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GENE EXPRESSION AND PROFILING OF HUMAN ISLET CELL SUBTYPES

A Master's Thesis Presented

By

DAVID MICHAEL BLODGETT

Submitted to the Faculty of the
University of Massachusetts Graduate School of Biomedical Sciences, Worcester
In partial fulfillment of the requirements for the degree of

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DEPARTMENT OF MEDICINE – DIABETES DIVISION

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Department of Medicine

25-JULY-2012

DEDICATION

This work is dedicated to all of the organ donors who made this research and all scientific research possible. Through organ donation, human lives can be saved immediately and we can use the material to perform our studies in order to help us treat and prevent future medical needs. The experiments in this thesis were performed using human pancreatic islet donations organized and allocated by the Integrated Islet Distribution Program. Thank you to all of the donors and their families who made this research possible.

ACKNOWLEDGEMENTS

ABSTRACT

Background: The endocrine pancreas contains multiple cell types co-localized into clusters called the Islets of Langerhans. The predominant cell types include alpha and beta cells, which produce glucagon and insulin, respectively. The regulated release of these hormones maintains whole body glucose homeostasis, essential for normal metabolism and to prevent diabetes and complications from the disease. Given the heterogeneous nature of islet composition and absence of unique surface markers, many previous studies have focused on the whole islet. Sorting islet cells by intracellular hormone expression overcomes this limitation and provides pure populations of individual islet cell subsets, specifically alpha and beta cells. This technique provides the framework for characterizing human islet composition and will work towards identifying the genetic changes alpha and beta cells undergo during development, growth, and proliferation.

Methods: Human islets obtained from cadaveric donors are dissociated into a single cell suspension, fixed, permeabilized, and labeled with antibodies specific to glucagon, insulin, and somatostatin. Individual alpha, beta, and delta cell populations are simultaneously isolated using fluorescence activated cell sorting. Candidate gene expression and microRNA profiles have been obtained for alpha and beta cell populations using a quantitative nuclease protection assay. Thus

far, RNA has been extracted from whole islets and beta cells and subjected to next generation sequencing analysis.

Results: The ratio of beta to alpha cells significantly increases with donor age and trends higher in female donors; BMI does not appear to significantly alter the ratio. Further, we have begun to investigate the unique gene expression profiles of alpha and beta cells versus whole islets, and have characterized the microRNA profiles of the two cell subsets.

Conclusions: By establishing methods to profile multiple characteristics of alpha and beta cells, we hope to determine how gene, miRNA, and protein expression patterns change under environmental conditions that lead to beta cell failure or promote beta cell development, growth, and proliferation.

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LIST OF SYMBOLS AND ABBREVIATIONS

NHP – non-human primates
PP – pancreatic polypeptide
GLUT4 – glucose transporter type 4
BMI – body mass index
IIDP – Integrated Islet Distribution Program
NIDDK – National Institute of Diabetes and Digestive and Kidney Diseases
FACS – fluorescence activated cell sorting
HBSS – Hank's balanced salt solution
rpm – revolutions per minute
EDTA – ethylenediaminetetraacetic acid
PBS – phosphate buffered saline
PFA – paraformaldehyde
BSA – bovine serum albumin
WB – wash buffer
VRC – vanadyl ribonucleoside complexes
PB – pacific blue
AF488 – Alexa Fluor 488
APC – allophycocyanin
α–GCG$^+$ – alpha cells
β–INS$^+$ – beta cells
δ–SST$^+$ – delta cells
FSC-A – forward scatter-area
SSC-A – side scatter-area
FSC-H – forward scatter height
qRT-PCR – quantitative real time polymerase chain reaction
qPCR – quantitative polymerase chain reaction
RNase – RNA degradation enzymes
qNPA – quantitative nuclease protection assay
HTG – High Throughput Genomics Molecular
FFPE – formalin fixed, paraffin embedded
RIN – RNA integrity number
miRNA – microRNA
BGI – Beijing Genomics Institute
DSN – double stranded nuclease
FBS – fetal bovine serum
RPKM – Reads Per Kilobase transcriptome per Million mapped reads

xii

PREFACE

All experiments and data presented in this thesis are original in nature. They will serve as the springboard for future publications. All figures are also original, however, data analysis of next generation sequencing data and subsequent presentations of data were performed with Jia Xu of the Bioinformatics Core and are shown with her consent.

We have performed many similar experiments on pancreatic islets isolated from nonhuman primates (NHP) in collaboration with Charles Roberts, Julie Carroll, and Kevin Grove at the Oregon Health & Science University. The benefit of these results is described in Chapter 5 as a future direction, however, the experimental data are not presented here.

I have worked with Dale Greiner, Dean Brostowin, Susanne Pechhold, and Rita Bortell to extract transplanted human islets from mouse kidney. We eventually hope to isolate the transplanted islets and measure gene expression changes that might explain beta cell proliferation. Currently, our yield of extracted islets precludes completion of these experiments and the results have not been included in this thesis presentation.

A detailed introduction precedes each experimental chapter. The Chapter 1 introduction serves as an outline to focus on the scientific and clinical significance of these studies.

CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

Pancreatic Islet Structure and Function

The human pancreas is a gland that performs both endocrine and exocrine functions. The endocrine portion includes the Islets of Langerhans, which are a heterogeneous arrangement of alpha, beta, delta, PP, and epsilon cells that secrete hormones[1,2]. The pancreatic islets help to maintain total body glucose homeostasis through the regulated release of insulin and glucagon from beta and alpha cells, respectively. In response to food consumption and gastric emptying, blood glucose levels rise and insulin secretion from beta cells increases. Increased insulin secretion mediates glucose transporter type 4 (GLUT4) translocation to the surface of fat and muscle cells thereby increasing glucose uptake out of the bloodstream and into the cells where it is metabolized. In times of fasting, when there is little consumed glucose in the bloodstream, alpha cells respond by secreting glucagon. Glucagon release initiates glycogenolysis in the liver and adds additional sugar to the bloodstream. In addition to regulating blood glucose concentrations, insulin and glucagon concentrations are controlled by negative feedback. When insulin levels are high, this inhibits glucagon release and promotes glucose uptake. When glucagon levels are high, beta cell release of insulin is inhibited and glucose is produced by glycogenolysis or gluconeogenesis, in extended fasting. By working together, these two molecules

maintain a nearly constant blood glucose concentration to ensure that the body has enough energy without permitting dangerous fluctuations in blood glucose levels[3]. In patients with type 1 diabetes, glucose homeostasis is interrupted by a lack of endogenous insulin. In type 2 patients, increased insulin resistance or decreased insulin sensitivity result in the manifestation of the disease.

Human Islet Transplant Successes and Limitations

In order to combat the destruction of beta cells in a patient with type 1 diabetes, the first human islet transplant occurred in 1990. Its success at establishing insulin independence for over three weeks provided the proof of principle to pursue clinical trials[4]. The Edmonton Protocol followed up on this success by transplanting islets into diabetic patients without the need for glucocorticoid therapy over the course of two or three islet infusion from multiple donors[5]. These transplants were completed with a 67% success rate at establishing insulin independence for a median of 15 months post-transplant. Unfortunately, however, complete insulin independence in transplant patients hovered around 10% after a five year post-transplant follow up[6]. A recent review in *Diabetes Care* (2012) analyzed 677 islet transplant cases that have occurred since 1999. This study showed that islet transplant success has improved markedly since the initial transplants took place. For islet donors who received this treatment between 2007-2010, approximately 44% maintained insulin independence for at least three years[7]. Overall, although islet transplants are not a true cure for type

1 diabetes, they have proven to increase C-peptide values, reduce hypoglycemic events, reduce average hemoglobin A1C values, and increase overall glucose stability[7,8].

As described, current attempts to cure diabetes by islet transplantation have had a lower than ideal success rate. In addition to a general shortage in transplantable islets, much of the inconsistency in islet transplant success is due to uneven donor islet quality. Chapter III will show how the demographics for islet donors are highly variable and uncontrolled. Donors are of different ages, have different body mass indexes (BMI), display varying ratios of beta to alpha cells, and isolated islets are cultured from organ donors who have experienced varying cold ischemia times, which can affect islet cell health and viability[8,9]. Furthermore, upon transplant, islets are still subject to the autoimmune attack that caused the initial case of diabetes, the standard alloimmune rejection, the lack of a native environment (given their infusion into the portal vein), and an overall absence of beta cell proliferation[8]. A gap exists in our knowledge and treatment between observing that islet transplants can remove insulin dependence and cure type 1 diabetes versus establishing a consistent method for successfully obtaining and maintaining to make it happen consistently.

Research Focus on Islets and Beta Cells

In an effort to prevent, treat, and cure both types of diabetes, research has focused on multiple aspects of diabetes pathophysiology. In type 1 diabetes,

researchers have investigated what initiates and perpetuates the autoimmune response that results in a process where autoreactive T cells destroy insulin producing beta cells, thereby causing diabetes. Studies have centered on the autoantigens, such as insulin or glutamic acid decarboxylase, that serve as targets for autoimmune response, regardless of whether they are specifically expressed in beta cells, islets, or simply within the endocrine system[10,11].

The focus on identifying the cause of type 1 diabetes, which also serves as a potential adversary to successful islet transplants, is also a key factor in efforts to generate new beta cells. Borowiak and Melton (2009) summarize the three potential avenues for programming beta cells and encouraging their proliferation and regeneration. This research is important in order to overcome the limitations discussed above that might restrict the success rate of human islet transplants in offering a sustainable, long-term, and permanent cure for type 1 diabetes. Current research focuses on generating beta cells *de novo* from embryonic cells or induced pluripotent stem cells. Since these stem cells can be programmed to potentially be any cell in the body, experiments have focused on replicating embryonic development by proceeding in an *in vitro* stepwise fashion using protein growth factors or small molecules to induce beta cell development[12]. Cells can also be induced to regain pluripotency and then reprogrammed to form beta cells. Fibroblasts, hepatocytes, and even alpha cells have been used in attempts to produce functional beta cells that will organize into islet cell clusters[12,13]. This strategy could be useful for avoiding alloimmune rejection

complications. Finally, given how current islet transplantation works and given the impaired beta cell function in type 2 diabetes, research is being performed to understand what transcriptional regulatory factors control the existence of beta cell progenitors that transiently exist during neonatal life, but apparently disappear following growth and development. If these genetic factors could be identified, understood, and induced to higher expression levels, it might be possible to induce beta cell proliferation and increase or sustain beta cell mass following transplantation or reduced beta cell function[12].

Throughout this thesis, our main goal is to be able to identify the genetic and regulatory components that control beta cell growth in response to environmental stimuli and demographic factors. We focus most of our efforts on using flow cytometry to obtain pure populations of islet cell subsets. Upon establishing these techniques, we have employed quantitative nuclease protection and next generation sequencing analyses. These methodologies will help us begin to establish the beta cell transcriptome and ways to detect changes in gene expression. By identifying which proteins are specifically expressed in the beta cell using these technologies, we aim to correlate changes in gene expression within the beta cell to observed increases in beta cell mass and proliferation.

CHAPTER II

PANCREATIC ISLET SORTING INTO CELL SUBSETS

Introduction

Pancreatic islets are comprised of numerous cell types that perform specific endocrine functions. Alpha and beta cells work together in measuring blood glucose concentration and respond by releasing glucagon and insulin hormones, respectively, in order to maintain a human blood glucose level of 5 mM. Due to this heterogenous arrangement, an increased interest in determining the gene expression profiles of pancreatic islet cell subtypes at baseline and following manipulation has developed. A major limitation to pursuing these studies has centered on the difficulty in obtaining pure populations of alpha and beta cells that contain high quality RNA[14]. In order to direct gene expression studies to individual islet cell subsets, it is necessary to separate alpha, beta, and delta cells away from other material in the single cell suspensions. Flow cytometry serves as one method that has been used in multiple different ways to accomplish this goal.

Researchers have taken advantage of the light scattering and autofluorescence measuring properties of flow cytometry analysis to separate the different cell types. Following hydrodynamic focusing of the cells into a single-file stream, the laser intercepts the cells causing light scatter. Forward scatter detectors measure

how much light in the laser path is blocked, which determines the size of the cell. Side scatter detectors measure how much light is scattered at a 90° angle from the laser path, which determines the density and granularity of the cell. The granular nature of insulin secretory vesicles results in a higher side scatter component in beta cells versus alpha and other islet cell types and allows for the enrichment of the beta cell population[15,16].

To further enhance the enrichment of beta cells, sorting protocols have capitalized on the high concentration of zinc that beta cells require for insulin secretion[17]. Newport Green is a non-toxic, zinc-specific dye that labels beta cells[18]. Through combination with a live-dead cell marker and a ductal cell-specific antibody, the beta cell population can be further enriched[19,20]. This technique eliminates dead and ductal cells by creating gates that remove cells with probe-specific fluorescent properties. Cells that are Newport Green positive and have a high side scatter component are sorted to provide an enriched population of beta cells. Transgenic mouse islet cells can be separated into alpha and beta cells via expression of cell specific, genetic reporter molecules, however, these techniques are not applicable to islets isolated from human donors[21-23]. The latest method to purify individual islet cell subsets seeks to use cell surface markers that are specific to each islet cell subtype, similar to the way immunologic cells are isolated[24]. Grompe, Streeter, and colleagues have immunized mice with whole or dissociated human islets and collected hybridomas that demonstrate specific reactivity to alpha cells, pancreatic

endocrine or exocrine markers, and ductal cells. By staining human islets with a combination of these antibodies, this group can isolate specific populations of each islet cell subtype[25,26].

Each of these sorting methodologies essentially increases the concentration of alpha or beta cells from the heterogeneous mixture found in pancreatic islets, however, these techniques are not directly specific to any particular cell type. Granularity, zinc-composition, and combinations of nonspecific surface markers appear to enrich cell types of interest, but they do not provide pure populations. This report shows how a method designed to detect mouse alpha, beta, and delta cells based upon intracellular hormone content designed by *Pechhold et al.* has been adopted and optimized to isolate human islet cell subsets[27]. By dissociating human islets into a single cell suspension, fixing the cells to maintain intracytoplasmic content, and staining with insulin, glucagon, and somatostatin specific antibodies, exceedingly pure populations of beta, alpha, and delta cells, respectively, have been isolated.

Methods

Treatment of Islets

Islet Distribution All human islets are received from the Integrated Islet Distribution Program (IIDP) coordinated by the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK), the City of Hope, and a series of Islet

Isolation Centers situated within University Hospitals across the United States. The IIDP provides purity information and indicates the number of days the islets were cultured following isolation. Additionally, they provide demographic information such as gender, age, and BMI for each donor.

Preparation Figure 2.1 provides a brief outline of islet treatment upon receipt: islets are dissociated into a single cell suspension, fixed and permeabilized, labeled with antibodies for intracellular hormones, and sorted using fluorescence activated cell sorting (FACS).

Dissociation Islets are washed in Hank's Balanced Salt Solution (HBSS – Mediatech (21-023, with calcium and magnesium) and sedimented at 1200 revolutions per minute (rpm) for 4 minutes at 8°C. To begin dissociation, islets are first resuspended in 10 mM ethylenediaminetetraacetic acid (EDTA – Mediatech (46-034)) dissolved in phosphate buffered saline (PBS – Sigma (D8537)) and sedimented. The pellet is resuspended in 1 mL of PBS and 300 μL of trypsin/EDTA (1:50 dilution of 20μg/mL trypsin, 10 mM EDTA in PBS). This resuspension incubates for 10 minutes at room temperature and is pipetted up and down periodically to help loosen up the pellet into single cells. The pellet is washed in PBS, pelleted again, and resuspended in 1 mL PBS. An aliquot is stained with trypan blue to count the cells and assess viability.

Fixation The cells are fixed at 4°C with 4% paraformaldehyde (PFA) for 5 minutes prior to the addition of 0.1% saponin (Sigma – S47036). The cells,

fixation, and permeabilization buffers are incubated at 4°C for 25 minutes, prior to dilution with PBS. Cells are washed twice with PBS (spin at 1500 rpm, 5 minutes, 4°C) in a polystyrene container. The increased speed and use of a polystyrene tube prevents cell loss. Finally, the cells are resuspended in wash buffer (WB) comprised of RNase-free 0.2% bovine serum albumin (BSA – Equitech (BAH67)), 0.1% saponin, dissolved in PBS, and distributed for staining.

Intracellular Hormone Staining Prior to staining, all antibody buffers are incubated for 1 hour with RNase inhibitors vanadyl ribonucleoside complexes (VRC) and Murine RNase Inhibitor (New England BioLabs – M0314). Staining buffer contains 1% BSA, 0.1% Saponin, 7.5 mM VRC, and a 1:10 dilution of Murine RNase Inhibitor, all diluted in PBS. A chicken IgGY anti-bovine insulin antibody (ABI) manufactured by Gallus Immunotech is used to staining intracellular insulin hormone. The antibody is used at a 1:10 dilution in labeling experiments involving cells from greater than 1,000 islets and at a 1:25 dilution in studies with cells from fewer than 1,000 islets. Cells are stained for 25 minutes at 4°C and washed twice with WB. During the incubation step, the glucagon (Sigma – G2654) and somatostatin (GeneTex – GTX71935) antibodies are Zenon labled (Invitrogen) with Zenon Pacific Blue (PB) for Mouse IgG1 (Z25041) and Alexa Fluor 488 (AF488) for Mouse IgG1 (Z25002), respectively. Anti-glucagon is incubated in Zenon at 3.5 µg antibody/µL of Zenon and anti-somatostatin is incubated with Zenon at 2.4 µg antibody/µL. The Zenon labeling reaction is performed following the manufacturer's instructions. Insulin-stained cells are

incubated with anti-glucagon labeled with PB and anti-somatostatin labeled with AF488 for 5 minutes at 4°C, followed by the addition of donkey anti-chicken allophycocyanin (APC) labeled secondary antibody (Jackson ImmunoResearch (703-136-155)) at a 1:16 final dilution. Cells are then washed twice with WB, a third time with PBS, and resuspended in 1% PFA. Cells are passed through a 0.37 mm filter prior to sorting.

Sorting Fluorescence activated cell sorting was performed in the University of Massachusetts Medical School Flow Core Laboratory using a BD Biosciences FACSAria II Cell Sorter. All fluorochrome, laser, dichroic mirror, and bandpass filter parameters and settings are listed in Table 2.1.

Table 2.1 Flow Cytometer Settings

Fluorochrome	Excitation Max λ (nm)	Emission Max λ (nm)	Laser and λ (nm)	Dichroic Mirror	Bandpass Filter
Alexa Fluor 488 (AF488)	495	519	Blue - 488 nm	505 Long Pass	525/50
Pacific Blue (PB)	403	455	Violet - 405 nm	none	450/50
Allophycocyanin (APC)	650	660	Red - 633 nm	none	670/30

This table describes the excitation and emission parameters for each fluorochrome and the flow cytometer settings used to detect each molecule.
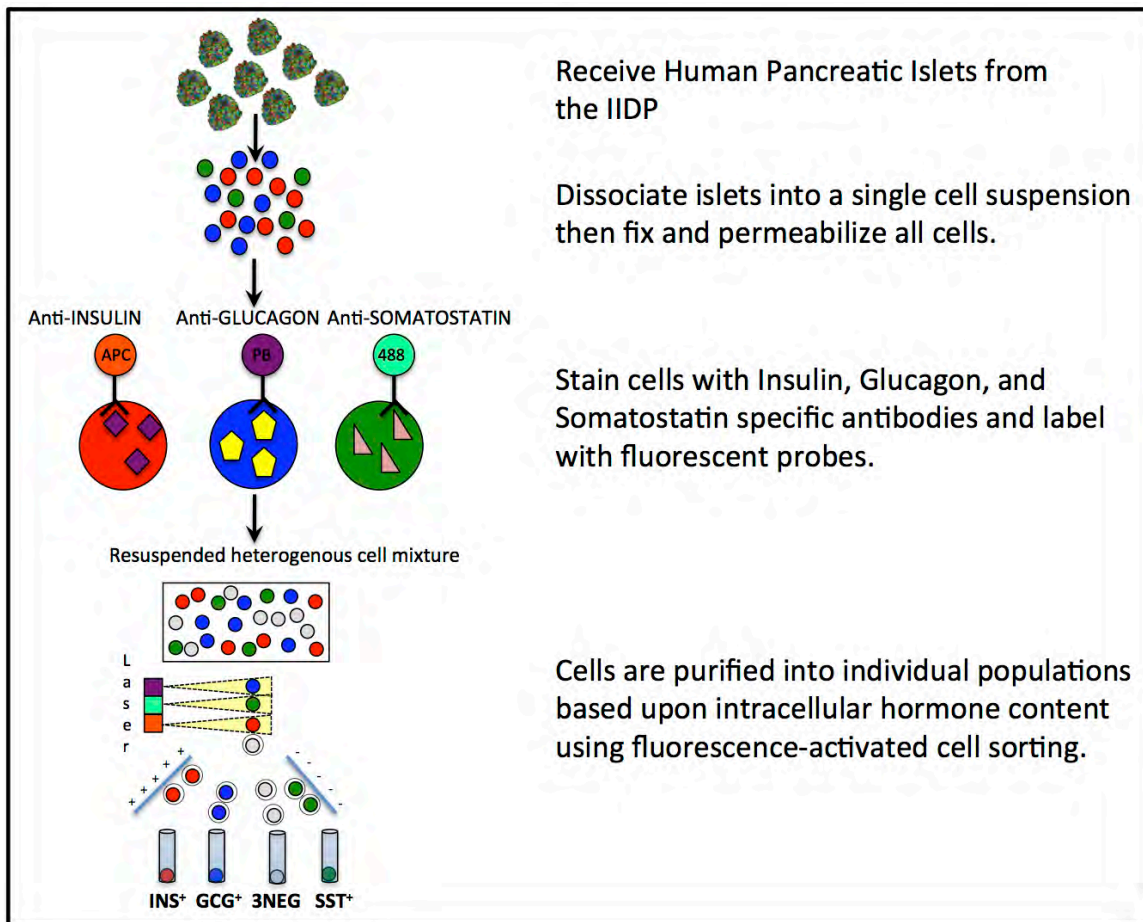
Figure 2.1 Islet Cell Purification Flow Diagram The human islet is comprised of a heterogeneous mixture of alpha (α–GCG$^+$), beta (β–INS$^+$), delta (δ–SST$^+$), and other cells, and they must be separated into individual populations before they can be studied independently[28]. We receive human islets from the IIDP, dissociate them into a single cell suspension, fix them with PFA, permeabilize them with saponin, and stain for intracellular hormone content. Using the hydrodynamic focusing mechanism of FACS, single cells are sorted into unique and pure populations.

Gating Hierarchy Figure 2.2 outlines the gating strategy used to isolate pure, single cell populations of human islet cell subsets. Debris is eliminated using a size gate in a forward scatter area (FSC-A) versus side scatter (SSC-A) plot (Figure 2.2A); particles outside of this gate are excluded from further analysis. Cells are next observed for insulin content in an APC (insulin) versus FSC-A plot (Figure 2.2B). All cells that form a distinct APC-positive population and can be distinguished from insulin negative cells are collected in the insulin gate. The cells excluded from the insulin gate (termed the inverse insulin gate) are plotted for PB (glucagon) versus AF488 intensity (somatostatin) (Figure 2.2C). All cells distinctly positive for PB or AF488 are collected in the glucagon and somatostatin gates, respectively. Doublets are excluded for each population by first observing each population on plot comparing FSC-Height (FSC-H) versus FSC-A. Doublet cells have a larger FSC-A component that does not correspond to the FSC-H component, due to their larger size, and they are consequently excluded from the insulin (Figure 2.2D) and glucagon (Figure 2.2E) positive cells. Second, the insulin positive cells are plotted against PB and AF488 (Figure 2.2F) and the glucagon positive cells are plotted against APC and AF488 (Figure 2.2G). The cells that are negatively labeled within each plot are the pure populations of each subset and they are dispensed for collection from the flow cytometer.
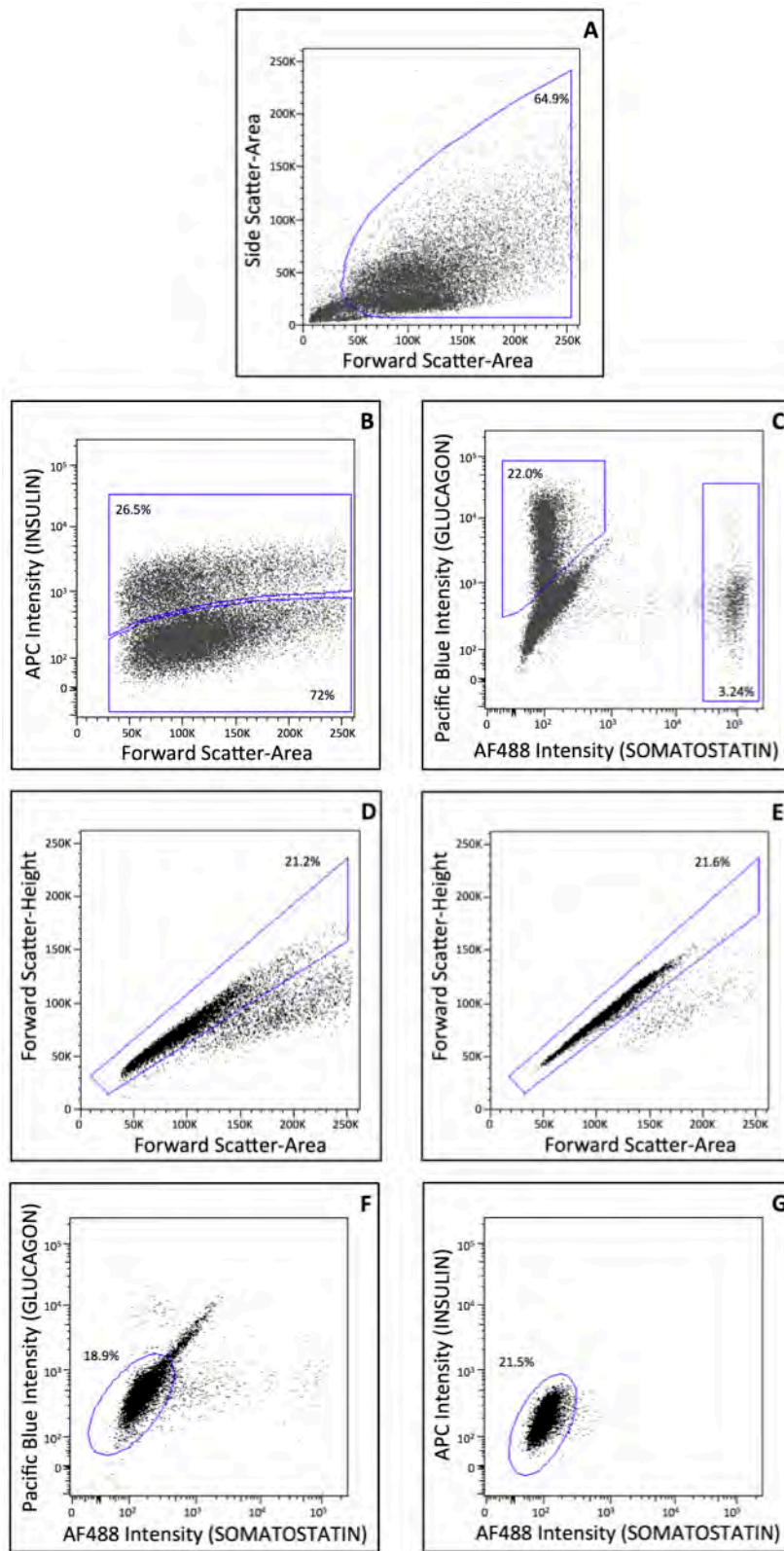
Figure 2.2 Fluorescence Activated Cell Sorting Gating Strategy Cells are sorted from a heterogeneous single cell suspension based upon their size, hormone content, and singlet nature. Viable cells are selected by gating out debris (A). Insulin positive cells are selected (B) and an inverse gate is used to identify glucagon and somatostatin positive cells (C). Doublets are excluded from insulin (D) and glucagon (E) positive cells. Double positive cells are eliminated by selecting against fluorophores that do not stain the population of interest – PB and AF488 for Insulin (F) and APC and AF488 for Glucagon (G). These populations are collected after sorting.

Results

The plots provided are representative images collected on 06-February-2012 (Figure 2.2 (A-G) and Figure 2.3 (A-C)) and 10-May-2012 (Figure 2.4 (A-C)). The cells that were collected on 06-February-2012 were subjected to further processing by RNA extraction and next generation sequencing (Chapter IV). Chapter III provides an analysis of each individual sort with respect to the alpha and beta cell composition and characterization relative to organ donor demographics.

The gating hierarchy and sorting strategy provide different ways to confirm that the isolated populations are pure and distinct. Table 2.2 shows the number and percentage of events collected for each population at each stage of the gating process. The two-tiered doublet exclusion gating strategy eliminates approximately 30% of insulin positive cells comprised of sample that is either double positive for hormone expression or did not completely dissociate. To show the pure nature of the beta cell population, the sorted insulin positive cells can be plotted against the glucagon (Figure 2.3A) and somatostatin (Figure 2.3B) positive cells, which show how the three populations are separate and distinct. Furthermore, consistent with the granular nature of insulin secretory vesicles, beta cells display a higher side scatter component than alpha and delta cells (Figure 2.3C). To further confirm that the sorting step was successful, a small fraction of each sorted population was reanalyzed via flow cytometry, see Table

2.3. Sorted populations of alpha (Figure 2.4A) and beta (Figure 2.4B) cells show greater than 97% purity. Additionally, reanalysis of the cell population that did not demonstrate any insulin, glucagon, or somatostatin signal showed that positively stained cells were not lost during the sorting procedure (Figure 2.4C).

Table 2.2 Gating Hierarchy Example

These tables show the number (top) or percentage (bottom) of events recorded for a whole islet analysis. The column titles correspond to the gating strategy described in Figure 2.2: inclusion in the size gate (A) and subsequent staining gates (insulin$^+$ (B), glucagon$^+$ (C), or somatostatin$^+$ (C)), doublet exclusion (insulin$^+$ (D), glucagon$^+$ (E)), and single positive cells (insulin$^+$ (F), glucagon$^+$ (G)). Of the 26,079 cells (27% of total cells) that positively stained for insulin, 18,544 (19% of total cells or 71% of cells that initially stained positively for insulin) would be collected as singlet cells that express only the insulin hormone.

Subset	Total Events	Size Gate	Individual Gates	Doublet Exclusion	Single Positive
Unsorted	151,569	98,367			
Insulin$^+$			26,079	20,897	18,544
Glucagon$^+$			21,644	20,829	20,723
Somatostatin$^+$			3,183	2,798	2,601
Unlabeled			47,461		

Subset	Total Events	Size Gate	Individual Gates	Doublet Exclusion	Single Positive
Unsorted	151,569	98,367			
Insulin$^+$			27%	21%	19%
Glucagon$^+$			22%	21%	21%
Somatostatin$^+$			3%	3%	3%
Unlabeled			48%		

Table 2.3 Purity of Reanalyzed Subsets

This table shows the percentage of events for four samples and it corresponds to the data presented Figure 2.4. The unsorted sample is a heterogeneous mixture of insulin, glucagon, and somatostatin positive cells. Following sorting, a small fraction of the purified populations are reanalyzed to determine purity.

Sample	Total Events	Size Gate Cells	Insulin$^+$	Glucagon$^+$	Somatostatin$^+$	Unlabeled
Unsorted	1,103,121	88%	27%	8%	1%	63%
Insulin+ Sorted	3,184	88%	**98%**	0%	0%	2%
Glucagon+ Sorted	1,765	83%	1%	**97%**	0%	2%
Unlabeled Sorted	3,509	93%	2%	0%	0%	**98%**
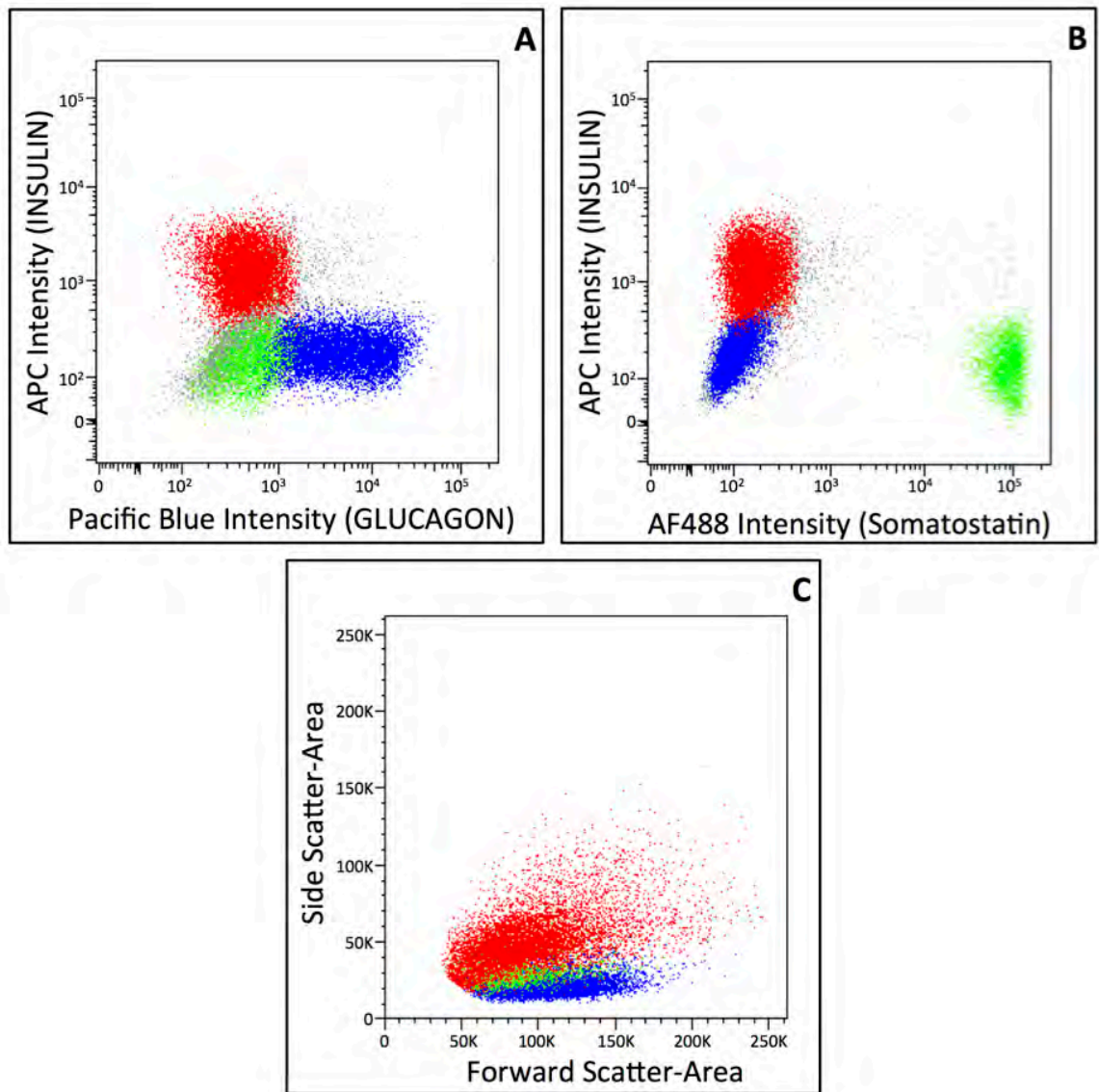
Figure 2.3 Fluorescence Activated Cell Sorting Islet Cell Subsets Gated and sorted cells are plotted to show individual populations (A, B) – insulin positive β cells (red), glucagon positive α cells (blue), and somatostatin positive δ cells (green). A traditional FSC versus SSC plot (C) shows that insulin positive cells have the highest degree of side scatter due to their granular nature.
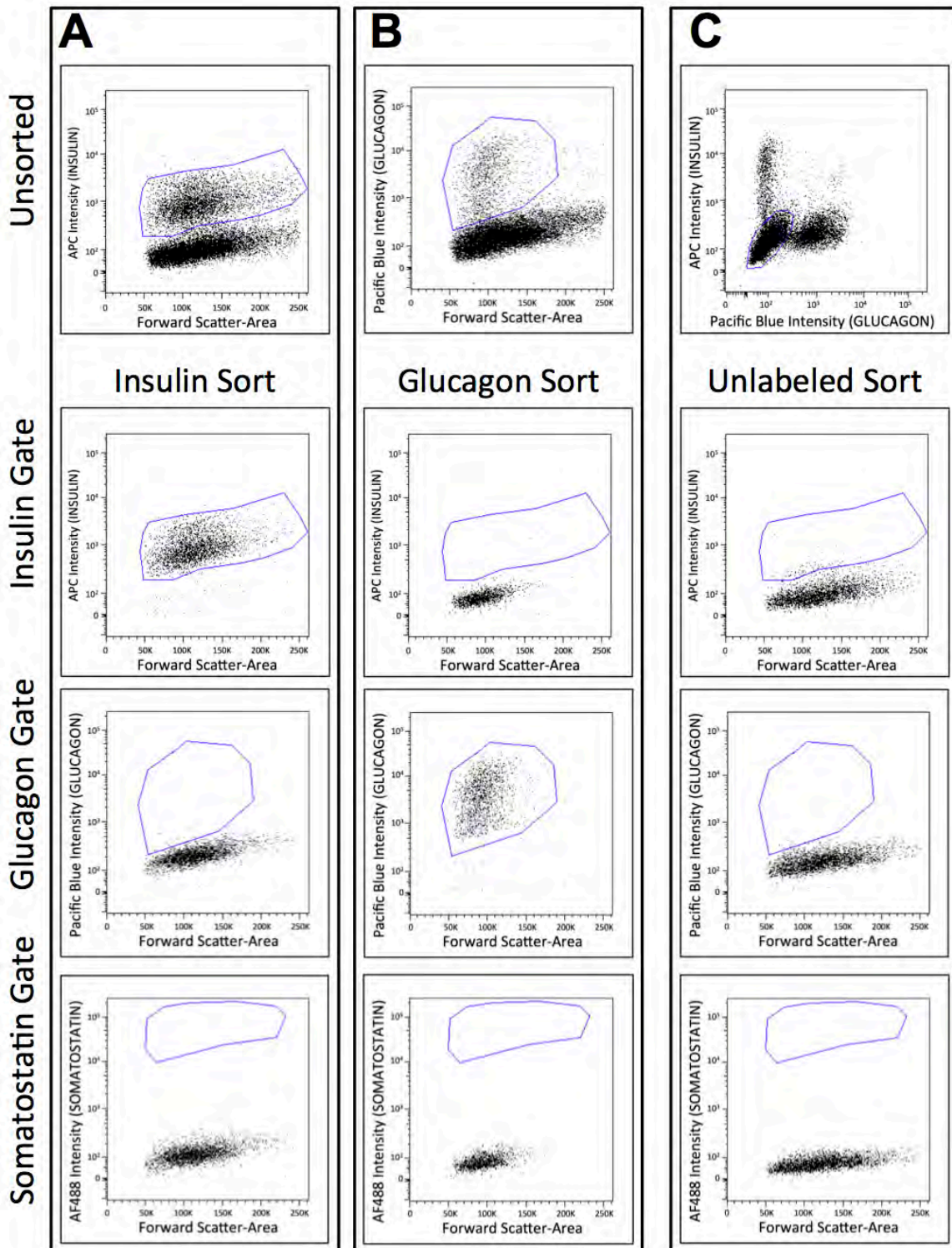
Figure 2.4 Reanalysis of Purified Subsets Following sorting, cells were reanalyzed to assess the relative purity of each population. The stained, but previously unsorted heterogeneous mixture of islet cells was used to design the gates (top row). The remaining rows show the same gates and are used to analyze the purity of the Insulin positive (A) – 98% pure, Glucagon positive (B) – 97.5% pure, and Unlabeled Cells (C) – less than 2.5% Insulin, Glucagon, or Somatostatin positive cells. The initial Somatostatin positive population is not shown. Please see Table 2.2 for a breakdown of subset population purities.

Discussion

The heterogeneous nature of pancreatic islets has long confounded analysis of individual cell subsets. Using flow cytometry, researchers have tried to isolate the beta cell population by taking advantage of their intrinsic high granularity and insulin-associated zinc concentration as well as by screening for subtype specific cell surface markers. These techniques have allowed for beta cell enrichment, but have not provided pure populations of islet cell subtypes. Assessments of purity have compared insulin to glucagon mRNA levels, but have not measured protein expression in the selected cell types[20,25,26]. Additionally, human islets cannot be manipulated to express cell type specific fluorescent reporter molecules, as has been reported for mouse[21,23].

Using a mouse model, Pechhold et al. developed a flow cytometry sorting technique that capitalized on the hormone expression pattern specific to the islet cell subtypes[27]. Confocal scanning microscopy had previously been used to analyze the hormone content and architecture of human, nonhuman primate, and mouse islets[28]. These studies allowed for analysis of cell structure and arrangement within islets and provided a method for separating out individual cell types for further study. Dissociated islet cells were fixed and permeabilized prior to exclusively labeling alpha cells with a glucagon specific antibody, beta cells with an insulin specific antibody, and delta cells with a somatostatin specific antibody. Labeling with fluorescently conjugated secondary antibodies permitted

sorting and collection of the individual cell types by flow cytometry. Given our interest in studying human cells, we have shown that this technique can be applied to human islets isolated from cadaveric donors such that we obtain individual alpha, beta, and delta cell populations that are greater than 97% pure.

The specificity and purity of alpha, beta, and delta cell populations highlight the major strength and advancement of this approach. Unlike with the other techniques, cells are sorted based upon the specific, intracellular hormone content and they represent a true isolation of a pure population, rather than just an enrichment. This method for sorting does have its drawbacks when compared to the other methods. Fixation and permeabilization result in a nonviable cell, following treatment. Cells sorted by surface marker labeling result in the isolation of viable cells that can be used in downstream experiments where attempts are made to culture or reprogram the enriched cell types[13,29,30].

One of the major reasons for performing isolation of islet cell subsets centers on performing gene expression analysis. To conduct these studies (quantitative real time polymerase chain reaction (qRT-PCR or qPCR), microarray analysis, next generation sequencing), RNA must be isolated from the cells. White and Kaestner (2009) discuss how it is difficult to obtain pristine RNA from the pancreas due to the endogenous presence of RNA degradation enzymes (RNases)[14]. This technique compounds this problem by fixing and permeabilizing the cell, which can introduce exogenous RNases (from buffers and antibodies) to

intracellular stores of RNA, resulting in the degradation of the most valuable resource. To overcome the issue of not having the full-length, intact RNA needed for more global qPCR or microarray studies, and turn a weakness into a strength, Pechhold and colleagues used a quantitative nuclease protection assay (qNPA) that could measure the expression levels of small, 50 nucleotide sequences of RNA. As will be discussed in Chapter IV, we have used this qNPA technique and optimized our staining and sorting protocol to attempt next generation sequencing of these sorted cells. By devloping this technique, we hope to make it possible to first establish the gene expression profiles (i.e. transcriptome) for individual islet cell populations. Second, we aim to measure how these profiles change in different donors, upon exposure of the islets to various stimuli that may promote proliferation Finally, we aim to pursue these same studies in a more controlled non-human primate (NHP) model system that more accurately replicates human islet morphology and physiology.

CHAPTER III

EPIDEMIOLOGY OF HUMAN ISLET COMPOSITION

Introduction

The islets of Langerhans are heterogeneous clusters of multiple hormone producing cell types that comprise the endocrine pancreas. Glucagon producing alpha cells, insulin producing beta cells, somatostatin producing delta cells, pancreatic polypeptide producing gamma cells, and ghrelin producing epsilon cells function in a coordinated fashion in order to maintain whole body glucose homeostasis[31]. Mouse islets are the most well-studied and demonstrate an arrangement where the beta cells form a core in the center of the islet, while the remaining four cell types surround this organized concentration of insulin producing the cells via an indiscriminate peripheral arrangement[28,31].

Compared to rodents, islets isolated from nonhuman primates and humans display a more variable islet structure in terms endocrine cell arrangement and relative ratios [28]. In these islets, alpha, beta, and delta cells are intermingled and can be found throughout the islet, both in the core and on the surface. Using laser scanning confocal microscopy through the whole islet, Brissova and colleagues showed that the beta cells are often found at the exterior of the islet[28]. Although their studies did not distinguish how islet structures changed within

different locations in the pancreas, each of the individual islets isolated demonstrated a similar heterogeneous pattern.

Both laser scanning confocal microscopy and analysis of histological sections have been used to compare the relative amounts of alpha, beta, and delta cells in the pancreatic islet. Similar to the structural differences, mouse and human islet composition shows different percentages of each cell type, with increased variability found in human islets. While mouse islets contained a vast majority of beta cells (75%, range 65-85%), the beta cell average in human islets was closer to half of the stained endocrine cell population (54%, range 30-75%) with a much wider variation among donors. Similar observations were found in alpha cells (19%, range 10-30% in mouse versus 35%, range 10-65%in human) and delta cells (6%, range 2-10% in mouse versus 11%, range 2-22% in humans)[28,31-33].

Given our intracytoplasmic staining of insulin, glucagon, and somatostatin proteins and analysis by flow cytometry, we aimed to show that our results for human islet composition was equally variable in showing the range of alpha, beta, and delta islets in donor islets. Additionally, we used the demographic information provided by the IIDP to assess how islet culture time and donor age, gender, and BMI affected the beta to alpha cell ratio in human islets.

Methods

Human islets were dispersed, fixed, stained, and sorted as described in Chapter II. Demographic information was collected from the IIDP donor information packets that generally included donor gender, age, BMI, history of diabetes, and duration of islet culturing prior to shipment. Total alpha, beta, and delta cell numbers were calculated from single stain analysis for insulin, glucagon, or somatostatin protein expression or from total stains that were simultaneously sorted for all three hormones. Results are presented as the percentage of each labeled cell subset (percentage of total labeled cells that are either insulin, glucagon, or somatostatin positive) or by comparing the ratio of beta to alpha cells in each given preparation. A two-tailed student's T-test was used to make all statistical comparisons: percentage or ratio of beta to alpha cells in all staining experiments, or stratified by gender, age, BMI, and days in culture. Power and sample size calculations were made using Java Applets for Power and Sample Size, an alpha error value of 0.05, and the data provided in Table 3.1, Figure 3.1, and Figure 3.2[34].

Results

Islet Composition Islet samples from 18 donors were analyzed via flow cytometry for glucagon and insulin content, 11 were also assessed for somatostatin content. Table 3.1 provides the demographic information for the donors and shows the percentage of positively labeled flow cytometry events that stained for

glucagon, and insulin positive cells. The population was 39% female, had an average age of 38.4 years, BMI of 30.3 kg/m^2, and 53.7% of total flow cytometry events corresponded to positively stained endocrine cells. Figure 3.1 shows the composition of the positively labeled cells: 41% (range 23 − 58.8%) stained positively for glucagon and are referred to as alpha cells, 54% (range 38.4 − 71.7%) stained positive for insulin and are referred to as beta cells, 5% (range 2.7 − 10%) stained positively for somatostatin and are referred to as delta cells. Using the data in Table 3.1 and an alpha error level of either 0.05 or 0.01, the power of this experiment is 0.95 or 0.82 respectively. As such, it is adequately powered to calculate the percentage of islet cells that are glucagon- versus insulin-positive.

Beta to Alpha Cell Ratios and Composition among Demographics Given the variability of the alpha and beta cell proportion of each islet donor, the beta to alpha cell ratio provides a way to examine how islet composition compares across the limited demographic groups. Within the whole donor population, the beta/alpha ratio is 1.49 (range 0.65 − 3.12). Islet donors were sorted into groups based upon gender (male, n=11 or female, n=7; Figure 3.2A, B), age (under 40, n =9 or over 40 n =9; Figure 3.2C, D), BMI (25 and under, n=5 or over 25, n=13; Figure 3.2E, F), and days in culture (under 3 or over 3; Figure 3.2G, H). No significant difference (p-value $\leq$ 0.05) was found within the gender, BMI, and days in culture groups. Currently, these experiments are not adequately powered to judge overall significance: gender (power = 0.47; n for each group $\geq$16 needed

for power $\geq$0.8), age (power = 0.68; n for each group $\geq$12 needed for power $\geq$0.8), and BMI (power = 0.35; n for each group $\geq$29 needed for power $\geq$0.8). Islets from female donors, however, tended to contain more Insulin$^+$ cells (Figure 3.2B) than male donors. Additionally, donors with higher BMI values displayed more variability in their islet composition (Figure 3.2F) than donors who displayed suggested BMI levels (less than or equal to 25 kg/m^2).

Table 3.1 Islet Donor Demographics

Gender	Age	BMI	% Glucagon[+]	% Insulin[+]	Ratio β/α
F	54	28.4	23.0%	71.7%	3.12
F	33	22.3	33.3%	61.4%	1.84
F	19	24.8	45.9%	48.8%	1.06
F	46	39.7	35.5%	61.6%	1.74
F	42	32.5	31.1%	63.6%	2.04
F	40	26	28.9%	66.5%	2.30
F	20	19	43.6%	48.6%	1.11
M	24	29.2	44.3%	45.7%	1.03
M	27	23.7	48.1%	48.5%	1.01
M	20	31.6	57.9%	39.7%	0.69
M	48	33.5	31.6%	63.3%	2.00
M	29	30.17	58.8%	38.4%	0.65
M	38	29.9	44.8%	50.0%	1.12
M	20	31.3	43.4%	51.3%	1.18
M	64	34.8	26.5%	68.2%	2.57
M	50	25	57.0%	39.0%	0.68
M	66	34	34.6%	56.4%	1.63
M	52	50	48.7%	48.7%	1.00
Average (n = 18)	38.4 (15.3)	30.3 (7.1)	40.9 (10.9)%	54.0 (10.4)%	1.49 (0.7)
Females (n = 7)	36.3 (13.1)	27.5 (6.9)	34.5 (8.7)%	60.3 (8.1)%	1.89 (0.7)
Males (n = 11)	39.8 (17.1)	32.1 (6.9)	45.1 (9.7)%	49.9 (9.7)%	1.23 (0.6)
p-value*	0.65	0.19	0.04	0.04	0.05

Average is reported with (standard deviation). *p-value is calculated using an unpaired, two-tailed, t-test and compares the female and male populations. Note: for whole sample, %Glucagon[+] versus %Insulin[+] p-value = 0.0008.
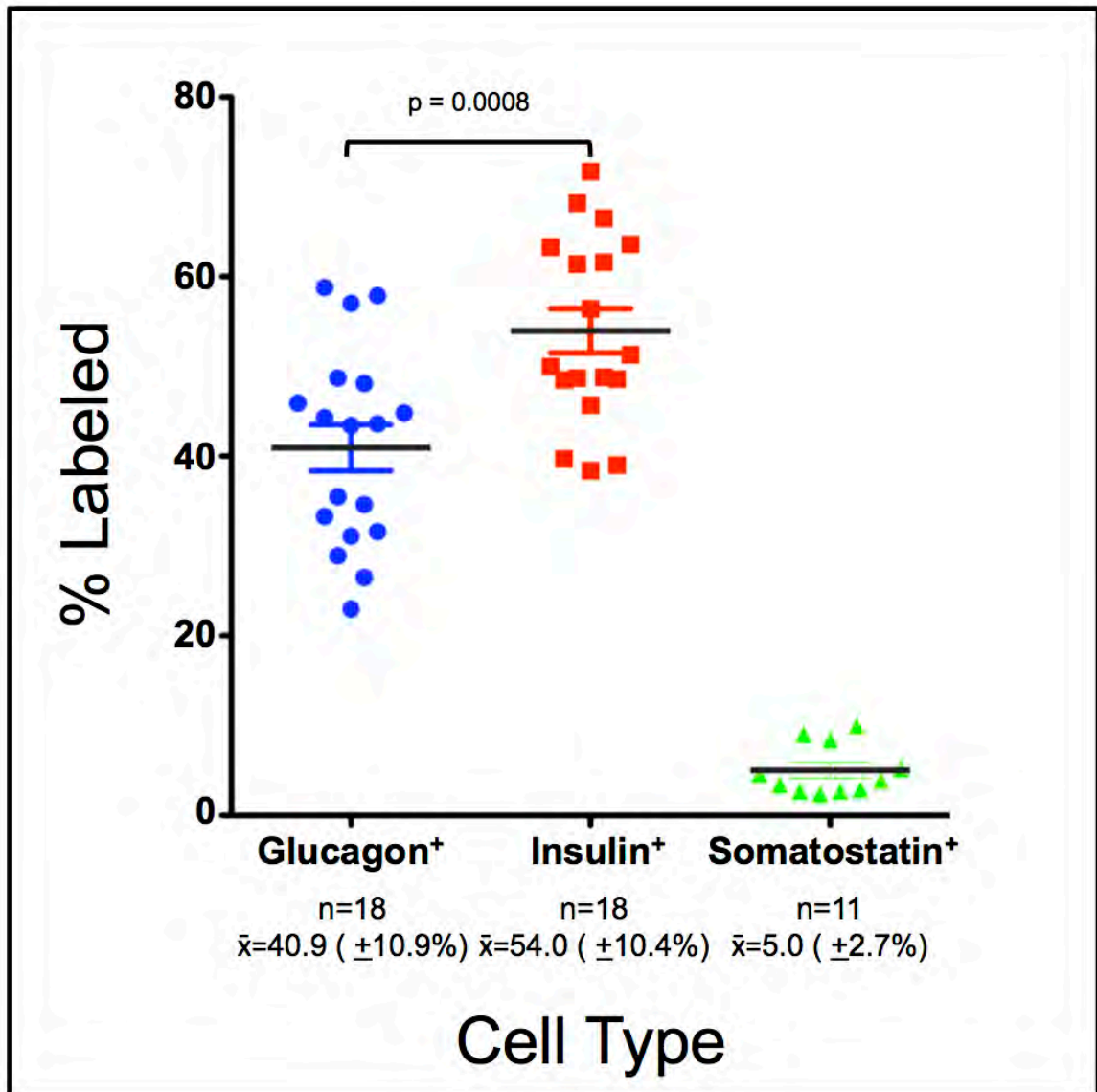
Figure 3.1 Human Islet Composition – Endocrine Cells Islets were stained with antibodies that recognize intracellular hormones glucagon (α-cells), insulin (β-cells), and somatostatin (δ-cells). The sample number, mean, and (standard deviation) are provided for each cell subset. β-cells comprise a higher percentage of labeled endocrine cells than do α-cells. Both α- and β-cells are expressed at a significantly higher level than δ –cells ($p < 0.0001$).
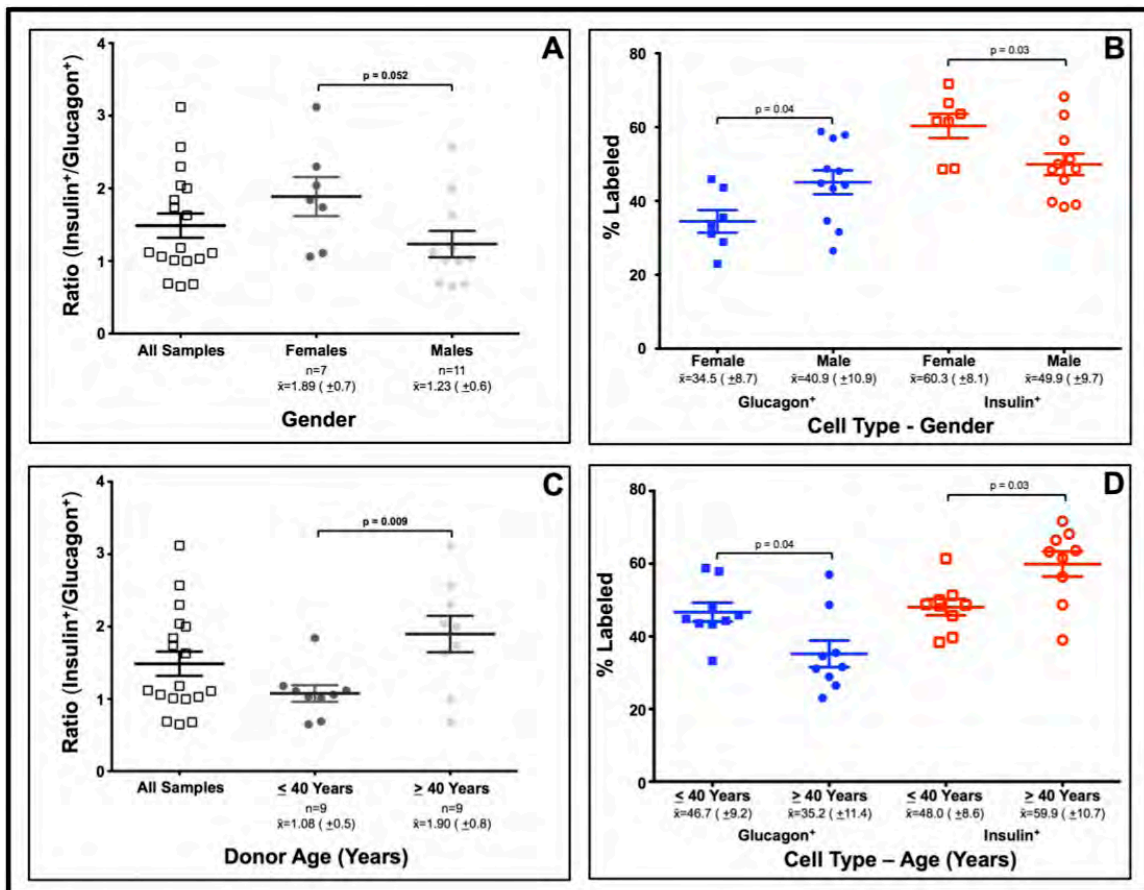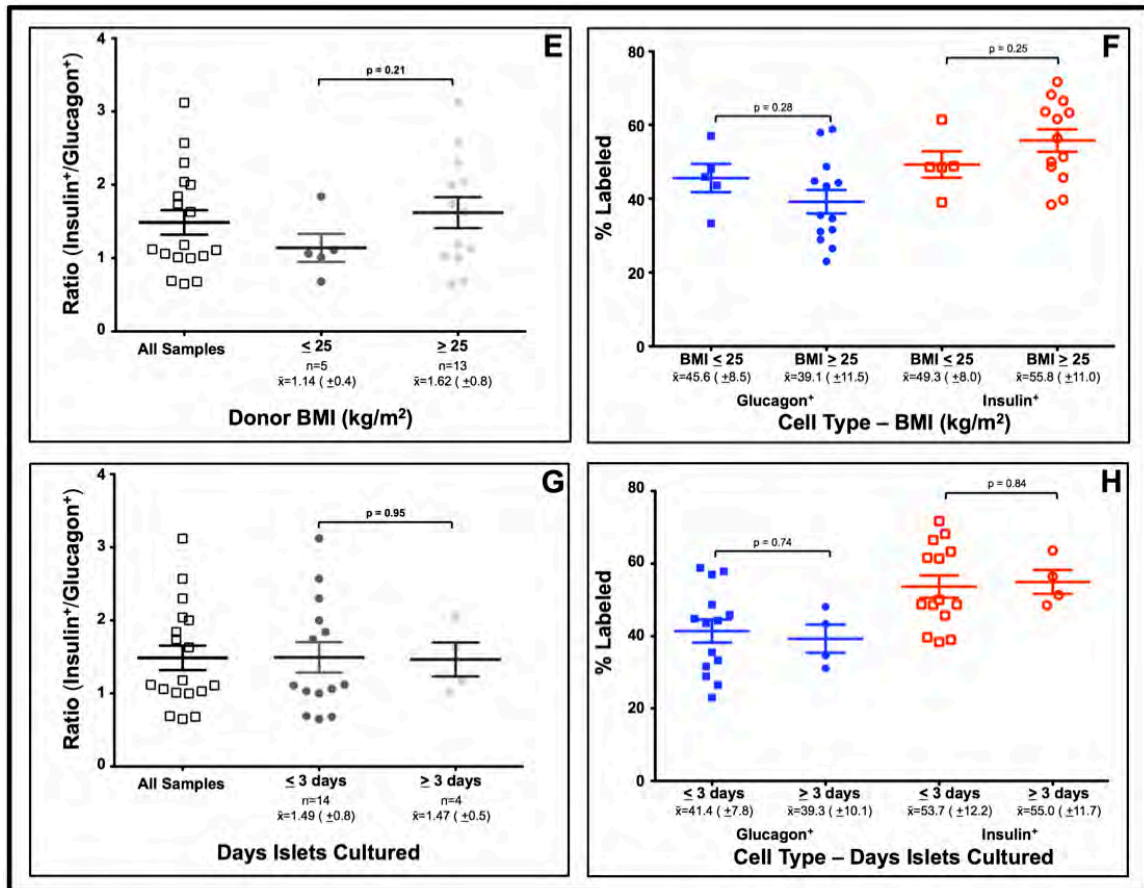
Figure 3.2 Human Islet Composition Across Demographic Variables Islet composition was converted to a beta/alpha cell ratio and plotted (A, C, E, G) or plotted as % Glucagon[+] and % Insulin[+] for each of the 18 analyses (B, D, F, H). Among the observed demographic variables, Gender and Age show significant differences between the beta/alpha cell ratio (C) and % Glucagon[+] and Insulin[+] between the two demographic groups (B, D). Donor BMI does not have significant difference on either islet measure (E, F). Technically speaking, the number of days islets are in culture does not change the beta/alpha cell ratio or the percentage of Glucagon[+] or Insulin^{+} cells (G, H).

Discussion

Human islet composition, viability, and quality are important factors to consider when assessing whether or not an islet preparation is appropriate for transplant into a type 1 diabetic patient[8,9,35,36]. It also speaks to the difficulty with using human samples in research studies. Gene expression of whole islets could vastly differ from donor to donor based upon the relative proportions of the heterogeneous cell types that comprise each particular islet donor who may be a different age, gender, weight, have different health conditions, or whose islets may have been isolated and cultured by a different isolation team for various periods of time[8,14,27,37]. Sorting by flow cytometry allows for the separation and examination of each cell type in terms of total islet composition and individual gene expression qualities.

These results show that analysis of single cell suspensions of human islets by flow cytometry detect the donor to donor variability equally as well as confocal microscopy[28]. This report details the cellular composition of human islets isolated from 18 donors, shows a similar characterization of heterogeneous variability as detected by confocal microscopy and histological staining, and further begins to examine whether or not there is a pattern to the factors that contribute to islet variability.

On average, it does appear that human islets have a higher percentage of beta cells to alpha cells, both of which far outnumber the contribution of delta cells to

whole islet composition. We found that of all events measured by the flow cytometer, an average of greater than 50% corresponded to positively labeled endocrine cells. This number could be lower than expected due to a wide spread of islet purity or a variable expression of insulin and glucagon within alpha and beta cell populations that may preclude clearly differentiating positively labeled cells from the background population. We have observed what appear to be two populations of insulin expressing cells following flow analysis. Restaining of cells that appear to be negative for endocrine cell markers with another insulin antibody shows that they do contain a portion of low expression insulin positive cells (data not shown).

The small sample size is a limitation in correlating the data with the broad range of the population. Based on the demographic information that is known, however, we were able to observe some statistically significant differences in beta/alpha cell ratios and other results that are trending in one direction versus another. First, it does not appear that the number of days in culture affects the beta/alpha cell ratio across donors. Different donor islets cultured for less than or greater than three days showed approximately the same beta/alpha cell ratio, however, we favor the more recently isolated islets. There is a trend toward a higher beta/alpha cell ratio in female donors. It was interesting to note the uniformity of the beta to alpha cell ratio in young (less than 40 years of age) donors. While this ratio was found to be about 1:1, donors over 40 years of age had almost two times more beta cells than alpha cells. Unfortunately, we cannot determine the

total number of beta or alpha cells isolated from each of these populations, however, the change in ratio is indicative of more insulin expressing cells. BMI does not appear to affect the beta to alpha cell ratio in a significant fashion, consistent with previous studies[38,39], although there is a trend toward a higher contribution of beta cells at higher BMI values. With more samples, we could more closely investigate the connection between age and BMI to determine if the older patients with high BMI had the highest (or lowest) beta to alpha cell ratios, which might explain pre-diabetes or undiagnosed type 2 diabetes[38,40].

This analysis shows that flow cytometry and hormone staining accurately measure human pancreatic islet composition. Human islets display a great degree of heterogeneous variability from donor to donor, not noticed in mice, and these differences could be attributed to the age, gender, or BMI of the donor subject. By analyzing and sorting these cells with flow cytometry, we obtain pure populations of each cell type, which can be used for downstream gene expression analysis and future comparisons.

CHAPTER IV

GENE EXPRESSION EXPERIMENTS & PROFILING RESULTS

Introduction

The arrangement and organization of the pancreatic islet has made it difficult to isolate unique populations of islet cell subtypes with intact RNA. Islets comprise less than 5% of total pancreas tissue and are located amongst exocrine tissue that contains numerous digestive enzymes and RNases[1,14]. As a result, human islets must first be separated from this largely irrelevant tissue, a process performed by hospital centers associated with the IIDP. Although these islet preparations are generally 80% pure, they do contain acinar tissue, ductal cells, and other debris that must be removed prior to separation. Finally, the heterogeneous construction of islets means that they must be dissociated into a single cell suspension prior to treatment and analysis. Up until this point, however, it has been difficult to obtain pure populations of alpha and beta cells that contain high quality RNA[14].

As was described in Chapter II, and throughout this thesis, we have developed a cell sorting strategy that allows us to separate pancreatic islets into their individual, pure, subset populations. Following intracellular hormone staining, we can obtain pure populations of alpha, beta, and delta cells. By understanding the specific cellular transcriptome of a cell type of interest, the molecular and cellular

controls that guide development and disease states can be investigated[41]. As an example, this chapter recounts how we stimulated human islets with exogenous glucose and an incretin mimetic, exenatide. By studying changes to the transcriptome, we can identify proteins, regulators, and pathways involved in biological processes such as increased glucose stimulated insulin secretion, glucagon inhibition, beta cell proliferation, and insulin resistance. This strategy will help us to understand the processes that might improve islet transplant success or more effectively treat or prevent type 2 diabetes.

This chapter will show how we initially used the qNPA to overcome the RNA quality limitation that prevents qPCR, microarray analysis, and next generation sequencing analysis. Second, we will show how we have streamlined the sorting and RNA extraction procedure in order to improve RNA quality. In this regard, we have successfully performed next generation sequencing on RNA extracted from purified beta cells and have worked to overcome the previous limitations that prevented gene expression analysis in human islets and islet cell subtypes.

Methods

Human islets were dispersed, fixed, stained, and sorted as described in Chapter II. Following sorting, pure populations of cells were treated in accordance with the protocol associated with the downstream assay.

HTG Lysis Sample Preparation for qNPA Cells were washed with PBS, sedimented at 1500 revolutions per minute (rpm) for 4 minutes at 8°C, and decanted. Cells were resuspended in lysis buffer supplied by HTG Molecular Diagnostics at greater than 200 cells/μL and frozen at -80°C until further use. Samples were subjected to qNPA as described previously[27]. Briefly, sorted cell lysates are incubated with gene specific 50 nucleotide probes and anneal with complementarity. S1 nuclease is used to digest unbound probes and mRNA and alkaline hydrolysis removes bound mRNA, to leave only the gene specific probe. The probes are transferred to the qNPA plate. The qNPA plates are designed so that each well has a 4x4, gene-specific, grid map printed on it. Each spot within the well corresponds to a specific gene and is programmed with linkers that recognize one of the 16 respective spots. The sample qNPA probes are added and binds to the programming linker. The detection linker hybridizes to the probe and the detection probe binds to it. After incubation with HRP substrate, photons are measured using the HTG Omix HD. Photon exposure is measured for 15, 30, and 60-second intervals. During data analysis, results are normalized to intensity per 1,000 cells per minute of exposure time. Genes that saturate the detector upon initial measurement are normalized by measuring the signal decay of genes within the dynamic range over 30, 60, and 120 minutes, and adjusted accordingly.

Exenatide Experiments Human islets were incubated overnight in culture with a titration of glucose and exenatide included within the culture medium. CMRL was

prepared with 5.5 mM D-glucose, 1x sodium pyruvate, 1x nonessential amino acids, and 10% fetal bovine serum (FBS). Control and 100 mM exenatide wells were cultured with 5.5, 8, or 16 mM final glucose concentration. Following overnight incubation, islets were removed from each well and processed as described previously.

Preparation for RNA Extraction Sorted cells were washed with PBS, sedimented at 1500 rpm for 4 minutes at 8°C, and decanted. Cell pellets were next treated according to the protocol described by the Qiagen formalin fixed paraffin embedded (FFPE) RNeasy Kit, starting at step 6, which disregards the deparaffination steps. In brief, pellets are resuspended in 150 µL of buffer PKD and 10 µL of proteinase K. Cells are incubated at 56°C for 15 minutes followed by 5 minutes at room temperature before a second incubation at 80°C for 15 minutes. Following a 3 minute incubation on ice, cells are spun at 13,200 rpm for 15 minutes at 20°C and the supernatant is removed and frozen at -80°C until further processing. Total RNA (greater than 18 nucleotides in length) is isolated using the QIAGEN miRNeasy FFPE kit (note: the sole difference between these two kits involves the concentration of ethanol mixed with the RNA during the binding step; the higher the ethanol concentration, the greater the affinity of the RNA for the column, which allows the microRNA species to bind and be isolated simultaneously with the total RNA). Briefly, cells are treated with DNase, incubated with an RNA binding buffer and 100% ethanol, spun through the RNeasy MiniElute Spin Column, washed with a QIAGEN buffer, spun without any

buffer to remove excess ethanol, and eluted in 30 µL of RNase Free water. Following elution, RNA is analyzed using the Agilent BioAnalyzer to assess RNA quality and concentration. Depending on the concentration of the RNA, it is analyzed on a pico (less than 5 ng/µL) or nano (greater than 5 ng/µL) chip.

microRNA Analysis HTG qNPA technology was used to obtain microRNA (miRNA) array data for alpha and beta cells. Following sorting of alpha and beta cell populations from two donors (a 20-year-old female with a BMI of 19 kg/m^2 and a 50-year-old male with a BMI of 25 kg/m^2), whole cell lysates were sent to HTG for processing via a miRNA specific qNPA chip) representing ~1,050 miRNA species (Sanger miRBase release 9.1). Each sample population was run in duplicate and miRNA species were only chosen to be positively expressing if both duplicates had signal intensity values above the negative controls within that specific experiment. In this way, we worked to eliminate false positive identifications.

Next Generation Sequencing RNA samples were shipped on dry ice to Beijing Genomics Institute (BGI) for library construction, validation, and sequencing. The BGI protocol for library development, normalization, and preparation mirrors the protocol described by Danos et al[42]. Briefly, RNA is digested with DNaseI and purified. RNA is fragmented followed by first and second strand cDNA synthesis and purification. Ends are repaired, adenylated, ligated to adaptors, and purified again. PCR amplification is completed using a pair of inner primers and the

library is validated. The library is then normalized with double strand nuclease (DSN) to remove a fraction of genes that are overexpressed. Following a final amplification using paired end primers, the library is purified, assessed for quality, and submitted for sequencing using the Illumina HiSeq2000, which is equipped to produce greater than 20 million 91 base pair, paired end reads of each library sample.

Results

qNPA Gene Detection The gene expression profiles of sorted alpha and beta cells were determined using the qNPA. Alpha cells were found to express PPIA, insulin, glucagon, PPY, CD2, Pax6, and β-actin, however, somatostatin, ngn3, amylase, CK19, Pax4, MafA, MafB, and p48 were not detected. Beta cells were found to express PPIA, insulin, glucagon, somatostatin, ngn3, PPY, CD2, Pax6, MafA, and β-actin, however, amylase, CK19, Pax4, MafB, and p48 were not detected (see Table 4.1 and Figures 4.1). In alpha cells, the glucagon gene expression level was found to be 72-fold higher than the insulin expression level; similarly in purified beta cells, the insulin glucagon gene expression level was found to be 101-fold higher than the glucagon expression.

Incubation with exenatide and glucose did not appear to have any effect on the relative proportions of alpha and beta cells in culture (Table 4.2). Across the four experiments, the relationship between the insulin to glucagon ratio at day zero did not consistently vary with the ratio calculated after overnight culture. Gene

expression changes in alpha cells were not detected as represented by glucagon (Figure 4.2A) and PPY (Figure 4.2B) gene expression levels across all glucose and exenatide concentrations. Unlike insulin (Figure 4.2C) and other beta cell genes, we detected increased expression of PPY in beta cells that correlated with increased glucose and exenatide concentration (Figure 4.2D). All gene expression levels were normalized to total plate intensity.

Table 4.1 Gene Expression Values for sorted Alpha and Beta Cells

Gene	Alpha Cell	Beta Cell
PPIA	74	54.5
INS	81.5	5752
GCG	5894.5	57
SST	n.d.	11
NGN3	n.d.	9
PPY	17.5	94
CD2	8	21.5
AMYLASE	n.d.	n.d.
CK19	n.d.	n.d.
PAX4	n.d.	n.d.
PAX6	46	30.5
MAFA	n.d.	24
MAFB	n.d.	n.d.
P48	n.d.	n.d.
BACTIN	171.5	239.5

Following completion of the qNPA, gene expression values for each gene in the purified alpha and beta cell populations were recorded using qNPA intensity units per minute, per 1,000 cells. n.d. indicates that the gene was not detected. Although this experiment was conducted four times, variations in initial runs of the control lane compromised the ability to compare raw values across runs. As a result, this table represents an average of two technical replicates for a representative experiment.

Table 4.2 Islet Composition upon Exposure to Increased D-glucose and/or Exenatide

Sample	% Glucagon[+]	% Insulin[+]	% Somatostatin[+]
Day 0	54.7%	43.0%	2.3%
CMRL (5.5 mM DG)	60.7%	33.0%	6.3%
CMRL (8 mM DG)	62.4%	31.8%	5.8%
CMRL (16 mM DG)	62.7%	30.5%	6.9%
EX CMRL (5.5 mM DG)	57.0%	37.3%	5.7%
EX CMRL (8 mM DG)	58.4%	34.9%	6.7%
EX CMRL (16 mM DG)	60.2%	32.6%	7.2%
average	59.4%	34.7%	5.8%
stdev	2.9%	4.3%	1.7%

Whole islets were dissociated, fixed, and stained as described previously immediately upon receipt (Day 0) or following a 20 hour overnight culture in glucose and exenatide as indicated: CMRL describes the culture media, (mm DG) describes the glucose concentration, and EX indicates that 100 mM exenatide was included within the incubation. Flow cytometry of each sample provided the necessary data to calculate the percentage of glucagon, insulin, or somatostatin positive cells. This experiment was conducted four times. Due to the variability in islet cell composition (as demonstrated in Figure 3.1), this table corresponds to one representative experiment.
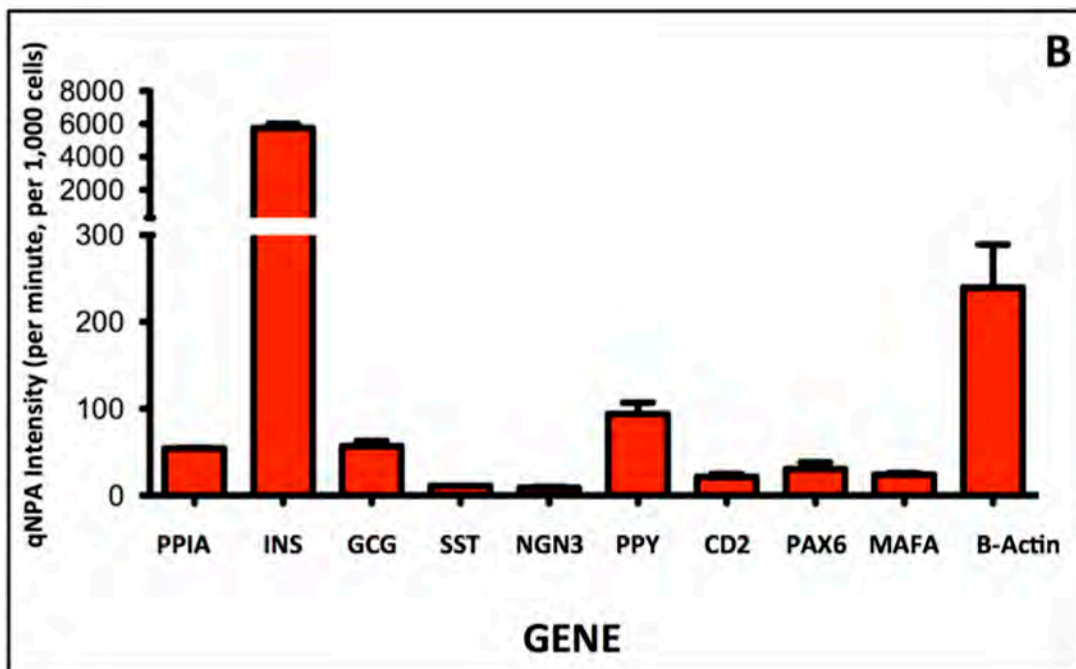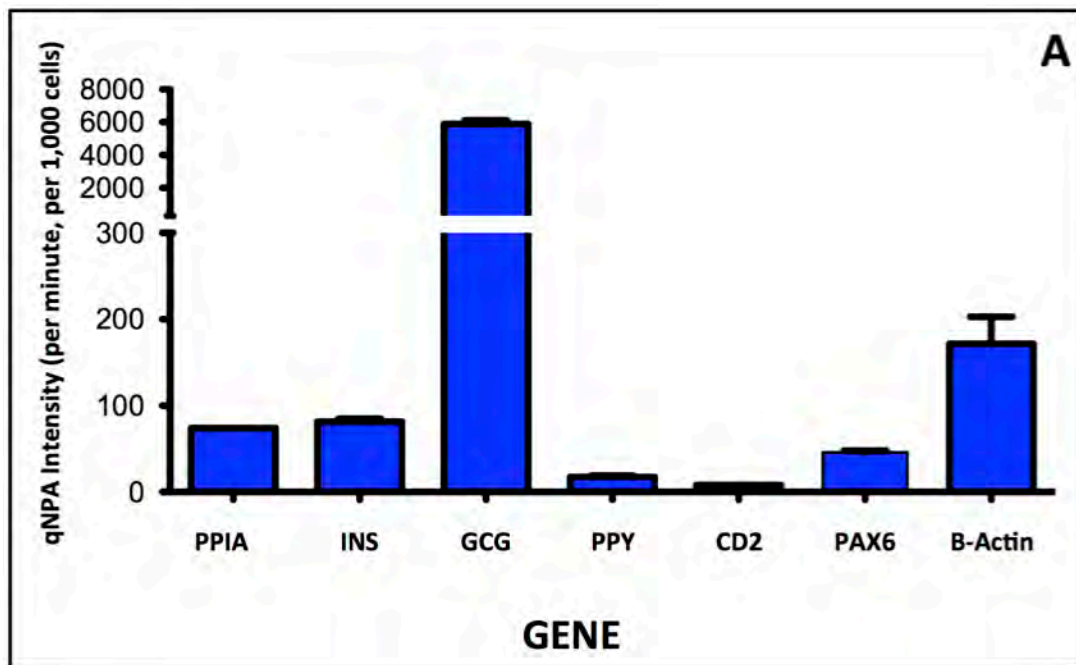
Figure 4.1 Gene Expression Values for sorted Alpha and Beta Cells The expression levels of the 15 genes listed in Table 4.1 were examined in sorted alpha (A) and beta (B) cells using the qNPA technique. The hormone specific to each subpopulation is the most widely expressed gene in each sample (Glucagon – A; Insulin – B). Glucagon is expressed at a 72-fold higher intensity than insulin in purified alpha cells. Similarly, insulin is expressed at a 101-fold higher intensity than glucagon in purified beta cells. These data serve as a representative image.
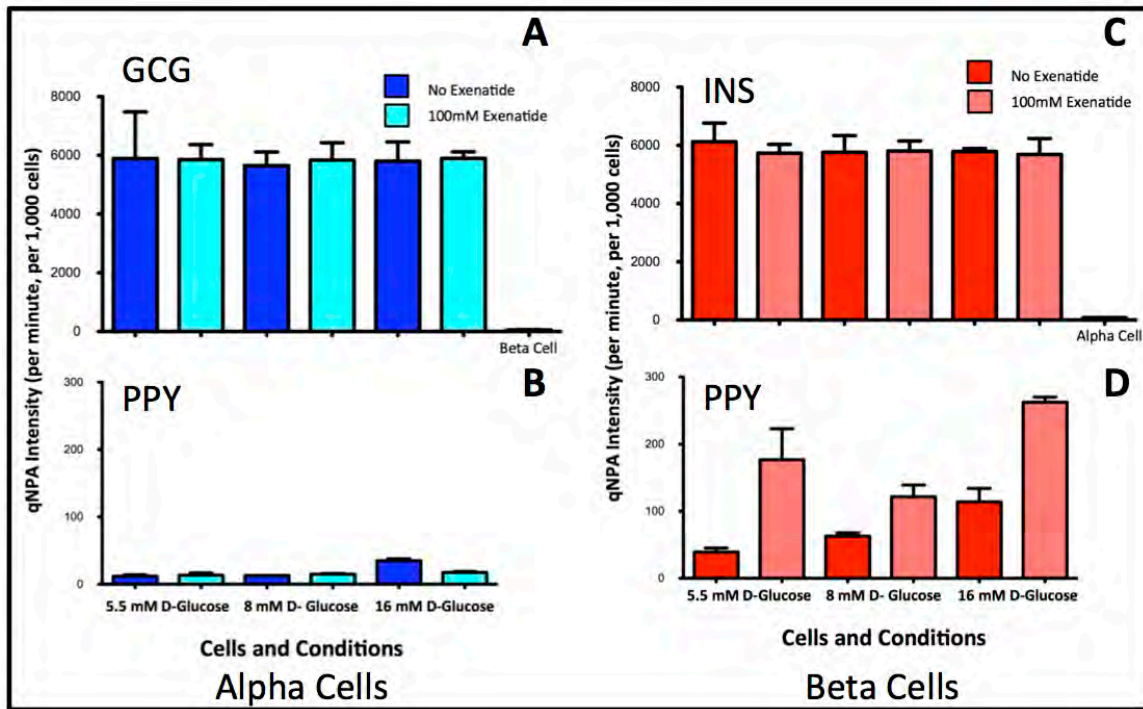
Figure 4.2 Effect of [D-Glucose] and Exenatide on Gene Expression Human islets were incubated in increasing [D-glucose] plus or minus exenatide. Glucagon and insulin gene expression remained unchanged (A and C) in alpha and beta cells respectively. Additionally, PPY (B) expression did not change in alpha cells, however, it showed an increase in beta cells (D) in response to incubation with exenatide and increased [D-glucose].

RNA Quality RNA has been isolated from dissociated whole islets and sorted alpha, beta, and unlabeled cells using multiple derivations of the Qiagen RNeasy kits. We have always been able to obtain RNA with an RNA Integrity Number (RIN) greater than 7 from whole, dissociated islets that have not been fixed, stained, or sorted (Figure 4.3A). Once islets are fixed, stained, and sorted, however, RNA extraction requires the RNeasy or miRNeasy FFPE kits. Additionally, we have found that RIN values suffer if RNA is washed in PBS and frozen (Figure 4.3B), resuspended in HTG or RLT lysis buffer and frozen, or if staining takes place in the absence of both VRC and recombinant RNase inhibitors. When staining takes place in the presence of both RNA inhibitors and following sorting, RNA is treated with proteinase K and heat, RIN numbers improve and 18S and 28S ribosomal RNA (rRNA) peaks can be detected following analysis on the Agilent Bioanalyzer. Although they vary from preparation to preparation, when working with approximately 200,000 sorted cells, we have consistently found RIN values greater than 3 and RNA yields greater than 200 nanograms in samples treated in this streamlined fashion (Figures 4.3C-D and Table 4.3). In the preparations we have used for RNA extraction and next generation sequencing analysis, we have been able to collect between 100,000 and 200,000 purified alpha and beta cells from approximately 10,000 islets, which provides a sufficient amount of RNA for next generation sequencing.

Table 4.3 Sorted Cell RNA Quantity and Quality

Sample	# of Cells	ng of RNA	RIN	Figure	Sequenced
Whole Islets	450,000	2069	7.7	4.3A	Yes
Beta Cells	200,000	519	3.2 - 4.9	4.3C	Yes
Alpha Cells*	230,000	~350	3.9 - 5.0	4.3D	Pending
Unlabeled Cells	485,000	2690	3.5 - 4.2	N/A	Yes

All islet RNA was isolated from the same donor and cell populations were sorted as shown in Figure 2.3. The donor was a 24-year-old male with a BMI of 29.2 kg/m^2.

*Alpha Cell RNA quantity is estimated. BGI calculated quantity of Whole Islet, Beta Cell, and Unlabeled Cell RNA to be approximately 5 times higher than measured here. As a result, Alpha Cell RNA quantity from this donor meets the necessary threshold and is now in processing for sequencing analysis.
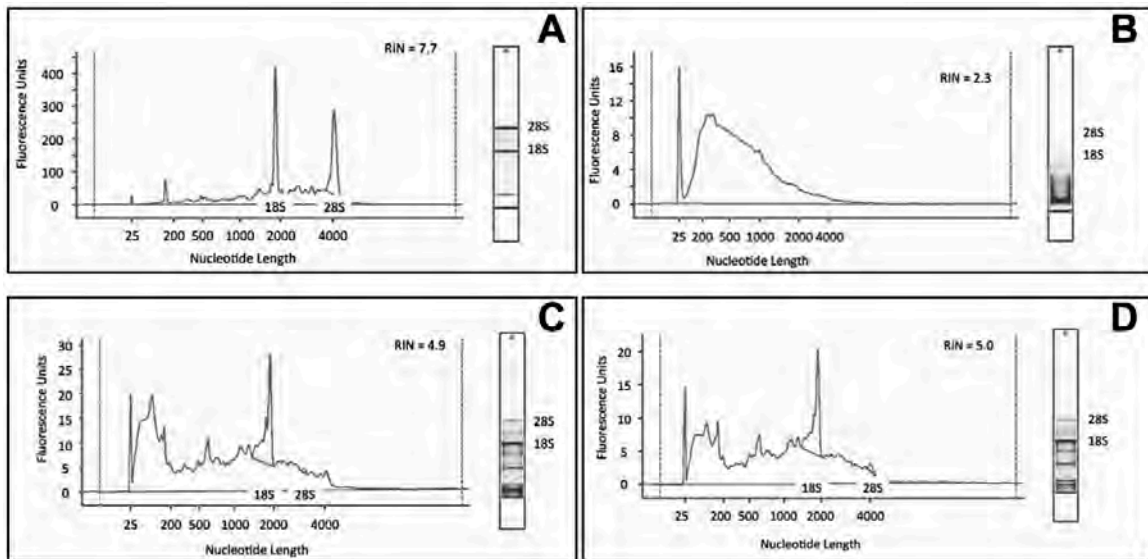
Figure 4.3 RNA Quality of Human Islet Cell Subsets Human islets were received from IIDP (donor 24 year old male with a BMI of 29.2 kg/m^2) and sorted via intracellular staining. RNA was extracted from whole islets prior to fixation (A), following fixation, staining, sorting, and freezing of the beta cell population (B), and following fixation, staining, sorting, proteinase K treatment, heat demodification, and freezing of the beta cell population (C) and alpha cell population (D). RNA quality and quantity were analyzed using a Pico chip on the Agilent Bioanalyzer 2000. The Bioanalyzer calculates the RIN Value based upon the intensity of the 18S and 28S ribosomal RNA peaks.

microRNA Analysis Panels A and B of Figure 4.4 show a comparison between the miRNA expression levels between alpha and beta cells in each donor (alpha versus beta in 20-year-old R^2 = 0.85; in 50-year-old R^2 = 0.77) as observed using the HTG Molecular miRNA qNPA chip. We found 22 miRNA species that were expressed at a greater than two fold difference in alpha versus beta cells in both samples. Additionally, we found 9 miRNA samples expressed at a greater than 2-fold difference across the two cell types to be unique to the 20-year-old donor and 15 miRNA samples to be unique to the 50-year-old donor. Panels C and D of Figure 4.4 show the similarity in miRNA expression across two alpha and beta cell populations. The expression levels for each miRNA species were plotted against each other for alpha cells (R^2 = 0.902) and beta cells (R^2 = 0.976). A comparison between the two samples shows that the alpha cells from the 50-year-old donor significantly ($p \leq 0.05$) expressed 4 miRNA species at a 2-fold higher level than the alpha cells from the 20-year-old donor and 3 species at 2-fold lower level. Within the beta cell population, only 2 species were expressed at significantly higher expression level in the 50-year-old. All miRNA expression levels can be found in the online Appendix (Table A.1).

A miRNA Profile Alpha versus Beta Cell (20 y.o. female)

y = 0.81x + 448
R² = 0.85

2x

0.5x

Not detected
in Alpha Cells

Not detected in
Beta Cells

Log Intensity Beta Cells

Log Intensity Alpha Cells

● Expressed in both samples
● Expressed in this sample only

B miRNA Profile Alpha versus Beta Cell (50 y.o. male)

y = 0.82x + 821
R² = 0.77

2x

0.5x

Not detected
in Alpha Cells

Not detected in
Beta Cells

Log Intensity Beta Cells

Log Intensity Alpha Cells

● Expressed in both samples
● Expressed in this sample only

C miRNA Profile Alpha Cells

y = 0.90x - 44
R² = 0.90

2x

0.5x

Not detected
in Donor 1

Not detected in
Donor 2

Log Intensity Alpha Cell (50 y.o. male)

Log Intensity Alpha Cell (20 y.o. female)

● Significant (p ≤ 0.05)

D miRNA Profile Beta Cells

y = 1.02x + 43
R² = 0.98

2x

0.5x

Not detected
in Donor 1

Not detected in
Donor 2

Log Intensity Beta Cell (50 y.o. male)

Log Intensity Beta Cell (20 y.o. female)

● Significant (p ≤ 0.05)

Figure 4.4 miRNA Profiling Results miRNA profiles are provided from two donors comparing the α- versus β-cell miRNA profiles from donor 1 (20 year old female, BMI 19 – A) and 2 (50 year old male, BMI 25 – B) and the two unique α-cell (C) and β-cell (D) populations. miRNA expression values were obtained using a qNPA chip (HTG Molecular) representing ~1,050 miRNA species (Sanger miRBase release 9.1). There were greater differences in miRNA expression levels within the α-and β-cell populations from the same donor; 22 miRNAs were found to be expressed at a $\geq$2-fold change in the individual cell types (A and B). The two donors showed similarity in miRNA expression levels among the α-cell (R^2 = 0.90 – C) and β-cell (R^2 = 0.98 – D) populations.

Next Generation Sequencing Results Our first sequencing sample has been analyzed by BGI and we have examine RNA isolated from whole islets, beta cells, and unlabeled cells. We have worked with the UMASS Bioinformatics Core to assess the data; the analysis presented here were created following core analysis of our raw data.

We generated greater than 23 million, 91 base pair, paired-end reads for each islet cell sample. Of these reads, greater than 60% of whole islet and beta cell sequences match gene sequence and approximately 20% and 40% of these gene reads match to protein coding exons in the beta and whole islet cell populations, respectively, see Table 4.4. Using pathway and gene set enrichment analyses, we are able to determine which genes are up or down regulated in the beta cell fraction versus whole islets[43]. We find that transcription factors such as PDX1, PAX6, and MAFA are enriched in the purified beta cell population versus the unsorted whole islets, see Table 4.5. Within the beta cell population, we found the insulin gene to be expressed at 170-fold higher intensity than the glucagon gene. Of the 18,840 genes detected, glucagon ranked 18,804 and somatostatin ranked 18,838 in terms of relative gene expression in the beta cell versus the whole islet. An entire list of genes expressed in beta cells, whole islets, and the unlabeled cells, as well as their relative expression levels (expressed in Reads Per Kilobase transcriptome per Million mapped reads (RPKM)) can be found in the online Appendix (Table A.2)[44]. Values were normalized following orthogonal straight line fit regression analysis using raw

read values to compare genes specifically detected in the beta cell population versus those only detected in whole, unsorted islets.

Table 4.4 Transcriptome Sequencing Mapping Results

Sample	Total Reads	Total Mapped	Coding RNA Mapping %	Gene %	Exon %	Intron %
Whole Islets	25,946,628	23,319,210	89.9%	69.9%	42.0%	44.7%
Beta Cells	27,532,604	24,371,357	88.5%	67.5%	20.2%	54.8%
Unlabeled Cells	27,041,150	25,220,839	93.3%	28.2%	10.6%	22.0%

RNA extracted from whole human islets (that were not fixed, stained, or sorted), purified beta cells, and unlabeled cells from the same donor (24-year-old male, BMI 29.2 kg/m^2) was subjected to next generation sequencing using the Illumina platform as described in the methods. This table provides the total number of reads generated for each sample and calculates the % of the reads that map to coding RNA and the gene (including a breakdown of intron versus exon regions; note intron and exon regions may overlap in reads that analyzed pre-mRNA species).

Table 4.5 Gene Set Enrichment Analysis of Top Beta Cell Genes

Gene Rank	Symbol	GeneID	exon length	Raw Reads Whole	Raw Reads Beta	RPKM Whole	RPKM Beta	Fold Change	Log Fold Change
40	NKX2-2	4821	2,080	292	609	6	325	46.42	5.54
51	PDX1	3651	2,573	2,554	4,479	43	1,931	44.33	5.47
113	IAPP	3375	1,976	2,286	3,379	50	1,896	37.49	5.23
157	PAX6	5080	6,966	2,789	3,950	17	629	34.67	5.12
219	NKX6-1	4825	1,116	410	538	16	535	31.99	5.00
411	SLC2A2	6514	3,439	96	185	1	60	27.54	4.78
584	MAFA	389692	2,373	425	470	8	220	25.42	4.67
656	GCK	2645	2,733	330	373	5	151	24.69	4.63
946	AKT3	10000	7,082	1,864	1,748	11	274	22.36	4.48
1783	FOXO1	2308	5,738	1,463	1,149	11	222	18.69	4.22
2501	FOXA2	3170	2,423	503	364	9	167	16.95	4.08
3290	INS	3630	644	132,160	78,710	8,800	135,538	15.40	3.94

A gene set enrichment analysis was performed to identify pathways in which a significant number of genes were enriched in beta cells versus whole, unsorted islets. The genes listed are representative of those categories and the table provides overall rank and exon length for each gene (identified by symbol and gene ID). Raw reads correspond to the number of reads measured by the sequencer. Normalized RPKM reads can be calculated as follows (e.g. INS, Beta Cells: $\left(\frac{gene\ specific\ reads*10^6}{(total\ reads*exonlength)/1000}\right) * 0.037$, where 78,710 is the number of insulin specific reads, 24,371,357 is the number of total reads, 644 is the exon length, and 0.037 is the universal normalization factor calculated by orthogonal straight line fit regression analysis.

Discussion

These results provide the overall message that human islets can be sorted into individual cell subsets and gene expression can be measured. The qNPA technique allows us to measure gene expression changes on a customized, preselected number of genes. The next generation sequencing results currently provide us with a transcriptome that lists the genes expressed along with relative expression values within each cell subset. These two observations extend work done with sorted mouse islets with regard to measuring gene expression changes[27]. They also extend previous studies that were focused on the whole islet and due to technological limitations, could not determine gene expression or gene expression changes within individual islet cell subsets[14,37,45]. In this way, these studies have provided a new technique and technology to understand the genetic changes responsible for observed phenotypes of beta cell function, proliferation, ineffectiveness, and destruction. By understanding which genetic changes are specifically happening within a beta cell, we will better be able to characterize and understand how a beta cell functions and is regulated.

The qNPA technique offers a powerful method for measuring changes in gene expression levels across multiple conditions using RNA that has been partially degraded and fragmented due to its endogenous environment and the cell sorting protocol. This technique measures gene expression directly from mRNA present in cell lysate. There is no need to extract RNA, convert it to cDNA,

amplify it, or otherwise treat the raw material in such a way that could cause RNA loss or bias the results[27]. There is no need for enrichment of RNA via selection of polyadenylated tails found on full length mRNA or depletion of unwanted ribosomal RNA that could compound the ability to measure genes expressed at low levels. The lack of selection and amplification prevents PCR biases against genes that are expressed at low levels (and are thus unable to be properly represented due to fold changes that exceed the dynamic range of measure technology). Since the assay readout is dependent upon detection probes specifically designed to multiple nucleotide sequences specific to the gene of interest, unwanted material is not measured without needing to be removed.

In this study, we have shown the ability to measure consistent levels of insulin and glucagon gene expression in beta and alpha cells, respectively, following treatment with an insulin secretagogue. Interestingly, the expression of pancreatic polypeptide is found to be upregulated in beta cells (but not alpha cells) treated with this same drug. Although we have not taken the next step, the identification of a change in a beta cell specific gene expression pattern in response to an external stimulus represents the power of the methodology. It sets us up to pursue hypothesis driven cell and molecular biology experiments in beta cells.

The qNPA suffers from its limited gene detection ability per assay, as the chips we designed could only measure 16 genes per assay; with a negative control

and two housekeeping genes amongst each detection. Given our interest in studying insulin and glucagon expression, this technique is also limited by its dynamic range and saturation by the most highly expressed genes. Reducing cell numbers per assay would preclude the detection of lower expressing genes. Additionally, changes within company infrastructure have also led to this technique becoming more expensive and the support staff less responsive. Although this technique has a lot of potential, the inability to rapidly (within 3 months) and inexpensively expand and change assays is a major limitation at a time when new gene expression patterns, observations, and questions arise quickly.

The next generation sequencing technique provides the ability to indiscriminately determine all of the genes that are specifically expressed in a particular cell population[41,46,47]. When using next generation sequencing, RNA quality is an important factor to consider. Much like PCR, isolated RNA must be extracted, converted to cDNA, prepared for sequencing, and then sequenced using the Illumina HiSeq2000. In initial studies, RNA quality had to be pristine in order to selectively enrich for mRNA (via polyA tail isolation) and selectively remove contaminating rRNA and tRNA (using various nucleotide sequences to subtract unwanted RNA from the total sample). Without full length RNA, these two techniques were not possible because unwanted reads easily outnumbered desired mRNA and the technological limitations of the number of reads that could be acquired per sample were prohibitive. As the technology has improved, costs

have decreased, the problematic RNA can be reduced via alternative techniques, and all mRNA can be read due to increasing the shear number of reads. This change has opened up next generation sequencing for samples such as ours, where the RNA is degraded and cannot be pretreated to enrich for only mRNA species[42]. These same problems can compromise the ability to perform quantitative PCR and microarray experiments and set next generation sequencing apart as a method for exploring transcriptomes[14]. While whole human islets have previously been subjected to next generation sequencing analysis, transcriptome analysis of purified alpha and beta cell populations has not been possible[48].

At the beginning of this study we were able to successfully isolate alpha and beta cell populations from whole islets, but RNA quality was still subpar. Through testing and limiting the RNA degradation that occurred during sample processing and streamlining the RNA extraction protocol to occur in a single day using a kit designed for FFPE samples, we have successfully isolated RNA of acceptable quality that is both unique to a specific cell subset and of adequate quality for RNA sequencing. The DSN treatment provides one of the major limitations of these studies. While treating with DSN is incredibly helpful in order to reduce contaminating rRNA sequence, it also reduces the total amount of sequence for genes that are naturally highly expressed in these cell subsets (read: insulin and glucagon as well as some housekeeping genes that are expressed at robust levels)[42]. This technique is beneficial because it allows us to detect even the

most lowly expressed genes by providing the read depth to measure those transcripts that would generally fall outside of the dynamic range of expression. The unfortunate consequence of this technique is that true quantitation has been compromised. We can still provide relative expression levels based upon the reported RPKM values, which allows for determining which genes are highly enriched in one sample versus another. We cannot, however, provide the absolute quantitative differences between two different genes, which would make precise measurement of gene expression levels across multiple environmental stimuli difficult. We are still working to determine if a change in the RNA quality isolated from whole, unsorted islets differs from whole, unsorted islets that undergo fixation, staining, and hydrodynamic focusing, but are not actually sorted.

At the writing of this thesis, we have shown that we can sequence RNA from sorted beta cells. Sequencing coverage appears to be fairly complete, as overall gene sequencing does not favor either the 3' or 5' end across single genes. Furthermore, sequencing quality looks identical in both whole islet (high RIN) and sorted beta cell (lower RIN) populations. Furthermore, we can show that exon coverage is similar across the whole gene and predominates over intron coverage. We believe that the presence of intron sequence is a result of our inability to select mature messenger RNA sequences via polyadenylated tail enrichment techniques and not due to genomic DNA contamination because we perform two separate DNase steps to completely remove all unwanted DNA. We

have extracted sufficient amounts (> 200 ng) of total RNA, both mRNA and miRNA with acceptable RIN values, from alpha and beta cells from two additional human islet donors. This will allow us to determine the differential gene expression between the two cell types and confirm the miRNA findings from the HTG technique. Since the time from RNA extraction to sequencing results is about 60 days, we hope to have these results in early September.

CHAPTER V

FINAL SUMMARY AND CONCLUSIONS

Pancreatic endocrine cells are co-located into clusters called the islets of Langerhans that are predominantly comprised of glucagon producing alpha cells, insulin secreting beta cells, somatostatin generating delta cells, and other cell types. The regulated release of these hormones maintains whole body glucose homeostasis, essential to prevent complications from diabetes (e.g. blindness, kidney failure, and cardiovascular disease). In type 1 diabetes, an autoimmune reaction destroys the beta cells and patients must monitor their blood sugar levels and inject insulin in order to maintain euglycemia. In type 2 diabetes, the beta cells fail to produce sufficient insulin to overcome the individual's decreased insulin sensitivity. Most studies to date have focused on whole islets, which are very heterogeneous. Recent focus has shifted to studying the individual islet cell subsets (i.e. alpha, beta, delta, PP, and other cell types). Unlike immunological cells, surface molecule reagents do not yet exist to specifically distinguish beta from alpha cells, although recent studies have made progress in this direction[25,26,49]. Furthermore, the composition of the pancreas as a 95% exocrine organ produces a hostile environment rich in RNase molecules that degrade RNA quality, a factor that has confounded complete gene expression studies.

We have worked to adapt flow cytometry and intracellular hormone staining to sort pure populations of individual human pancreatic islet cell subsets. We have

characterized the relative expression and proportion of insulin producing beta cells to glucagon producing alpha cells in human donors based upon age, BMI, and gender. Although the current sample size is small, these types of studies, which are a byproduct of our sorting technique, could provide clues to how pancreatic islet composition contributes to the interruption of whole body glucose homeostasis. In addition, we have used a quantitative nuclease protection assay, modified from previous mouse studies, to measure gene expression in low quality RNA[27]. Finally, we have worked to improve RNA quality. By streamlining our sorting and RNA extraction protocols, we have improved RNA quality and performed next generation sequencing analysis to begin to define the transcriptome of human islet cell subtypes, most notably the human beta cell.

As described at the end of Chapter IV, additional RNA samples from human (and nonhuman primate) whole islets and sorted alpha and beta cells have been sent out for next generation sequencing. These samples will be very important to assess our approach's accuracy and reproducibility. We will soon have sequenced the transcriptome of three distinct whole islet, alpha cell, and beta cell populations that were isolated from three different donors. Analysis of the nonhuman primate rhesus macaque (*Macaca mulatta*) will provide gene sequence currently unavailable from the draft genome of this species[49]. By carrying out these experiments, we will fill a gap in the availability of sequence data for this species, which will help make it a more amenable model system to molecular biological and genetic analysis. Finally, the NHP samples that we have

sent for sequencing include a whole islet NHP preparation that was not fixed, stained, or sorted (unprocessed) versus a whole islet NHP preparation that was fixed, stained, and mock sorted through the flow cytometer as a heterogeneous mixture of cells (processed). This experiment will provide us with the ability to determine if sample processing changes the nature and quality of the sequences that can be detected in the sample.

The following plan outlines how we will assess the NGS technology as a useful tool for analyzing transcriptomes from fixed and sorted cells. The outlined experiments specifically focus on pancreatic islets and their associated, sorted cell subtypes Our validation and findings, however may be directly applied to other samples previously stored as formalin fixed, paraffin embedded tissue. These FFPE samples comprise a vast resource for patient or subject samples related to a specific disease or clinical trial. Through our studies, we aim to interrogate, develop, and establish a NGS protocol for these types of samples that have traditionally been precluded from downstream quantitative gene expression analysis due to poor RNA quantity and quality[50]. As advances are made in improving the quantitative capabilities, turn around time, and expense of NGS, we hope that our analysis will permit analysis of these RNA samples.

VALIDATING OUR APPROACH FOR ISLET CELL SUBSET TRANSCRIPTOME SEQUENCING

1.) Compare sequencing results between unprocessed and processed "whole islets" that were derived from the same donor.

- Compare total number and identity of genes expressed in the two samples.

- Compare the gene rank lists for each sample.

- Plot expression levels for all genes against each other and calculate correlation coefficient.

- Compare internal ratios of select genes between the two samples.

The comparisons will allow us to determine if processing the islets by fixing, permeabilizing, staining, and sorting qualitatively and quantitatively alters observed gene expression characteristics. We hypothesize that the processed and unprocessed samples will display no qualitative differences, however, as described below, quantitative biases could arise for the most highly expressed genes.

Given the stated limitations introduced by double stranded nuclease treatment in precluding quantitative gene expression analysis, we could also compare sequencing results from unprocessed and processed "whole islets" using traditional sequencing preparation steps:

- Enrich each sample for polyadenylated species using immobilized oligo (dT) – average 50-fold enrichment of mRNA[51] or

- Deplete ribosomal RNA contaminants by using immobilized sequences designed to selectively hybridize with ribosomal RNA. By immobilizing rRNA species, desired mRNA (regardless of polyadenylated tail status) can be enriched and purified, increasing the number of mRNA specific reads in NGS analysis[52].

- Perform sequencing as described with DSN treatment step.

This study would allow us to determine if traditional mRNA enrichment methods would eliminate the need to perform DSN and provide us with better data for gene quantification. In this scenario, we would compare mapped gene transcripts across all three samples (both processed and unprocessed) to determine the relative affects of DSN, polyA enrichment, ribosomal RNA depletion on an RNA sample of high integrity and then see if these results were reproduced in the processed sample. We would look to see if transcripts were biased toward either the 3' end of a gene or toward genes comprised of longer transcripts in one treatment or the other. We would also determine how many transcripts were mapped in each sample, compare the intronic versus exonic composition of each read, and identify any gross differences brought about in one treatment versus the others. If ribosomal rRNA depletion or

enrichment based upon poly adenylation status increased mRNA composition of the sample without introducing any of the biases discussed above, a much larger percentage of the NGS reads we generate would be specific to expressed genes. Eliminating the need to perform DSN (thus increasing the number of mRNA-specific reads) coupled with barcoding each sample with a unique tag would allow us to combine samples for sequencing within the same lane – together, these steps would markedly reduce the cost and reagents needed to perform each experiment[27,53].

APPLYING OUR APPROACH TO BETTER UNDERSTAND PANCREATIC ISLET BIOLOGY

2.) Compare sequencing results among all three samples of each population.

- We have noted that the ratio of alpha cells to beta cells varies from donor to donor.
- Which genes are consistently expressed in alpha cells or beta cells and how do relative expression levels differ?

This analysis will allow us to determine this technique's reproducibility by measuring the percentage of similar genes detected across multiple donors.

3.) Compare sequencing results between alpha and beta cell populations.

- Plot expression levels for all genes against each other and calculate the correlation coefficient.

- Perform gene set enrichment analysis (GSEA) for each population.

- Compare results to published literature in order to assess relative quantities of known genes.

These analyses will allow us to compare the differential gene expression between alpha and beta cells. Since alpha and beta cells are both secretory endocrine cells we wish to observe the similarities and differences between their transcriptomes.

MEASURING GENE EXPRESSION CHANGES IN DIFFERENT CELL SUBSETS

4.) Quantitating and validating results using gene expression assays

- Retest expression and relative expression ratios using qNPA or Nanostring technology – compare to NGS results.

- Assess utility of qPCR to confirm these results as related to starting mRNA quality.

- Measure gene expression changes among donors with specific characteristics (gender, BMI, diagnosed as type 2 diabetics), islets that have been treated with small molecules (GLP-1 analogues, sulfonylureas, high glucose), or in model systems where diet, exercise, age, and lifestyle can be controlled (NHP or human islets transplanted into appropriate mouse models).

Double stranded nuclease treatment currently compromises quantitation via next generation sequencing. Currently, each next generation sequencing

reaction is fairly expensive and the turn around time for results (both generating the sequencing files, assembling the transcriptome, and then analyzing the results for specific genes of interest (with appropriate data quality assessment, result normalization across donors, and elimination of biases)) can be quite extensive. While the technology continues to develop and advances are made in the turn around time and quantifying ability of NGS, we will benefit from developing and modifying existing tools to validate our NGS results and obtain quantitative data. Since our end goal is to establish techniques to quantitate gene expression changes among specific genes, we can use the NGS results that have been generated using double stranded nuclease treatment as a guide. This will allow us to design Nanostring (limit 800 candidate genes) or qNPA (limit 47 candidate genes) assays to quickly and reliably assess gene expression changes in a more cost effective manner that allows us to determine the effect of double standed nuclease treatment[27,53]. By using GSEA algorithms from sequencing results on sorted islet cell subtypes, we can choose a wide spectrum of genes that are abundantly expressed in each cell type. By measuring how the expression levels of these genes change in response to environmental stimuli hypothesized to increase beta cell mass, we can begin to identify the regulatory processes that control beta cell growth, development, maturation, redifferentiation, dedifferentiation, or death.

By establishing a reliable next generation sequencing platform, in conjunction with focused gene expression analysis, we will be able to study the dynamic nature of the beta cell transcriptome. This framework will allow us to measure differential gene expression between islet cell subtypes, among different developmental or disease states, or in response to environmental stimuli that regulate beta cell proliferative capacity and function. The data form specific islet cell populations will enable us to develop specific hypotheses regarding which genes, regulators, and pathways control glucose homeostasis. Improving our understanding of how individual islet cell subsets develop, grow, mature, and become susceptible to negative external stimuli, will elucidate the physiology and pathophysiology of islets, specifically the beta cell.

We will apply this technique to the NHP model system where more control can be exerted over islet donor age, diet, environment, quality of life, and pre-analysis metabolic phenotype. With scheduled islet isolations, we can minimize any gene expression changes associated with dysfunction, apoptosis, and necrosis induced by cold ischemia. By continuing to perform targeted human studies (e.g. control versus type 2 diabetics) and applying these to the model system, we will investigate how the transcriptome of beta cells changes under various conditions and identify pathways that dictate beta cell survival. Long term, this information may be applied to studies involving stem cell differentiation, islet transplantation, and bariatric surgery to understand which changes that occur in the beta cell may result in a positive and potentially therapeutic outcome.

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