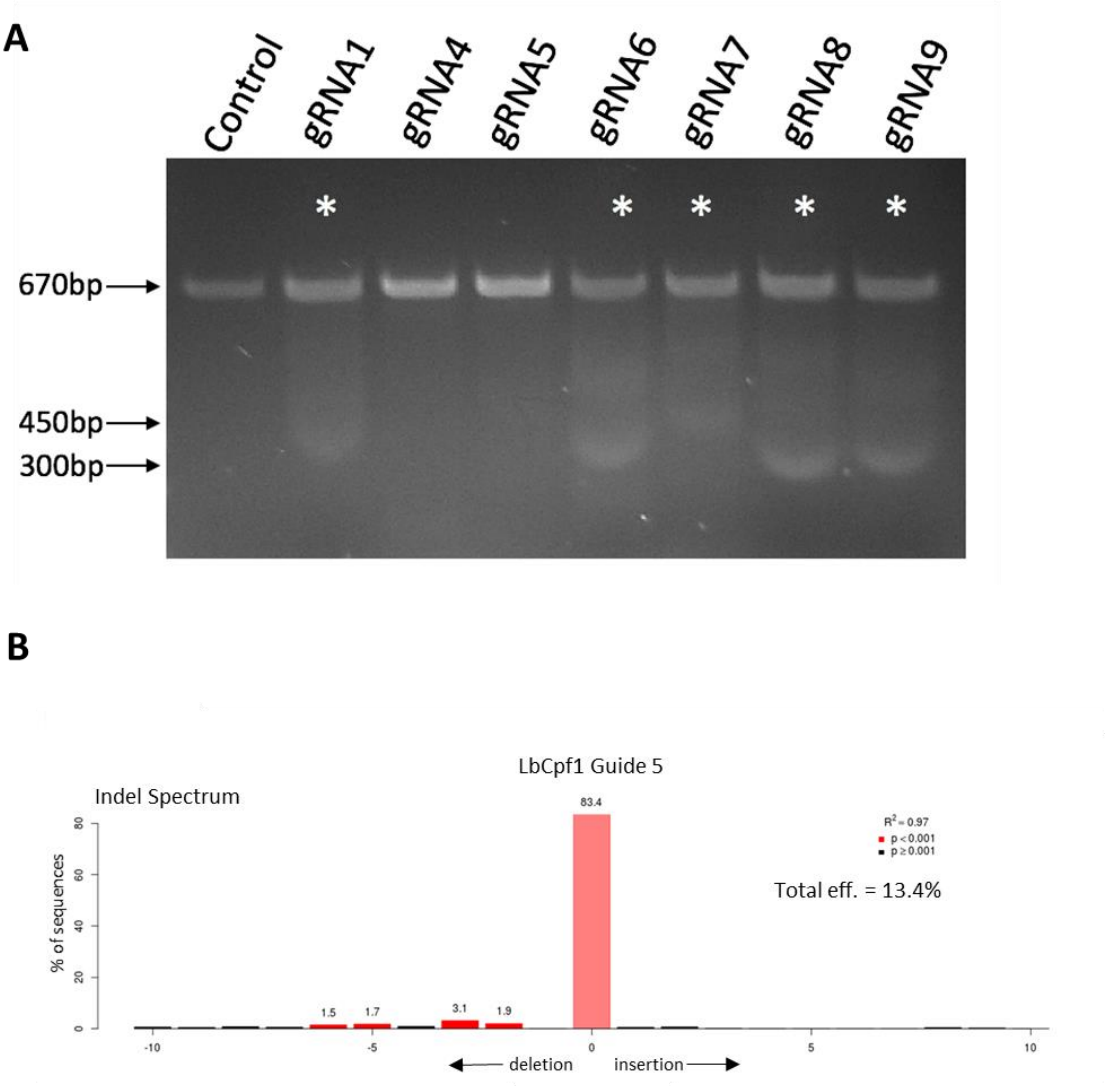

Figure 2. gRNA cutting efficiency.

(A) Surveyor assay in HUES8 cells testing 7 guides for the INSULIN locus. Guides 1, 6, 7, 8, and 9 produced faint bands demonstrating some amount of indel formation. (B) TIDE analysis in 293T cells for Cpf1 guide 5 chosen for targeting the GLUCAGON locus.

Analysis of Small Molecule HDR Enhancers

Though extensive guide testing had been done for both the *INSULIN* and *GLUCAGON* loci, there was still great difficulty in obtaining positively targeted knock-in clones. For this reason, two homologous recombination enhancing small molecules, RS-1

and L755507, were tested to try and increase the efficiency. HUES8 cells were electroporated with the *INSULIN* targeting vector and gRNA7 with the addition of either 7.5uM RS-1 or 5uM L755507 or no compound for 24hrs post transfection (Xcessbio biosciences). As seen in Figure 3A, no colonies were positive for 5' homology arm insertion across the control, RS-1, and L755507 plates. The *INSULIN* guide 7 was then ligated into the eCas9 vector instead of the Cas9 vector and the experiment was repeated. This time, all conditions produced positive clones with 2/20 (10%) positive clones in the control, 7/36 (20.5%) with L755507, and 11/29 (37.9%) with RS-1 (figure 3B). Based on this data, while eCas9 alone was sufficient to produce a small number of positive clones, it was necessary to promote HDR in conjunction with eCas9 for successful targeting of the HUES8 line. RS-1 was selected as the HDR enhancing drug for the generation of the *INSULIN* and *GLUCAGON* reporter lines given it had the highest efficiency.

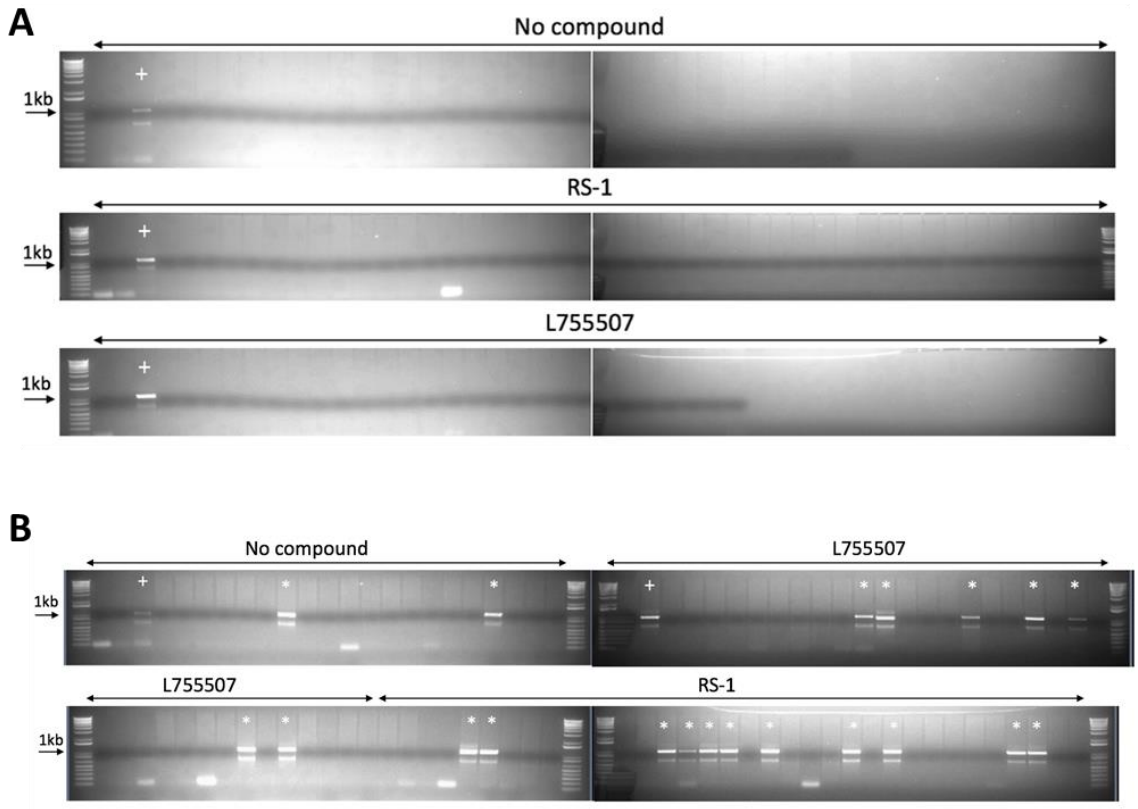

Figure 3. Small molecule HDR enhancers increase knockin efficiency when used with eCas9.

(A) 5' PCR results for INSULIN targeting show no correct integration when used with a normal Cas9 protein in HUES8 cells. (B) eCas9 addition increases the efficiency of the knock-in, giving 2/20 positive control colonies (10%), 7/34 (20.5%) positive L755507 colonies, and 11/29 (37.8%) positive RS-1 colonies.

Generation of Reporter Cell Lines

INSULIN Red Nucleus (IRN) Line

The first reporter line generated targeted the 3' UTR of the *INSULIN* gene (Figure 4). Notable elements include: the modification of mCherry to have three tandem nuclear localization signal sequences (3X NLS) in its C-terminus, the addition of a GS-bridge in front of the P2A, and a beta globin poly-adenylation signal after the 3X NLS. After stable integration, Cre recombinase could be used to remove the poly-adenylation signal and the

selection cassette. Clones were selected with 1ug/mL puromycin for 7 days and 40 resistant clones were genotyped using PCR. Of those 40, 12 were double positive for 5' and 3' insertion of the construct (30%) and were screened for homozygous or heterozygous insertion. 9 clones were confirmed heterozygous and 2 were sequenced to ensure the untargeted allele was unmodified. Of the 2 clones that were sequenced, one contained an inserted "G" base pair within the site of the guide sequence while the other clone contained no indels in the untargeted allele. Please find the primers and PCR conditions used in Appendix I. The two heterozygous clones were found to have a normal karyotype after clonal expansion. The cell line was named HUES8-IRN (*INSULIN* Red Nucleus).

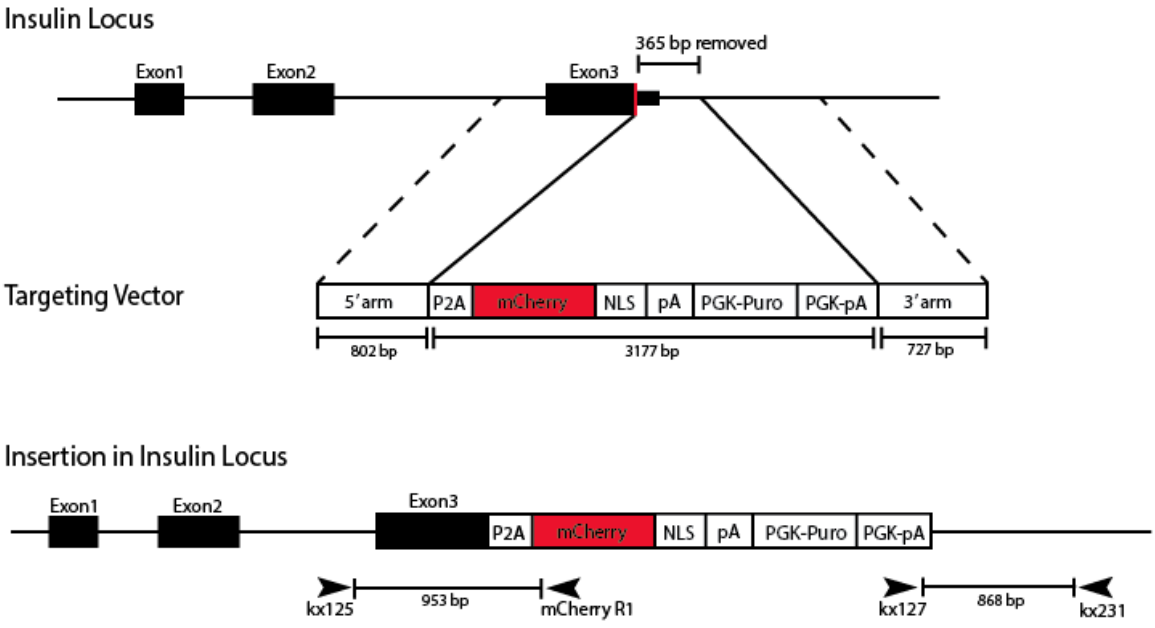

Figure 4. Schematic of the targeting of mCherry to the *INSULIN* gene.

A fluorescent reporter (mCherry) was targeted to the 3' UTR of the INSULIN gene so that its expression would be under the direct control of INSULIN expression.

To validate the fidelity of the HUES8-IRN reporter for *INSULIN* expression, the two clones were differentiated in planar culture towards SC-β cells using the version 4 β-cell protocol adapted from Pagliuca et. al. Epifluorescence of the mCherry reporter was observed beginning in mid-stage 5 of the protocol and was captured at stage 6 day 3 (Figure 5A). This confirmed that the reporter expression was able to be turned on at the correct time and was cell specific. Although an NLS was added to the construct to ensure reporter expression was nuclear, much of the expression appeared to be cytoplasmic when observed under fluorescent light for reasons not entirely known. At stage 6 day 6, cells were fixed and immunostained for C-peptide and mCherry (Figure 5B) showing a strong localization between the two populations of cells. FACS analysis (Figure 5C) of C32 showed a small (4.96%) population of mCherry+ cells which overlapped with C-peptide+ cells (3.29%) which further provided evidence that the mCherry expression was linked to C-peptide-expressing cells. There was a noticeable population of cells (1.67%) that were mCherry+ but C-peptide- though the cause is unknown and could potentially be background noise. Given that the protocol was done in 2D 6 well plates, the efficiency was lower (~5%) than for normal differentiations in 3D spinner flasks (~20%).

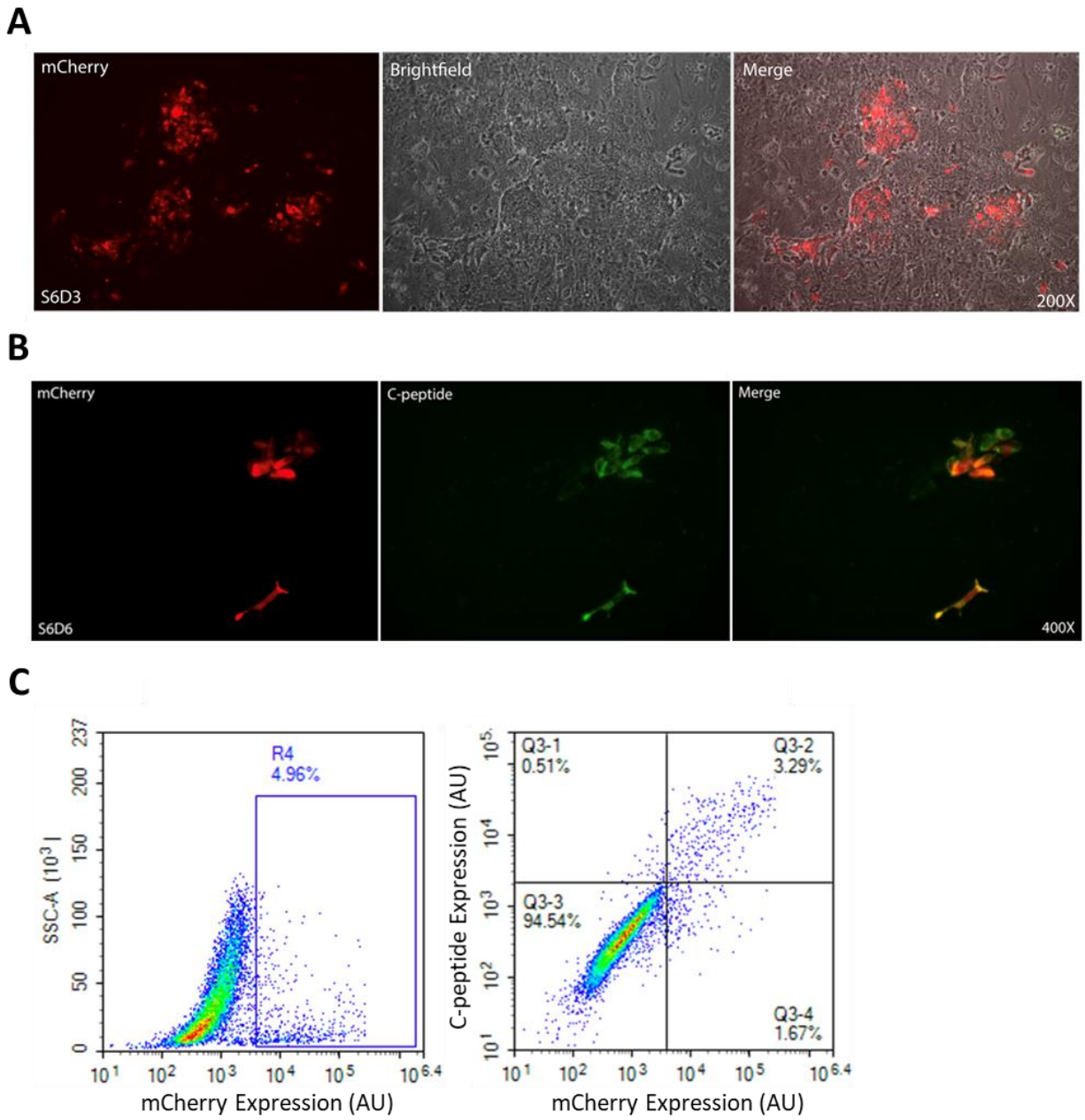

Figure 5. HUES8-IRN C32 validation.

(A) Epifluorescence of mCherry during stage 6 day 3 of the β-cell protocol. (B) Immunostaining analysis of stage 6 day 6 of the β-cell protocol. Cells were co-stained with mCherry and C-peptide. (C) FACS analysis of mCherry and C-peptide showing a 4.96% population of mCherry cells of which 3.29% is co-positive for C-peptide.

GLUCAGON Red Nucleus (GRN) Line

The second reporter was generated by targeting the 3'UTR of the *GLUCAGON* gene (Figure 6) in the iPS 1016 cell line. After drug selection, 48 resistant clones were genotyped using PCR and 9 double positive (18.7%) ones were screened for homozygous or heterozygous insertion. Four heterozygous clones were sequenced to ensure the untargeted allele was unmodified. These clones were then karyotyped and confirmed to be normal. The line was named 1016-GRN (*GLUCAGON* red nucleus). To validate the fidelity of the 1016-GRN reporter for *GLUCAGON* expression, the 4 clones were differentiated using an unpublished α-cell protocol developed in the Melton lab containing modifications from protocol by Rezania et al. One additional modification was the addition of LDN for a second day during stage 4 of the protocol to induce the formation of polyhormonal (*INSULIN*- and *GLUCAGON*-expressing) cells (Figure 10). mCherry fluorescence was detected at stage 6 day 5 of the α-cell protocol (Figure 7A). Cells were immunostained with GLUCAGON and mCherry antibodies to observe localization between the two (Figure 7B) using α-cell protocol and showed a strong overlap. This was further characterized by FACS analysis at stage 5 day 5 showing a 93.5% co-positivity between mCherry and *GLUCAGON*-expressing cells (Figure 7C).

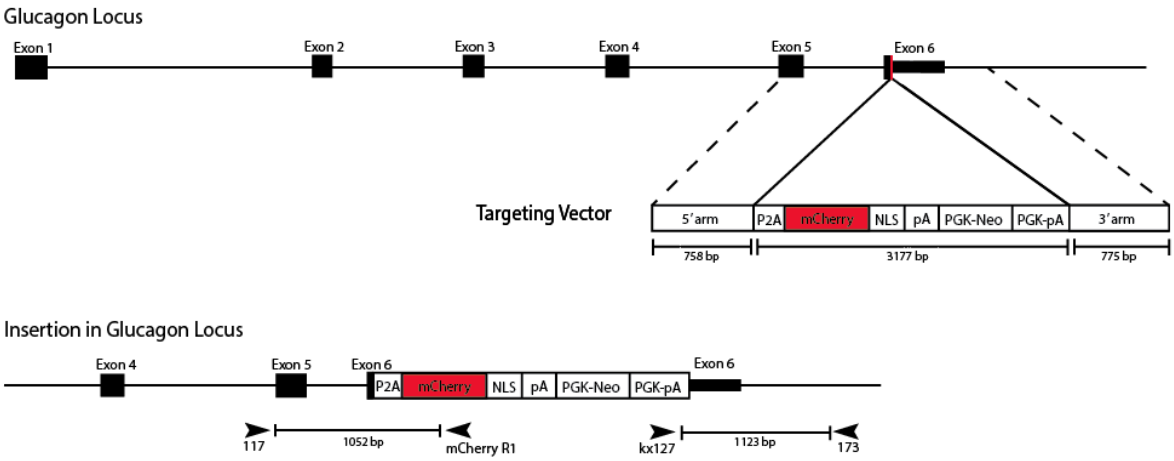

Figure 6. Schematic of the targeting of mCherry to the *GLUCAGON* gene.

A fluorescent reporter (mCherry) was targeted to the 3' UTR of the GLUCAGON gene so that its expression would be under the direct control of GLUCAGON expression.

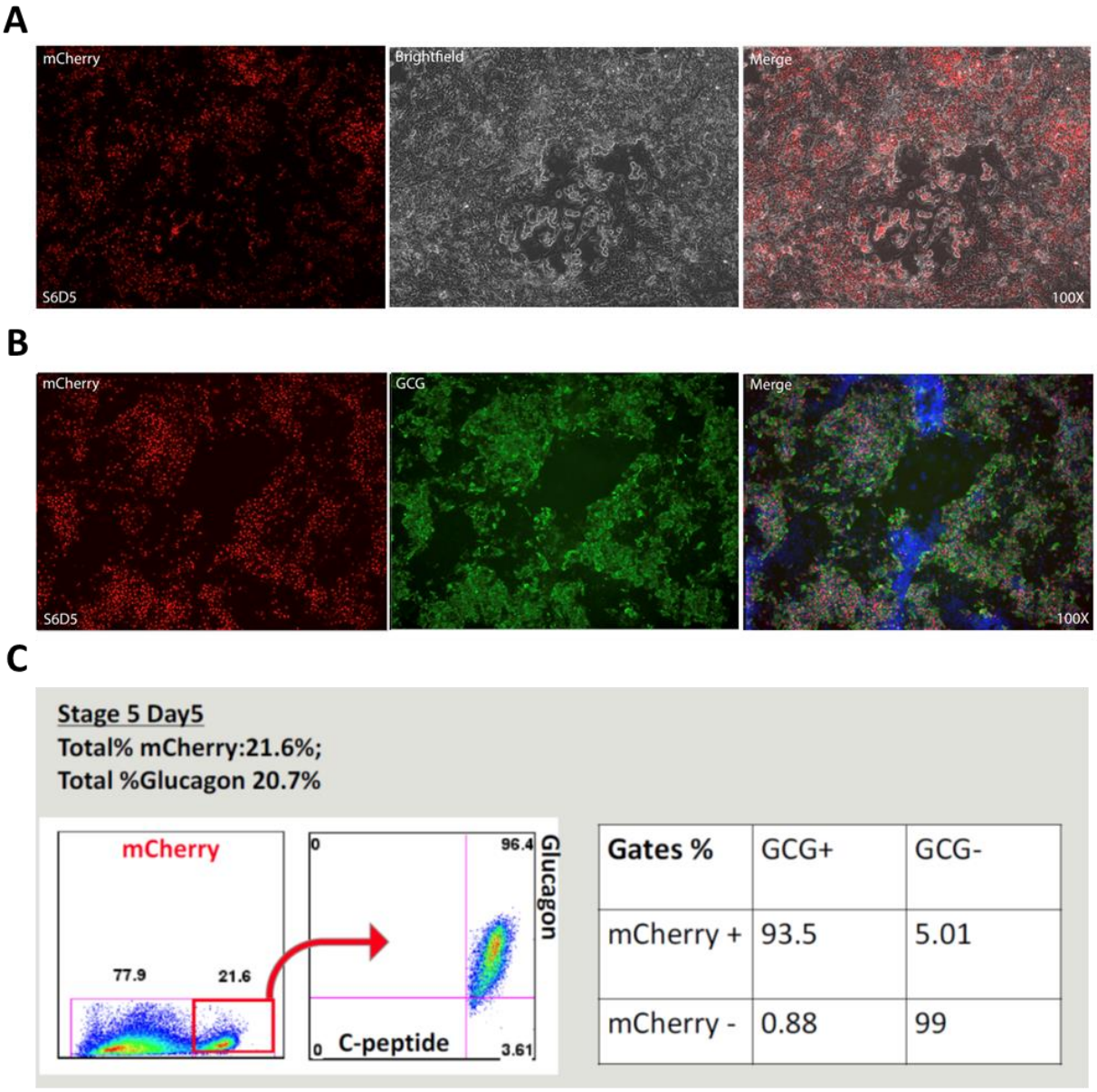

Figure 7. 1016-GRN validation.

(A) Epifluorescence analysis of 1016-GRN cells at Stage 6 day 5 using the SC-α directed differentiation protocol, 100X. (B) Immunostaining analysis of 1016-GRN cells at Stage 6 day 5 using the SC-α directed differentiation protocol, 100X. (C) FACS analysis at S5D5.

Double reporter line: *INSULIN* Red Nucleus-*GLUCAGON* Green Nucleus (IRN-GGN)

A dual reporter for both *INSULIN* and *GLUCAGON* was generated to sort for mono-hormonal cell populations during the differentiations. Previously, a 1016-IRN line was made and validated so the *GLUCAGON* locus was targeted within this line using an eGFP fluorescent protein instead of mCherry (Figure 8). After drug selection, 48 resistant clones were genotyped using PCR and 1 double positive one (2%) was screened for homozygous or heterozygous insertion. The clone was heterozygous was sequenced to ensure the untargeted allele was unmodified. This clone was then karyotyped and confirmed to be normal. The line was named 1016-IRN-GGN (*INSULIN* Red Nucleus-*GLUCAGON* Green Nucleus).

To validate the fidelity of the 1016-IRN-GGN dual reporter for *GLUCAGON* and *INSULIN* expression the line was differentiated using an unpublished, updated version of the SC-β directed-differentiation protocol from Pagliuca *et. al* and an unpublished SC-α directed-differentiation protocol from the Melton lab. The presence of eGFP and mCherry fluorescence at stage 6 day 1 of the β-cell protocol and stage 6 day 1 of the α-cell protocol was assessed and many mCherry/GFP+ cells were observed (Figure 9A). Cells were then immunostained with GLUCAGON and GFP antibodies to observe localization (Figure 9B). As expected, there were far more GFP+ cells in the α-cell protocol than the β-cell protocol although mCherry+ cells seemed to outnumber GFP+ cells in both differentiation protocols. This could also be because the GFP+ expression is nuclear while the mCherry expression is more cytoplasmic.

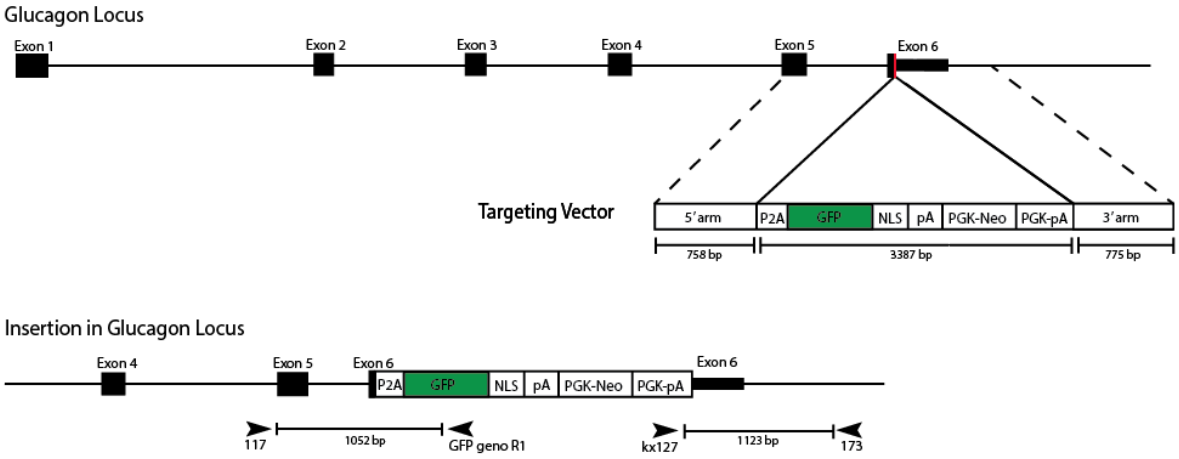

Figure 8. Schematic of the targeting of eGFP to the *GLUCAGON* gene.

A fluorescent reporter (eGFP) was targeted to the 3' UTR of the GLUCAGON gene in the 1016-IRN line so that its expression would be under the direct control of GLUCAGON expression.

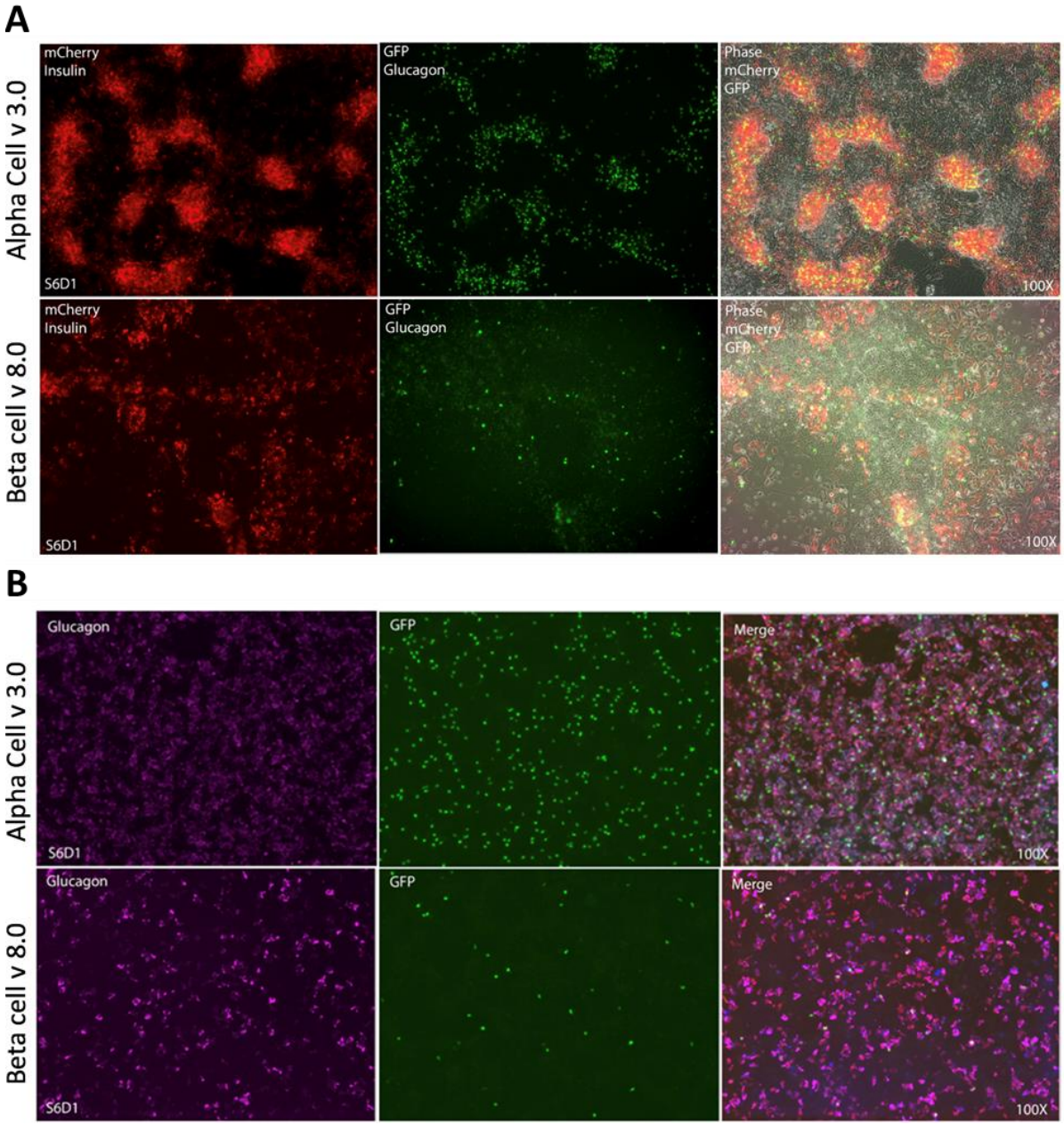

Figure 9. 1016-IRN-GGN validation.

(A) Epifluorescence analysis of 1016-IRN-GGN cells at Stage 6 day 1 using the SC-α and SC-β directed differentiation protocols, 100X. (B) Immunostaining analysis of 1016-IRN-GGN cells at Stage 6 day 1 using the SC-α and SC-β directed differentiation protocols, 100X.

Optimization of the SC-α cell Protocol

LDN Addition for Polyhormonal Induction

While the protocols used for these experiments were developed for 3D cultures, 2D experiments were carried out in 15cm plates due to the time-consuming process of spinner adaptation. For the sake of having plenty of SC-α cells for the reaggregation and GSIS experiments, improvements had to be made to the α-cell protocol. The goal was to increase the formation of *GLUCAGON*-expressing cells enough so that multiple 15cm plates could be used in lieu of spinner flasks, allowing for more replications of the experiment. LDN was added for an extra day on S4D2 to induce the formation of more polyhormonal cells in the protocol (Figure 10A). When compared to the control, the additional LDN caused an increase in the frequency of mCherry+ cells by mid-stage 4 all the way into the beginning of stage 6. The additional LDN did not affect cell viability when compared to the control, showing that the prolongation of LDN in the protocol for 2D cultures can induce more *GLUCAGON*-expressing cells without causing cell death.

Use of Conditioned Medium in Stage 6

Previous observations made during the 2D SC-α directed-differentiation protocol showed a large amount of cell death during stage 6. Cells in this stage are given S3 medium in addition to PdBU to push polyhormonal *INSULIN*/*GLUCAGON*+ cells into monohormonal *GLUCAGON*+ cells (unpublished protocol). While this was not observed in 3D cultures, it became a problem because it was previously observed that cells need to at least reach 21 days into stage 6 to start becoming mono-hormonal. Since SC-α and SC-β cell differentiations were being carried out in sync for the sake of the reaggregation

experiment, conditioned medium from the SC-β cells was filtered and used as S6 medium for SC-α cells on feeding days. There is literature to suggest that the use of conditioned medium during culture can help promote growth and viability in different cell types (Pancholi *et al.,* 1998 and Purmessur *et al.,* 2011). Medium was taken from the SC-β plate and passed through a 22μM and mixed with PdBU. When compared to previous versions of the protocol, SC-α cells receiving SC-β cell conditioned medium were able to survive longer even after being replated on stage 6 day 5 (figure 10b). Some cell death was still observed, but the conditioned medium plates were able to survive 6 weeks into stage 6 while control plates were completely dead by stage 6 day 21. Though it is not certain as to why the conditioned medium had a protective effect on the α-cell differentiations, there was no negative effect on the populations of mCherry+ cells when viewed under a fluorescent microscope.

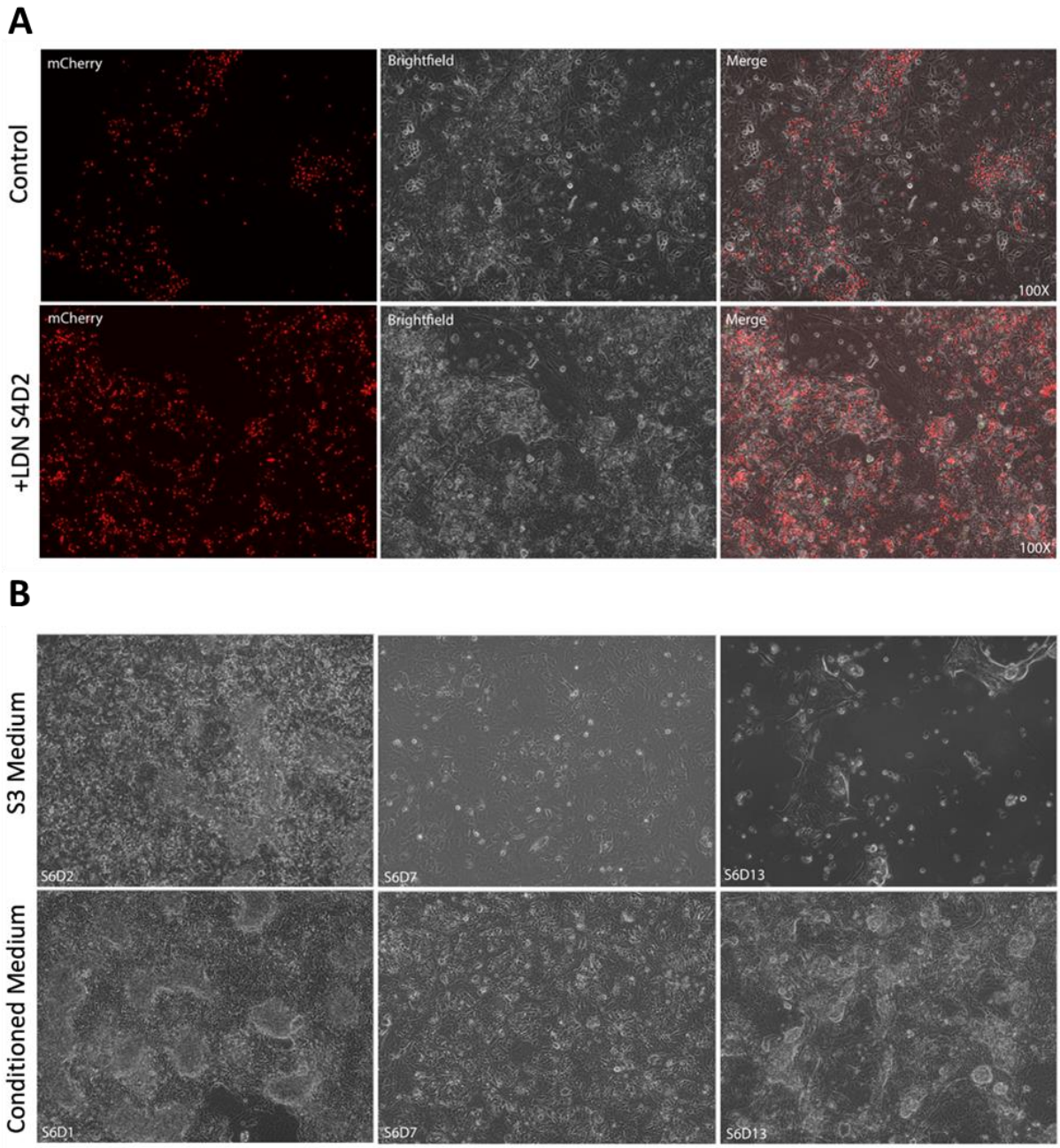

Figure 10. Optimizations to the SC-α cell protocol.

(A) Epifluorescence analysis of 1016-IRN cells at early Stage 6 of the SC-α directed differentiation protocol, with or without LDN addition at S4D2, 100X. (B) Time lapse of phase images taken through stage 6 of the SC-α directed differentiation protocol with and without conditioned medium, 100X.

GSIS of α- and β-organoids

The 1016-IRN and GRN lines were differentiated in 15cm plates towards either a β-cell or α-cell fate, respectively. These differentiations were taken into at least stage 6 day 21 before being sorted by mCherry fluorescence. Sorted cells were then seeded into a v-bottom 96 well plate into β-cells only (sorted *INSULIN*+ cells), α-cells only (sorted *GLUCAGON*+ cells), or mixed (1:1 or 2:1 β- to α-cell) populations at 5000 cells per well. This allowed the cells to form aggregates which were fed every 48-72 hrs until GSIS was done on them roughly a week later. The aggregates were challenged with either low glucose (2.8mM), high glucose (16.7mM), or KCl (30mM in 2.8mM glucose) along with normal human Islets and the supernatant was collected for INSULIN secretion while the cells were lysed for INSULIN content. An INSULIN ELISA was performed on all samples to assess the amount of INSULIN secreted for each condition.

For cells that were sorted on or around stage 6 day 21, GSIS results indicated that this was not a sufficient amount of time for these cells to have become mono-hormonal. Total INSULIN secretion for each condition was in some cases almost 10 times higher in the sorted cell populations than in the control Islets (Figure 11A). This is likely because the cells are still polyhormonal and tend to dump large amounts of INSULIN since they do not yet possess the regulatory genes to control INSULIN secretion (Ali Rezania, personal communication, 2018). When looking at the fold change in INSULIN secretion between low and high glucose it would seem as though the 1:1 ratio of cells produces a higher change when compared to islets and other sample conditions. However, this experiment was repeated three times and there was high variability between each batch of differentiated cells with one outlier batch pushing the 1:1 ratio stimulation index average

to be higher than it was. In addition, an outlier value of 33% INSULIN secretion in low glucose for the β-cell only condition caused the INSULIN secretion average for that condition to become skewed which in turn lowered the stimulation index for that condition. This made it difficult to assess whether the coupling of α- and β-organoids gave an improvement in INSULIN secretion.

There were two additional runs for this experiment in which β-cell differentiations were allowed to continue eight weeks into stage 6 before GSIS. One batch was done with the 1016-IRN line in 2D and the other batch was done with the double 1016 IRN-GGN line in 3D spinner culture. Due to the lack of available sorted *GLUCAGON*+ cells, 20nM or 100nM of exogenous GLUCAGON protein was added to the β-organoids during GSIS. These concentrations were selected based on published data which demonstrated that cells incubated with GLUCAGON protein experienced a 4.3-fold increase in cAMP levels at 100nM concentrations, with 20nM being the half-max accumulation (Li *et al.,* 1997). The addition of this exogenous GLUCAGON also provided a positive control to see if the matured β-organoids can respond to GLUCAGON at all.

In both batches the INSULIN secretion was drastically lower than the cells that were sorted around stage 6 day 21 which implies that these cells were most likely monohormonal at this point. However, when compared to normal human Islets, the β-cells secreted a fraction of their INSULIN when compared to the KCl depolarization (Figure 12A). In addition, the addition of exogenous GLUCAGON did little to help INSULIN secretion between low and high glucose concentrations as seen through the stimulation index values (Figure 12B). There was little explanation for this discrepancy until unpublished work from E. Rosado-Olivieri in the Melton lab showed an

accumulation of pro-INSULIN in the 1016 cell line compared to normal HUES8 and islet cells (Figure 13). The amount of pro-INSULIN in these cells could implicate an issue in INSULIN processing or misfolding that could potentially explain the GSIS failure with these monohormonal cells. One additional observation was that in all GSIS runs there seemed to be a lower than average change in INSULIN secretion between low and high glucose even in the islet controls, suggesting that the batches of cadaveric Islets were either less than functional, or that there could have been issues in the design of the GSIS assay.

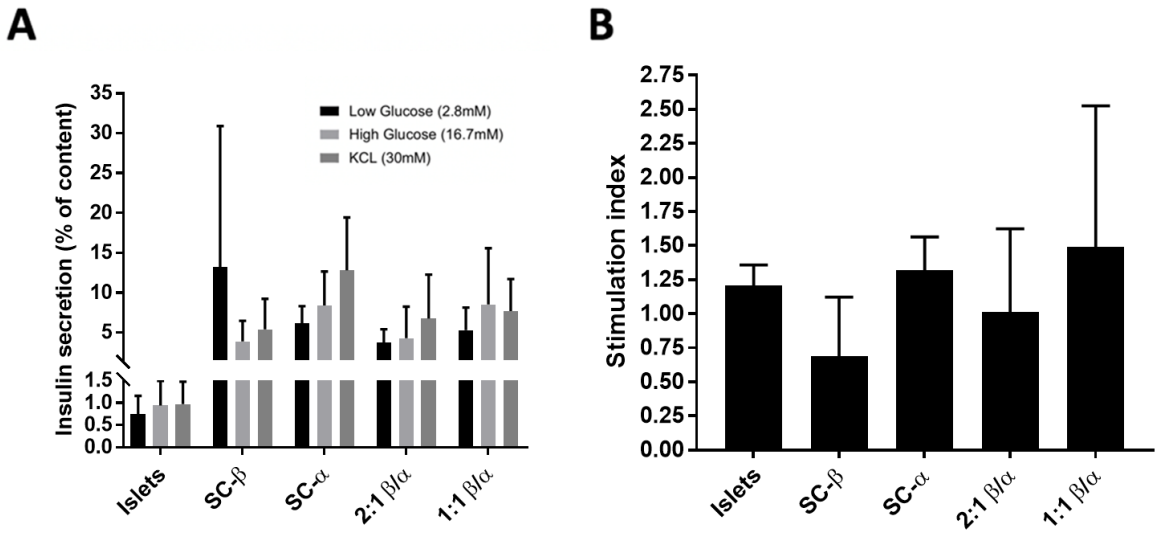

Figure 11. GSIS performance of polyhormonal cell populations.

(A) INSULIN secretion for each GSIS condition for each cell population. SC-derived cells secrete significantly more INSULIN than the Islets which suggests these populations were polyhormonal and not far along into the differentiation. (B) Stimulation index of fold change in INSULIN secretion between low and high glucose conditions.

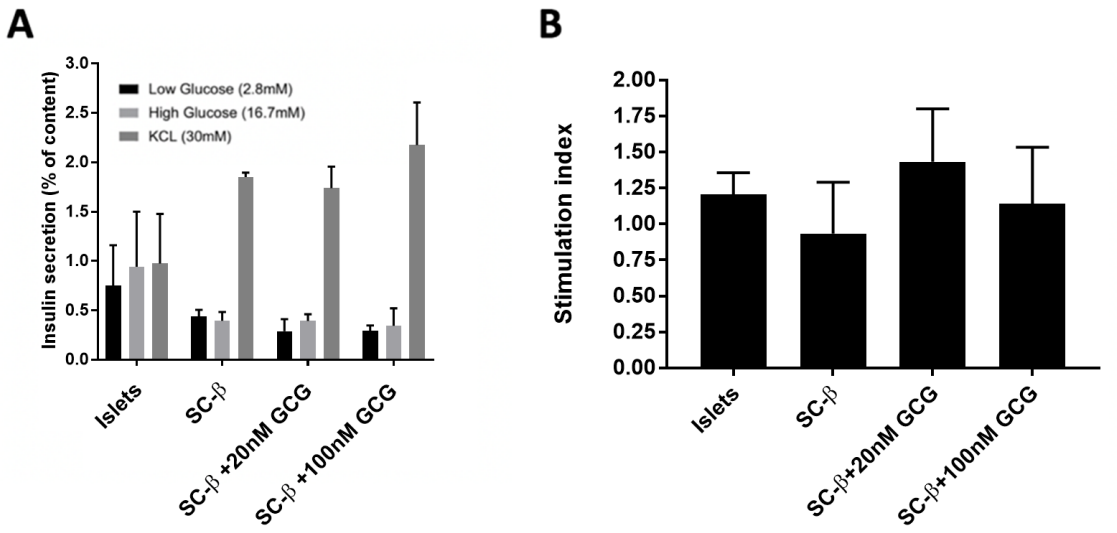

Figure 12. GSIS performance of monohormonal cell populations.

(A) INSULIN secretion for each GSIS condition for each cell population. SC-derived cells secrete significantly less INSULIN than the islets from low to high glucose. (B) Stimulation index of fold change in INSULIN secretion between low and high glucose conditions.

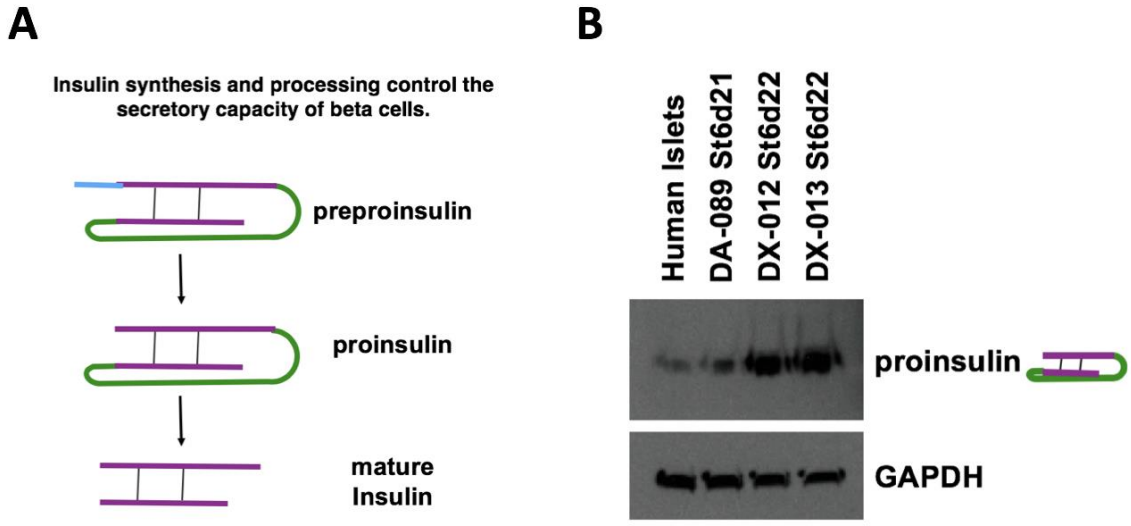

Figure 13. Unpublished observations of 1016 cell line.

(A) Schematic of INSULIN processing in normal β-cells. (B) Western blot by E. Rosado-Oliveri showing pro-INSULIN accumulation during β-cell differentiations in 1016 cells (DX-012 and DX-013) compared to HUES8 cells (DA-089) and Islets.

Results summary

Targeting of the *INSULIN* and *GLUCAGON* genomic loci was successful due to the optimization of the CRISPR/Cas9 system and use of homologous recombination enhancing small molecules. eCas9 produces more positively targeted colonies than unmodified Cas9 and RS-1 is the most efficient small molecule for HDR. The *INSULIN*, *GLUCAGON*, and dual *INSULIN-GLUCAGON* reporter lines had high fidelity and were able to be successfully differentiated into *INSULIN*- and *GLUCAGON*-expressing cells. 2D differentiations for both α- and β-cells proved to be less than efficient at producing mono-hormonal cell populations and the α-cell protocol proved to be toxic in stage 6 unless supplemented with conditioned medium from the β-cell differentiations. GSIS performance was unsatisfactory as the fluorescence sorted populations of cells tended to be polyhormonal and secreted far more INSULIN than normal Islets due to their immaturity. β-cell differentiations that were continued weeks into stage 6 did become mono-hormonal but GSIS performance was unsatisfactory due to very low INSULIN secretion from low to high glucose. Low GSIS performance was also observed in the islet control samples, though the total amount of INSULIN secreted was closer to the average for Islets than any of the SC-differentiated samples. Later observations revealed that the 1016 cell line has an accumulation of pro-INSULIN which could suggest defective INSULIN processing or misfolding.

Chapter IV.

Discussion

Type I diabetes is a debilitating and sometimes fatal disease that affects 15-30 million people worldwide (World Health Organization, 2012). Though exogenous INSULIN injections are the primary method of controlling glucose, they can never reach the level of efficiency found in endogenous INSULIN secreted from β-cells. While cell replacement therapy presents a window of opportunity for patients, the current supply comes from cadaveric Islets which are limited in both quality and quantity. Patient-derived iPSC differentiated β-cells would be the ultimate solution for these issues, providing unlimited numbers of cells while avoiding the immunosuppressant obligations of cadaveric transplants. To reach this stage, there needs to be a fully optimized differentiation protocol to produce all the cells needed for anything from β-cells to whole Islets.

The purpose of this study was to take two differentiation protocols for α- and β-cells, respectively, and aggregate cells together in hopes that the aggregates would mimic the INSULIN secretion of normal human Islets. To achieve this goal, fluorescent reporter lines targeted to the *INSULIN* and *GLUCAGON* genes were generated in the 1016 induced pluripotent stem cell line using CRISPR/Cas9 gene editing. These lines were then differentiated and sorted for mCherry fluorescence, isolating populations of *INSULIN*- and *GLUCAGON*-expressing cells that were then aggregated together in 96

well plates. These aggregates were subjected to GSIS and INSULIN secretion and content were analyzed with an ELISA and compared to normal human Islets.

To generate the reporter lines, guide RNAs were tested and optimized to select the ones that produced the most indels, and thus the highest cutting efficiency. For *INSULIN*, gRNA7 proved to be the most efficient at cutting, especially after being ligated into the eCas9 protein. While most cell lines can be edited with the normal version of the Cas9 protein, stem cells (specifically HUES8 cells) seem to be much more resistant and thus need extra effort. This is especially the case for creating knock-in lines since homology-directed repair (HDR) is not the primary mechanism of DNA damage repair (Song *et al.*, 2016). For *GLUCAGON*, the 3' UTR was too AT rich for the NGG PAM sequence for cas9 so Cpf1 was used in its place due to its TTTN PAM sequence. Using TIDE analysis, gRNA5 with a 13.4% cutting efficiency was selected for *GLUCAGON* targeting. Differences in the two strains of Cpf1 were observed with LbCpf1 being about 1-2% more efficient at cutting than the AsCpf1 strain for the same guide sequence.

After selecting the most efficient guides, two small molecule homologous recombination enhancing drugs were used to try and increase the number of positively targeted clones. RS-1 and L755507 were able to give 37.8% and 20.5% HDR for the *INSULIN* 5' homology arm compared to 10% for no compound at all. However, in HUES8 cells, these efficiencies were only seen in cells that were electroporated with eCas9 guides. For cells electroporated with regular Cas9 guides there was no improvement in HDR in any of the sample groups and no positive clones were obtained. In a separate experiment, an NHEJ inhibitor SCR7 was used with regular Cas9 and no drug resistant colonies were obtained (data not shown). For this reason, we propose that

to create knock-in lines in stem cells it is necessary to promote double stranded breaks (gRNA) and to stimulate HDR (RS-1, L755507, etc.). For certain genes like *INSULIN*, eCas9 alone is sufficient to obtain a small number of positive clones but given the variability it is better to obtain as many as possible.

Using the optimized guides and RS-1 compound, three reporter cell lines were generated: HUES8-IRN, 1016-GRN, and 1016 IRN-GGN. All lines showed a high fidelity of reporter expression driven by the targeted gene. A 1016-IRN line was previous generated by Jeff Davis and José Rivera-Feliciano of the Melton lab and was used in this study as a source of the SC-β cells for the GSIS experiment and as a template for the dual IRN-GGN reporter line. While a HUES8-IRN line was generated in this study, the differentiation efficiency was low, and the line did not adapt well in 3D culture. For this reason, the 1016 lines were used for the GSIS experiment as the retargeted HUES8 lines were not ready by the time the experiment started. In addition, *GLUCAGON* still proved difficult to target in HUES8 which is why the 1016-GRN line was used instead.

For the sake of time and to spare a laborious process of 3D adapting each reporter line, 15 cm plates were used to 2D differentiate the 1016 IRN and GRN lines with the belief that it would allow for more replications of the experiment without losing time for the adaptation. Though 2D was less efficient, there were few problems translating the β-cell protocol. For the SC-α cell protocol, the addition of LDN at Stage 4 day 2 of the protocol induced a greater population of polyhormonal *GLUCAGON*-expressing cells which did not have deleterious effects on cell viability. However, cell viability problems were encountered in stage 6. Cells began to die at an alarming rate once they entered stage 6 and began receiving PdBU to drive monohormonal expression. To combat this,

conditioned medium from the stage 6 β-cell protocol was filtered through a 22uM filter and added to PdBU to serve as the S6 media for the SC-α cell differentiations. This seemed to stall the cell death without compromising the number of *GLUCAGON*-expressing cells when looked at under the fluorescent scope. Though it is not known why the cells began to die in stage 6 it may have to do with the way the α-cells are arranged in a normal islet (Cabrera *et al.,* 2006). Observations under the microscope showed fluorescent cells start to clump together in stage 5 and were typically the last ones to die off in stage 6. It was hypothesized that a secreted activity present in the conditioned medium had positive effects on the clustered cells, keeping them alive.

Once both 2D protocols were optimized, the IRN and GRN reporter lines were differentiated towards an α- or β-cell fate, sorted by fluorescence, and then reaggregated into 5000 cell populations for GSIS. While the theory was that populations of α- and β-cells together would enhance INSULIN secretion during GSIS, the data did not demonstrate this. In all conditions the amount of INSULIN secreted was anywhere from 5-10x higher than in a normal islet. After discussion with Ali Rezania, it was thought that these cell populations were still entirely polyhormonal and would therefore dump INSULIN at an erratic rate given their lack of maturity and regulatory genes controlling INSULIN secretion. This can be supported by the review of pancreas development conducted by Gittes (2009) explaining that polyhormonal cells are a hallmark of early embryonic endocrine cells. In the three batches of sorted cells around stage 6 day 21, there was high variability and all samples secreted far more INSULIN than the islet controls. In addition, when calculating the stimulation indices for each sample, none seemed to have a large change in INSULIN secretion from low to high glucose. Thus, we

reasoned that SC-differentiated cells needed more time to become mature which was difficult given the cell viability issues with the SC-α cell protocol.

Of the two batches of cells that were carried well into stage 6 (7 weeks and 8 weeks respectively) the GSIS performance was drastically different than the other batches. For these populations, only a fraction of INSULIN was secreted in comparison to the islet controls and KCl challenge values. Since these cells were mono-hormonal we speculate that the regulatory genes controlling INSULIN secretion were turned on thus accounting for the drastic change between the two experiments. What was puzzling about this result was that administration of exogenous GLUCAGON protein did little to stimulate the INSULIN secretion from low to high glucose. Shortly after, unpublished data from E. Rosado-Olivieri showed that in differentiations of the 1016 cell line there was a large accumulation of pro-INSULIN which was not seen in HUES8 differentiations and islet controls. Taken together this suggests that there could potentially be an issue with INSULIN processing or misfolding that could account for the failed GSIS results. An additional explanation could be a flaw in the assay design given that the fold changes for INSULIN secretion between low and high glucose were also low in the islet controls. However, compared to the SC-differentiated samples it still appeared that the INSULIN stored in the islets was able to be released given the small difference in INSULIN secretion between the high glucose and KCl challenges. This also points to a possible issue in glucose sensitivity for the 1016 line.

Given the issues with the 1016 cell line it is difficult to extrapolate whether the incorporation of isolated α-cells can improve the GSIS function of β-cells or if it is an unnecessary addition. Ideally this study should be repeated in HUES8 targeted cells and

in 3D spinner culture to avoid working with a line shown to have a pro-INSULIN accumulation and α-cell stage 6 viability issues. In addition, the 3D culture conditions would more likely allow for the isolation of true mono-hormonal cell populations. Given the time frame for which this study was completed, there was a serious limitation given the flaws of the cell line used. However, the generated lines can still be used to optimize differentiation protocols and perhaps even be repaired if the pro-INSULIN issue can be solved.

The generation and co-culture of α- and β-organoids should have improved the pancreatic function during glucose challenges when compared to β-organoids alone. Given the homeostatic relationship between INSULIN and GLUCAGON we speculated that combining the cell types together would create a stronger, islet like structure which could be transplanted for efficient glucose control in Type I diabetic patients. Pancreatic Islets rely on a vast network of paracrine and autocrine signaling which is not well understood and thus stem cell derived endocrine cells may need the support of additional endocrine or exocrine cell types (Caicedo et al., 2013). Given the unique structure of Islets, β-cells come into contact with both α- and delta cells preventing regions comprised of a single endocrine cell type. Therefore, it is possible that organoids of both cell types may need to be generated and co-cultured with β-organoids for successful GSIS stabilization.

In addition, in human Islets, the endocrine cells are distributed along blood vessels which may contain more smooth muscle than rat Islets. This difference could result in vascular tissue being a potential site for important paracrine signaling. Co-culture with contractile vascular cells could be another option to produce fully functional

islet organoids. Though ultimately the α- and β-organoid co-culture proved to be unsuccessful in enhancing glucose responsiveness, it nonetheless provides a basis for the incorporation of multiple pancreatic cell types for stem cell derived transplants. A robust approach to cell replacement therapy may prove to be the most effective avenue for the treatment of diabetes.

Appendix

SC-directed β-Cell Differentiation Protocol v8.0

Stage	Day	Media	Factors	Dilution	Final [ ]	Vol/250 ml	Vol/300 ml
Stage 0	1(24hrs)	mTeSR—half feed					
Stage 0	2(48hrs)	mTeSR					
Stage 1	1	S1	Activin- A	1:100	100ng/ml	2.5 ml	3.0 ml
			CHIR99021	1:3333	1.4ug/ml	75 ul	90 ul
Stage 1	2	S1	Activin- A	1:100	100ng/ml	2.5 ml	3.0 ml
Stage 1	3	N/A	----------	----------	----------	----------	----------
Stage 2	1	S2	KGF	1:1000	50ng/ml	250 ul	300 ul
Stage 2	2	N/A	----------	----------	----------	----------	----------
Stage 2	3	S2	KGF	1:1000	50ng/ml	250 ul	300 ul
Stage 3	1	S3	KGF	1:1000	50ng/ml	250 ul	300 ul
			LDN193189	1:5000	200nM	50 ul	60 ul
			Sant-1	1:4000	0.25uM	62.5 ul	75 ul
			RA	1:5000	2uM	50 ul	60 ul
			PDBU	1:2000	500nM	125 ul	150 ul
			RhoK Inh	1:1000	10uM	250 ul	300 ul
Stage 3	2	S3	KGF	1:1000	50ng/ml	250 ul	300 ul
			Sant-1	1:4000	0.25uM	62.5 ul	75 ul
			RA	1:5000	2uM	50 ul	60 ul
			PDBU	1:2000	500nM	125 ul	150 ul
			RhoK Inh	1:1000	10uM	250 ul	300 ul
Stage 4	1	S3	KGF	1:1000	50ng/ml	250 ul	300 ul
			Sant-1	1:4000	0.25uM	62.5 ul	75 ul
			RA	1:100,000	0.1uM	2.5 ul	3.0 ul
			RhoK Inh	1:1000	10uM	250 ul	300 ul
			Activin A	1:2000	5ng/ml	125 ul	150 ul
Stage 4	2	N/A	----------	----------	----------	----------	----------
Stage 4	3	S3	KGF	1:1000	50ng/ml	250 ul	300 ul
			Sant-1	1:4000	0.25uM	62.5 ul	75 ul
			RA	1:100,000	0.1uM	2.5 ul	3.0 ul
			RhoK Inh	1:1000	10uM	250 ul	300 ul
			Activin A	1:2000	5ng/ml	125 ul	150 ul
Stage 4	4	N/A	----------	----------	----------	----------	----------
Stage 4	5	S3	KGF	1:1000	50ng/ml	250 ul	300 ul
			Sant-1	1:4000	0.25uM	62.5 ul	75 ul
			RA	1:100,000	0.1uM	2.5 ul	3.0 ul
			RhoK Inh	1:1000	10uM	250 ul	300 ul
			Activin A	1:2000	5ng/ml	125 ul	150 ul
Stage 5	1	BE5	Sant-1	1:4000	0.25uM	62.5 ul	75 ul
			Beta-Cellulin	1:5000	20ng/ml	50 ul	60 ul
			XXI	1:10,000	1uM	25 ul	30 ul
			Alk5i	1:10,000	10uM	25 ul	30 ul
			T3	1:10,000	1uM	25 ul	30 ul
			RA	1:100,000	0.1uM	2.5 ul	3.0 ul
Stage 5	2	N/A	----------	----------	----------	----------	----------
Stage 5	3	BE5	Sant-1	1:4000	0.25uM	62.5 ul	75 ul
			Beta-Cellulin	1:5000	20ng/ml	50 ul	60 ul
			XXI	1:10,000	1uM	25 ul	30 ul
			Alk5i	1:10,000	10uM	25 ul	30 ul
			T3	1:10,000	1uM	25 ul	30 ul
			RA	1:100,000	0.1uM	2.5 ul	3.0 ul
Stage 5	4	N/A	----------	----------	----------	----------	----------
Stage 5	5	BE5	Beta-Cellulin	1:5000	20ng/ml	50 ul	60 ul
			XXI	1:10,000	1uM	25 ul	30 ul
			Alk5i	1:10,000	10uM	25 ul	30 ul
			T3	1:10,000	1uM	25 ul	30 ul
			RA	1:400,000	0.1uM	0.625 ul	0.75 ul
Stage 5	6	N/A	----------	----------	----------	----------	----------
Stage 5	7	BE5	Beta-Cellulin	1:5000	20ng/ml	50 ul	60 ul
			XXI	1:10,000	1uM	25 ul	30 ul
			Alk5i	1:10,000	10uM	25 ul	30 ul
			T3	1:10,000	1uM	25 ul	30 ul
			RA	1:400,000	0.1uM	0.625 ul	0.75 ul
Stage 6	Odds	S3	----------	----------	----------	----------	----------
Stage 6	Evens	N/A	----------	----------	----------	----------	----------

SC-directed α-cell differentiation protocol v3.0

Stage	Day	Media	Factors	Dilution	Final [ ]	Vol/250mL	Vol/300mL
Stage 1	1	S1	Activin-A CHIR99021	1:100 1:3333	100ng/mL 1.4µg/mL	2.5mL 75µL	3.0mL 90µL
Stage 1	2	S1	Activin-A	1:100	100ng/mL	2.5mL	3.0mL
Stage 1	3	N/A	--------	--------	--------	--------	--------
Stage 2	1	S2	KGF	1:1000	50ng/mL	250µL	300µL
Stage 2	2	N/A	--------	--------	--------	--------	--------
Stage 2	3	S2	KGF	1:1000	50ng/mL	250µL	300µL
Stage 3	1	S3	RA	1:5000	2µM	50µL	60µL
Stage 3	2	S3	LDN193189 RA	1:5000 1:5000	200nM 2µM	50µL 50µL	60µL 60µL
Stage 4	1	S3	LDN193189	1:5000	200nM	50µL	60µL
Stage 4	2	S3	--------	--------	--------	--------	--------
Stage 4	3	N/A	--------	--------	--------	--------	--------
Stage 4	4	S3	--------	--------	--------	--------	--------
Stage 4	5	N/A	--------	--------	--------	--------	--------
Stage 5	1	S3	Alk5 inhibitor	1:10,000	10µM	25µL	30µL
Stage 5	2	N/A	--------	--------	--------	--------	--------
Stage 5	3	S3	Alk5 inhibitor	1:10,000	10µM	25µL	30µL
Stage 5	4	N/A	--------	--------	--------	--------	--------
Stage 5	5	S3	Alk5 inhibitor	1:10,000	10µM	25µL	30µL
Stage 5	6	N/A	--------	--------	--------	--------	--------
Stage 5	7	S3	Alk5 inhibitor	1:10,000	10µM	25µL	30µL
Stage 6	Odds	S3	PdBU	1:2000	500nM	125µL	150µL
Stage 6	Evens	N/A	--------	--------	--------	--------	--------

Tables

IRN targeting

Purpose	**Primer name**	**Orientation**	**Sequence**
INS 5' arm	kx125	Fwd	GGGGTCAGGTGCACTTTTT
INS 5' arm	mCherry geno R1	Rev	CGCATGAACTCCTTGATGATGG
IRN 3' arm	kx127	Fwd	GGTCCCTCGAAGAGGTTCAC
IRN 3' arm	kx231	Rev	AAAGGTGCAGATTGGTTTGG
INS intact allele	INS-seq3	Fwd	ACTGTGTCTCCCTGACTGTGTC
INS intact allele	INS SURV Rev1	Rev	TAGAACCTGGGAGGGCTAGG

GRN targeting

Purpose	**Primer name**	**Orientation**	**Sequence**
GCG 5' arm	GCG 5' ver fwd2	Fwd	CACTTGTGCATGTTACCAGTGG
GCG 5' arm	mCherry geno R1	Rev	CGCATGAACTCCTTGATGATGG
GCG 3' arm	kx127	Fwd	GGTCCCTCGAAGAGGTTCAC
GCG 3' arm	173	Rev	CAAGGCATGATGGGCAAACT
GCG intact allele	43	Fwd	AGAGTAGTGAGAACTGGACACC
GCG intact allele	38	Rev	GTGGCTACCAGTTCTTCTAT

IRN-GGN targeting

Purpose	**Primer name**	**Orientation**	**Sequence**
GCG 5' arm	GCG 5' ver fwd2	Fwd	CACTTGTGCATGTTACCAGTGG
GCG 5' arm	eGFP geno R1	Rev	CAGCTTGCCGTAGGTGGCAT
GCG 3' arm	kx127	Fwd	GGTCCCTCGAAGAGGTTCAC
GCG 3' arm	173	Rev	CAAGGCATGATGGGCAAACT
GCG intact allele	43	Fwd	AGAGTAGTGAGAACTGGACACC
GCG intact allele	38	Rev	GTGGCTACCAGTTCTTCTAT

gRNA Sequences for Targeting

Gene	**Guide Sequence**
INSULIN	GCCGACAGGCATGGCCGCTTT
GLUCAGON	ATCTCTAGGAAATAACTATA

Definition of Terms

Cas9: CRISPR endonuclease with an NGG PAM sequence which produces a blunt ended cut in double stranded DNA and is primarily used in molecular biology for gene editing

Cpf1: CRISPR endonuclease with a TTTN PAM sequence which produces a staggered cut in double stranded DNA and is used for its ability to edit regions of genomic DNA that are high in A/T nucleotides

CRISPR system: Clustered Regularly Interspaced Short Palindromic Repeats, family of sequences generated by bacteria as a defense mechanism against foreign invaders, specific sequences are obtained from viruses that have previously attacked the bacterium and RNA harboring those sequences guides the endonuclease to recognize and cut exogenous DNA

Electroporation: a physical transfection method that uses an electrical pulse to create temporary pores in cell membranes through which substances like nucleic acids can pass into cells.

FACS analysis: Fluorescence activated cell sorting, a type of flow cytometry which separates heterogeneous populations of cells into 2 or more groups based on fluorescent signaling

GLUCAGON: Hormone secreted by pancreatic α-cells when blood glucose levels are low to increase blood glucose concentration

gRNA: guide RNA, sequence which guides the CRISPR endonuclease to the region of interest and then binds using complementary base pairing which allows the protein to cut the DNA

GSIS: Glucose stimulated insulin secretion, the process by which pancreatic β-cells secrete insulin based on concentrations of glucose, this can be used as a test for the functional analysis of differentiated β-cells

Homology directed repair: mechanism of repairing double stranded DNA breaks through the use of homologous recombination

Homologous Recombination: type of genetic recombination where nucleotide sequences are exchanged between two similar or identical strands of DNA

Indel: term used to describe an insertion or deletion of bases in the genome of an organism

Insulin: hormone secreted by pancreatic β-cells when blood glucose levels are high to reduce blood glucose concentration

L755507: a small molecule compound which acts as a β3-adrenergic receptor agonist and has been shown to increase the rate of homologous recombination in cells

NHEJ: non-homologous end joining, a method of double stranded DNA damage repair which does not require the use of a homologous template and directly ligates broken strands which can be error prone and potentially result in the formation of indels within the DNA sequence

NLS: Nuclear localization sequence, sequence of amino acids that tags a protein for import into the cell's nucleus

PAM sequence: protospacer adjacent motif, a 2-6 base pair sequence which immediately follows the DNA sequence targeted by the CRISPR system and is essential for the recognition and cutting of the target sequence, examples are the NGG and TTTN PAM sequences of the Cas9 and Cpf1 endonucleases in which N represents any nucleotide

Pancreatic Islet: regions of the pancreas containing its endocrine cells, importance in the maintenance of blood glucose levels and contain alpha (α-), beta (β-), gamma, epsilon, and PP cells

Pluripotent stem cell: refers to the ability of a stem cell to differentiate into any of the endoderm (interior stomach lining, gastrointestinal tract, the lungs), mesoderm (muscle, bone, blood, urogenital), or ectoderm (epidermal tissues and nervous system) germ layers, can be used in molecular biology for the directed differentiation of any cell type

Reporter cell line: a fluorescent protein in a plasmid which is integrated into a cell genome to measure the expression of a certain gene

RS-1: a small molecule compound which enhances homologous recombination in cells by stimulation of the RAD51 protein

Surveyor assay: assay that detects base substitutions and indels produced by gene editing and can provide an estimate of the efficiency of the editing

TIDE analysis: assay to determine the frequency of cuts generated in a pool of cells by gene editing through the analysis of trace sequences from PCR products flanking the targeting region

Targeting: a molecular biology technique which allows for the modification of a specific gene of interest

UTR: untranslated region, region of genome which can be upstream (5’ UTR) or downstream (3’ UTR) of the coding region of an mRNA strand and are not translated into protein

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