Exploring $\beta$-cell Function and Heterogeneity in Obese SM/J Mice

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WASHINGTON UNIVERSITY IN ST. LOUIS

Division of Biology and Biomedical Sciences
Developmental, Regenerative, and Stem Cell Biology

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Exploring β-cell Function and Heterogeneity in Obese SM/J Mice
by
Mario Miranda

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ABSTRACT OF THE DISSERTATION

Exploring β-cell function and heterogeneity in obese SM/J mice

by

Mario Miranda

Doctor of Philosophy in Biology and Biomedical Sciences
Developmental, Regenerative, and Stem Cell Biology
Washington University in St. Louis, 2021
Heather A. Lawson, Chair

Pancreatic β-cells perform glucose-stimulated insulin secretion, a process required to maintain systemic glucose homeostasis. Obesity promotes glycemic and inflammatory stress, causing β-cell death and dysfunction, resulting in diabetes. Efforts to improve β-cell function in obesity have been hampered by observations that β-cells are highly heterogeneous, varying in morphology, function, and gene expression. There is great need to understand the breadth of β-cell heterogeneity in health and obesity to improve diabetic therapies.

High fat-fed SM/J mice spontaneously transition from hyperglycemic-obese to normoglycemic-obese with age, providing a unique opportunity to study β-cell adaptation. Here, we show that as they resolve hyperglycemia, obese SM/J mice dramatically increase circulating and pancreatic insulin levels while improving insulin sensitivity. Immunostaining of pancreatic sections reveals that obese SM/J mice selectively increase β-cell mass but not α-cell mass. Functional assessment of isolated islets reveals that obese SM/J mice increase glucose stimulated insulin secretion, decrease basal insulin secretion, and increase islet insulin content. Next, we integrate pancreatic islet single cell and bulk RNA sequencing data to identify β-cell
subpopulations based on gene expression and characterize genetic networks associated with β-cell function in obese SM/J mice. We assign roles to 4 β-cell subpopulations, whose composition is influenced by age, sex, and diet. Network analysis identified fatty acid metabolism and β-cell physiology gene expression modules associated with the hyperglycemic-obese state. We identify subtype-specific expression of Pdyn and Fam151a as candidate regulators of β-cell function in obesity. These results establish that improved β-cell function underlies the resolution of hyperglycemia, which may be driven by changes in subpopulation structure and decreased fatty acid metabolism.
Chapter 1: Introduction

By

Mario A Miranda, Juan F Macias-Velasco, Heather A Lawson

Adapted for dissertation from the published manuscript:

1.1 Overview

Pancreatic β-cells perform glucose-stimulated insulin secretion, a process at the center of type 2 diabetes etiology. Efforts to understand how β-cells behave in healthy and stressful conditions have revealed a wide degree of morphological, functional, and transcriptional heterogeneity. Sources of heterogeneity include β-cell topography, developmental origin, maturation state, and stress response. Advances in sequencing and imaging technologies have led to the identification of β-cell subtypes, which play distinct roles in the islet niche. This chapter reviews β-cell heterogeneity from morphological, functional, and transcriptional perspectives, and considers the relevance of topology, maturation, development, and stress response. It also discusses how these factors have been used to identify β-cell subtypes, and how heterogeneity is impacted by diabetes. We examine open questions in the field and discuss recent technological innovations that could advance understanding of β-cell heterogeneity in health and disease.

1.2 Heterogeneity in β-cells, a need for greater understanding

Pancreatic islet β-cells are optimized to secrete insulin, a process critical for maintaining appropriate blood glucose concentration. This requires coordination of glucose sensing and metabolism, ATP production via oxidative phosphorylation, insulin biosynthesis, and [Ca2+]-dependent insulin release⁴. In type 2 diabetes, peripheral insulin resistance promotes initial β-cell expansion, but ultimately results in β-cell death and dysfunction²⁴. Therapies include Metformin, sulfonylureas, and injection of exogenous insulin, which do not improve long-term β-cell function, and have been associated with weight gain, cancer, and hypoglycemic events⁵⁷. Current research strategies include ameliorating disrupted β-cell signaling pathways, removing dysfunctional β-cells, and improving the β-cell microenvironment⁸¹². Triggering β-cell
neogenesis, transdifferentiation of other cell types, and inducing β-cell replication are also being explored. Many of the therapeutic agents that improve β-cell function are in various stages of development. These strategies require understanding the physiological mechanisms controlling β-cell function and are complicated by observations that not all β-cells are alike.

Cell sorting, imaging, and sequencing technologies have allowed for analysis of multiple β-cell properties, revealing that β-cells vary in morphology, function, and gene expression. Drivers of heterogeneity include β-cell topography, developmental origin, maturation state, and stress responses. Several groups have proposed that distinct β-cell subtypes exist, and play unique roles within the islet. The importance of heterogeneity is reinforced by observations that β-cells are subject to variable fates in diabetes including apoptosis, replication, and dedifferentiation. Characterization of β-cell heterogeneity has led to questions about its role in diabetic etiology and therapy. This review examines current understanding of the breadth and sources of β-cell heterogeneity and its relevance to diabetes. Examining heterogeneity lends itself to questions about the applications and limitations of β-cell plasticity, which can focus research aimed at improving β-cell function.

1.3 β-cells are morphologically and functionally heterogeneous

Histological assessment of the pancreatic islets show β-cells vary in basic morphology, including larger cells with large nuclei that localize near the islet center. Increased nuclear size is attributed to polyploidy; β-cells are the only endocrine cell type observed in tetra- and octoploid states. The cause of polyploidy in β-cells is linked to metabolic stress, as mean nuclear size and incidence of polyploidy increase in diabetes. β-cells exhibit morphological variation in other organelles, with central β-cells exhibiting enlarged endoplasmic reticulum (ER)
and Golgi apparatuses following short glucose exposure. Other features that distinguish central from peripheral β-cells include mitochondrial morphology, number of insulin granules, and number of cell-cell contacts. Most β-cells exhibit polarity, consisting of an apical face where primary cilia project into extracellular space, a lateral face where glucose uptake and insulin secretion occur, and a basal face connected to vasculature, forming a rosette-like structure around capillaries. Further, β-cells with atypical cellular organization have also been identified, including nonpolar cells and cells with apical mRNA localization. β-cells display observable cell cycle variability, with <0.5% of adult human β-cells and 2% of adult mouse β-cells displaying markers of mitosis. This replication rate is increased in obesity, pregnancy, and with glucocorticoids. Clearly, β-cells vary in basic cellular features, and experiments reveal that β-cell function is equally diverse.

Glucose-stimulated insulin secretion has been characterized in depth. Glucose is converted to pyruvate through glycolysis initiated by glucokinase (Gck), then promotes ATP production through the Citric Acid Cycle and oxidative phosphorylation, which triggers membrane depolarization and [Ca^{2+}]-dependent insulin release. Fluorescence-activated cell sorting (FACS) based on metabolic activity of individual β-cells using NADPH autofluorescence revealed 25% of rat β-cells do not increase activity in high (20mM) glucose conditions. A follow-up study examined metabolic responders and non-responders at 7.5mM glucose. Responders enter first phase insulin secretion at lower glucose concentration and secrete more insulin, despite having similar insulin stores. This is attributed to preferential secretion of newly synthesized insulin over stored insulin and is supported by work showing insulin biosynthesis is stimulated by glucose and that responsive cells are enriched for proinsulin-rich granules.
Thus, glucose sensitive cells produce and release mature insulin granules rapidly, resulting in elevated proinsulin/insulin ratio. Dispersed human β-cells display variation in insulin secretion, with 20% of cells secreting 70-90% of insulin\textsuperscript{60,61}. Sorting β-cells based on metabolic activity at 16.7 mM glucose revealed metabolically responsive β-cells are larger in size\textsuperscript{62}. Controlling for cell size shows metabolically active and inactive cells secrete similar amounts of insulin. Thus, insulin secretion is influenced by cell size, linking β-cell morphology and insulin secretion. This evidence was corroborated by observations that larger, central β-cells degranulate faster than peripheral cells \textit{in vivo} in response to hyperglycemic conditions\textsuperscript{36}. Observations that insulin secretion varies between β-cells has been confirmed by a variety of techniques including patch-clamp measurements and 3D imaging\textsuperscript{63-65}.

Glucose sensitivity is associated with higher expression of glycolytic and protein biosynthesis genes and correlates with the glucose phosphorylation activity of $Gck$\textsuperscript{66,67}. Treatment with glyceraldehyde bypasses glucose phosphorylation to induce metabolic activity in glucose-unresponsive cells at low glucose concentrations but cannot exceed maximum glucose-induced insulin biosynthesis, suggesting $Gck$ activity is the rate limiting step in insulin synthesis and secretion\textsuperscript{68}. Giordano \textit{et al.} assessed the stability of glucose sensitivity during repeat glucose exposure using a hemolytic plaque technique to measure insulin secretion\textsuperscript{69}. They found 75% of β-cells were consistently responsive or unresponsive following 3 glucose exposures, while 25% of β-cells switched between states, suggesting glucose sensitivity fluctuates in a subset of cells. Glucose sensitivity is predictive of susceptibility to mitotic stimuli and protection against oxidative damage and apoptosis, hinting at how cells would fare in diabetes\textsuperscript{51,56,70}. Collectively,
these observations highlight the breadth of phenotypic variation in \( \beta \)-cells, motivating studies aimed at understanding the mechanisms underlying these differences.

**1.4 Heterogeneity in gene and protein expression**

Many genes in the insulin secretion pathway display heterogeneous expression and have a direct impact on \( \beta \)-cell function. Glucose import through \( \text{Glut2} \) is the first step in the insulin secretion pathway and is inhibited in STZ-induced diabetes in mice\(^{71} \). Heterogeneous \( \text{Glut2} \) expression has been reported in several studies and may be indicative of maturation state and replicative potential\(^{25,45,72–76} \). The rate-limiting enzyme \( \text{Gck} \) is variably expressed among \( \beta \)-cells, correlating with glucose sensitivity, \( \text{Glut2} \) levels, and number of insulin granules\(^{67,77} \). Metabolic perturbations influence \( \text{Gck} \) expression, which precede changes in \( \text{Ins2} \) expression, suggesting a regulatory role in insulin production\(^{78} \). Variation in insulin gene expression and protein levels has been observed in many studies\(^ {23,38,74,78,79} \). Central \( \beta \)-cells contain less insulin protein than peripheral \( \beta \)-cells, a distinction that disappears with fasting\(^ {78} \). Using an insulin promoter construct linked with GFP, Katsuta et al. used FACS to separate \( \beta \)-cells containing low, medium, and high levels of insulin protein\(^ {38} \). Medium level \( \beta \)-cells comprised 70% of cells, and the low insulin population had diminished insulin gene expression and secretion. Variable expression of these components may directly contribute to the heterogeneity seen at the phenotypic level.

Other gene markers of \( \beta \)-cell heterogeneity with a less direct connection to insulin secretion have been identified. These include cell-cell adhesion genes like the sialylated form of neural cell adhesion molecule (PSA-NCAM)\(^ {80} \). FACs sorting \( \beta \)-cells into PSA-NCAM-high and -low populations revealed that expression correlates with glucose-stimulated insulin secretion\(^ {73,81,82} \). PSA-NCAM-low cells have less \( \text{Ca}^{2+} \) influx following glucose exposure,
reduced ATP levels, and decreased expression of Glut2 and Gck. Morphologically, PSA-NCAM knockout cells differ in f-actin distribution, altering cell polarity. The proportion of PSA-NCAM-high cells increases in diabetes. Whether NCAM is heterogeneously expressed in humans is unknown. β-cells can be sorted based on expression of E-cadherin, which promotes islet formation and compaction. Like NCAM, sorting β-cells into E-cadherin-high and-low populations revealed that higher expression is associated with increased glucose-stimulated insulin secretion and insulin expression. Further, glucose induces E-cadherin expression and causes dispersed β-cells to aggregate in vitro. Other variably expressed genes include Npy, Th, Vmat2, and Dickkopf-3.

Several studies have identified β-cell subpopulations based on variation in global gene expression. Baron et al. found two subpopulations in nondiabetic donors driven by variable expression of ER-stress response genes including HERPUD1, HSPA5 and DDIT3, which correspond with low expression of genes associated with β-cell function including UCN3, MAFA, and NEUROD1. This heterogeneity was lost in hyperglycemic samples. Similarly, Muraro et al. identified 3 β-cell populations, one of which had elevated expression of stress response genes HERPUD1 and HSPA1B. Importantly, Muraro et al. validated these findings using RNA-FISH on intact islets to rule out isolation-induced stress. Xin et al. identified four subpopulations in human β-cells, one of which differentially expressed 431 genes. This subpopulation had low insulin expression and elevated expression of ER-stress and UPR-associated genes. Fang et al. profiled 9,964 β-cells from healthy and diabetic donors and identified a subpopulation enriched for expression of heat shock proteins. Lastly, Segerstolpe et al. identified 5 subgroups based on expression of RBP4, FFAR/GPR120, and ID family
transcription factors\textsuperscript{93}. \textit{RBP4} expression is associated with insulin resistance, while ID transcription factors are associated with cell cycle activity\textsuperscript{94,95}. Several groups have failed to detect β-cell subpopulations; however, many were limited by the number of β-cells analyzed\textsuperscript{96–101}. Despite little consensus in the genes that may drive heterogeneity, an emerging pattern suggests β-cells differ in expression of genes related to stress response.

The limitations of single cell technology explain some of the discordance among studies. These shortcomings are highlighted in a meta-analysis from Mawla and Huising, who examined discrepancies across five single cell RNA sequencing studies\textsuperscript{90,91,93,97,99,102}. Pooling β-cells across all studies revealed 2 populations of β-cells driven by differential expression of 52 genes. These include genes associated with other endocrine and exocrine cell types, but not stress response. Only 3 genes had previously been associated with β-cell heterogeneity: \textit{RBP4}, \textit{DLK1}, and \textit{HERPUD1}. When subset into cells from healthy donors, only 24 genes were differentially expressed across β-cells. Among these, only \textit{NPY} has been previously associated with β-cell heterogeneity. This lack of correspondence among studies is a concern. Only 86 genes were detected in all β-cells within the aggregated data. This contrasts with the hundreds of housekeeping genes required for cellular function\textsuperscript{103}. β-cells express at least 18,000 genes, thus 99.5% of genes are inconsistently captured\textsuperscript{21,93}. With current methodology, the capture of most genes is likely due to technical artifacts or random chance, and not intrinsic biological variation\textsuperscript{104}. Methods for preprocessing and noise removal are being developed to aid in quality control, including tools that remove cell doublets, remove ambient RNA, assess transcript coverage, and provide imputation for gene dropout\textsuperscript{105–108}. In human studies, donor age and isolation procedure likely contribute to discordance among studies but are seldom controlled for.
Further, many groups report differential expression of genes between subsets of β-cells, but effect size is rarely reported or discussed. As technological advances increase single cell sequencing read depth, profiling of thousands of cells all but guarantees statistically significant differential expression based on p-values alone. Discussions about whether these differences are meaningful will be increasingly important, and will be aided by measuring effect size\textsuperscript{109}.

Dorrell \textit{et al.} used FACS to identify 4 subpopulations based on expression of human β-cell surface markers CD9 and ST8SIA1\textsuperscript{110}. They designated these populations as β1–4, where β1/β2 cells are positive for ST8SIA1 and β3/β4 are ST8SIA1 negative. There are 125 genes differentially expressed across groups, while gene ontology enrichment analysis revealed β1/β2 cells are enriched for protein secretion, while β3/β4 cells are enriched for neurogenesis. This corresponds with β1/β2 populations having greater glucose-stimulated insulin secretion, while β3/β4 have elevated basal insulin secretion. The proportions of these populations are altered in diabetic individuals, suggesting they differ in susceptibility to metabolic stress, with the caveat that inter-patient variation is much higher than variation between healthy and diabetic donors. How these markers of heterogeneity are tied to β-cell function remain areas of interest. It is possible that these populations have separate roles within the islet niche, with β1/β2 cells specializing in glucose-stimulated insulin secretion, and β3/β4 cells specializing in basal insulin secretion.

The potential existence of specialized basal insulin secreting β-cells is supported by Farack \textit{et al.}, who used smFISH to identify a subpopulation of mouse β-cells with elevated insulin expression\textsuperscript{23}. These cells, termed “extreme” β-cells, represent ~7% of β-cells, and localize near the islet center. Extreme β-cells have a distinct polarization pattern, high pro-insulin
and ribosomal RNA content, and less mature insulin protein. They have elevated expression of *Ins1, Iapp, Chga,* and *Pcsk2* which are associated with insulin secretion. Thus, the authors suggest that these cells are producing and quickly secreting high levels of insulin, so they maintain a high proinsulin/insulin ratio despite having other markers of β-cell maturity. The proportion of extreme β-cells increase in *db/db* mice, suggesting they may play a role in glycemic stress response. These findings are summarized in Figure 1.1. Could these extreme β-cells overlap with the β3 and β4 populations identified by Dorrell *et al.* Or with PSA-NCAM-low and E-cadherin-low cell populations? Answers to these questions may provide insight into how to remedy the dysregulated glucose-stimulated insulin secretion seen in diabetic islets. Discussed below are 4 factors that influence β-cell behavior: topology, development, maturation, and stress response. These factors provide insight into how β-cell heterogeneity is maintained, and how it may be harnessed for therapeutic purposes.

### 1.5 Topography influences β-cell heterogeneity

In addition to β-cells, islets contain α-cells, δ-cells, ε-cells, γ-cells, as well as non-endocrine cells including endothelial cells, macrophages, glia, fibroblasts, and pericytes. Mice and humans have different islet compositions, which may explain why few discoveries from rodents validate in humans. Mouse islets are comprised of 60-80% β-cells and 15-20% α-cells. β-cells localize to the center, or core, of the islet, while α- and δ-cells localize to the periphery, or mantle. Human islets are comprised of 50% β-cells and 40% α-cells. Recent imaging efforts suggest human islets comprise small repeating clusters of the β-cell core, α-cell mantle pattern. Further, location within the pancreas influences islet cell composition. Islets in the pancreatic head are enriched for γ-cells, while islets enriched in α-cells are found in
the neck. The proportion of β-cells per islet increases from head to tail of pancreas\textsuperscript{115–117}. Islets in the tail of the pancreas degranulate faster than head islets \textit{in vivo} following prolonged glucose exposure, and have higher rates of replication, insulin synthesis, and secretion\textsuperscript{36,118–120}. Human islets near the pancreatic head tend to be less spherical than those found in the tail, which may influence cell-cell communication\textsuperscript{121}. NCAM plays a role in islet organization, as NCAM knockout mice have altered islet cell composition, including α-cell infiltration into the islet core\textsuperscript{80}. Both humans and mice display altered islet composition in diabetes, including deviation from the mantle-core arrangement\textsuperscript{122,123}. β-cell loss is more pronounced in γ-cell-rich islets within the head of the pancreas in diabetes\textsuperscript{120}. β-cells in the splenic region of the pancreas undergo proliferation during diabetes, while those near the head do not\textsuperscript{56}.

As previously discussed, β-cells near the islet core exhibit morphological and functional differences from those in the periphery\textsuperscript{31,36,78,124–126}. These differences are attributed to the homo- and heterotypic connections maintained by each cell, a result of the mantle-core architecture of the islet, in which core β-cells are enriched for homotypic contacts\textsuperscript{127}. Dispersed β-cells secrete less insulin in response to glucose than β-cells in an intact islet\textsuperscript{128,129}. β-cells coupled with other β-cells secrete more insulin than individual β-cells, while β-α-cell couplings produce more insulin than individual β-cells, but less than β-β-cell coupling\textsuperscript{60,129,130}. Peripheral β-cells have more heterotypic contacts with somatostatin-secreting δ-cells, which inhibits insulin secretion\textsuperscript{124,131–134}. Importantly, comparisons between core and peripheral β-cells in islets cultured \textit{ex vivo} are confounded by the rapid onset of hypoxia in the islet core following isolation, particularly in large islets\textsuperscript{135–137}. It is critical that observations comparing each population are verified using complementary methods. Primary cilia influence β-cell function by
facilitating communication with neighboring cells, and further exploration is needed to see if they play a role in maintaining heterogeneity\textsuperscript{138,139}. The morphological and functional differences between core and peripheral β-cells may be derived from connections to other β-cells, α-cells, or δ-cells, which in turn is influenced by the location of the islet within the pancreas (summarized in Figure 1.1C).

Within each islet, small clusters of β-cells synchronize Ca\textsuperscript{2+} fluctuations\textsuperscript{140}. This is attributed to gap junctions between β-cells coordinating insulin release and may explain why coupled cells secrete more insulin than isolated cells\textsuperscript{39,124,141,142}. Gap junctions are heterogeneously expressed across the population\textsuperscript{39,40,141,143}. A subpopulation of hyperconnected β-cells has been proposed\textsuperscript{24}. These “hub” cells were identified by quantifying the number of significant pairwise correlations of glucose-stimulated Ca\textsuperscript{2+} oscillations that each cell maintained. These cells represent 1-10\% of all β-cells and control the activity of “follower” cells by coordinating Ca\textsuperscript{2+} signaling through gap junctions. These cells express high levels of Gck and genes involved in glucose oxidation, but low levels of insulin, and have accelerated and extended Ca\textsuperscript{2+} signaling during glucose stimulation. Ablation of hub cells disrupts Ca\textsuperscript{2+} synchronization across the islet, while ablating follower cells had no effect. These cells are sensitive to diabetic insults, which could explain why Ca\textsuperscript{2+} coordinated insulin release is disrupted in diabetes. These findings are summarized in Figure 1.1E. Salem \textit{et al.} built upon this model, showing transplanted islets contain hyperconnected β-cells, termed “leader” cells, that trigger a wave of calcium release across the islet \textsuperscript{144}. The role of hub/leader cells have been challenged, because the mechanisms through which hyperpolarizing hub cells inhibit Ca\textsuperscript{2+} coordination among other cells is unclear\textsuperscript{145}. The relevance of such a subpopulation has been questioned, as disrupting Ca\textsuperscript{2+}
signaling in ~70% of β-cells does not alter insulin secretion, suggesting other mechanisms are of greater importance. Further, knockout of the gap junction protein Cx36 in mice results in altered calcium synchronization, but has minimal effect on overall insulin release, suggesting Ca\textsuperscript{2+} signaling is influential but not critical for β-cell function.

Vascularization and innervation also vary amongst islets and provide a template for heterogeneity. Capillaries provide nutrients, oxygen, and ECM support to islets, which improve insulin secretion and promote cell survival. Small islets have 2-3 vascular penetrations, large islets have >3, resulting in β-cells receiving unequal access. Islets in the pancreatic periphery receive capillary branches, while islets at the center directly contact large blood vessels. Several groups have proposed the existence of two classes of islets based on vascularization, high-perfused and low-perfused. High-perfused populations represent 75% of islets, have higher glucose-stimulated insulin secretion and greater rates of β-cell replication, but are susceptible to cytokine induced cell death and hypoxia. These differences are attributed to capillaries increasing oxygen tension within β-cells. The low-perfused islets appear to serve as reserves and are recruited to activity when metabolic demand increases. β-cells organize themselves around capillaries in rosette structures, with insulin granules localizing towards the capillary and driving polarity. Evidence suggests blood typically flows from islet core to mantle, from β-cells, to α-cells, then δ-cells. This may bias directionality of cell-cell communication among endocrine cells. Innervation also impacts islet development, maturation, mass, and function. While only a small number of β-cells receive axon terminals, which tend to reside in the islet periphery, neurotransmitters including serotonin and GABA promote β-cell replication. Human islets have less innervation than...
mouse islets, which may explain why they have lower regenerative capacity\textsuperscript{168}. However, it is unclear if differences in biological age between mice and human donors confound these findings. Clearly the location of the β-cell within the islet, and the islet within the pancreas, influences β-cell exposure to nutrients and potentially harmful milieu. As β-cell heterogeneity is explored in greater depth, topology may aid in understanding why cells behave differently.

### 1.6 Developmental origin influences β-cell heterogeneity

The pancreas arises from two epithelial evaginations in the foregut, termed the dorsal and ventral buds, which give rise to the head and tail regions, respectively\textsuperscript{169}. Despite differences in blood supply and innervation, both buds contain endocrine progenitor-precursor cells, which give rise to nascent β-cells\textsuperscript{169–172}. Using chimeric mice, Deltour et al. showed that individual islets contain β-cells with different developmental origins, suggesting β-cells are not monoclonal within the islet\textsuperscript{173}. Nascent β-cells undergo an initial period of replication, peaking at 20 weeks gestation\textsuperscript{174}. β-cell replication remains high at birth, then declines rapidly with age, resulting in a several-fold expansion of β-cell mass between birth and adulthood\textsuperscript{175}. β-cell telomere length diminishes over time, particularly during the first two months of life, when β-cell proliferation is at its highest\textsuperscript{176}. Further, 4-month-old mice display greater variability in telomere length among β-cells compared to 12-day old mice, suggesting replication is not uniform across the population. In adult mice, β-cell replication is the principle mechanism for maintenance of β-cell mass, driven by a small number of mitotically competent cells, which declines with age\textsuperscript{47,177–179}. The number of replicating β-cells increases under metabolic stress and inflammation, but this effect is also restricted by age\textsuperscript{48,51,175,180–182}. Several other markers have been associated with β-cell aging and functional decline, including expression of IGF1R and P53BP1, and increased lipid
storage\textsuperscript{183,184}. The degree of heterogeneity displayed by these markers is unknown. While β-cell replication plays a central role in generating and maintaining β-cell mass, the notion that it is the sole mechanism for β-cell replacement has been challenged.

Insulin positive cells are observed in ductal tissue following partial pancreatectomy and overexpression of gastrin and TGFα in ductal cells\textsuperscript{185–188}. Prolonged glucose infusion induces ductal clusters to become PDX1 positive, with some expressing insulin\textsuperscript{189}. These extra-insular β-cells are also proliferative, as they are observed in aggregates <50 μm\textsuperscript{190–192}. Like low-perfused islets, ductal cells may provide a reserve pool of insulin production during glycemic stress. Several groups have identified a pancreatic multipotent progenitor (PMP) population\textsuperscript{25,76,193,194}. These cells express insulin but lack Glut2 expression, likely preventing them from performing glucose-stimulated insulin secretion. Interestingly, these cells have the capacity to proliferate and generate multiple endocrine cell types, including functional β-cells. Further, replication of these cells is induced by hyperglycemic conditions, suggesting they too may play a role in glycemic stress response. The existence of PMPs has been heavily challenged, as other studies fail to identify such populations, even following substantial β-cell loss\textsuperscript{177,195,196}. Much of the argument centers on the weaknesses of lineage tracing, reviewed in\textsuperscript{197}, and the low likelihood that these cells contribute meaningfully to functional β-cell mass. What relationship, if any, exists between ductal-derived β-cells and PMP’s remains to be characterized.

α-cells have been shown to acquire β-cell-like properties in response to extreme β-cell loss and genetic perturbations\textsuperscript{198–202}. However, the utility of these observations is questioned because of lack of evidence that this phenomenon occurs in normal islet homeostasis. A study by van der Meulen et al. addressed some of these criticisms by identifying a Glut2-low population
of β-cells residing within the islet periphery. Using lineage tracing, they showed that these “virgin” β-cells do not express Glut2 because they are an intermediate in α-to-β-cell transdifferentiation. These cells do not import meaningful amounts of glucose following 50 minutes of exposure and are resistant to STZ-induced β-cell death, likely due to lack of Glut2 expression. These cells transition into Glut2-high β-cells over time, making them indistinguishable from conventional β-cells. A recent study challenged that Glut2-low cells represent an α-β transitional cell, and that massive β-cell loss induces transdifferentiation. Feng et al. used an STZ model of diabetes to assess the fates of Glut2-high and Glut2-low β-cells using single cell RNAseq. While Glut2-low β-cells survive STZ treatment, they do not mature into Glut2-high cells in 9 months post-treatment, contradicting van der Meulen et al.’s findings. Further, they did not find evidence for α-β-cell transdifferentiation at the transcriptional level. However, these mice were given insulin pellets to ensure long-term survival, which may confound interpretation. Regardless of their relevance to normal insulin homeostasis, the transdifferentiation of ductal or α-cells into β-cells remains an appealing therapeutic approach for diabetes. Neither ductal or α-cell populations are reduced by diabetes, providing a steady source of potential β-cells. Further, α-cells display a high degree of resistance to viral and metabolic induced stress, suggesting that β-cells derived from α-cells could retain these properties.

The potential sources for β-cells are summarized in Figure 1.1A. At this junction, it is not clear how β-cells from different developmental sources display meaningful heterogeneity. Given the influence of the β-cell microenvironment on function, there is no reason to believe β-cells from dorsal or ventral islets, β-cell replication, differentiation of PMPs, or transdifferentiation of β-cells would behave identically. Understanding how β-cells functionally mature will provide insight into how β-cell location, age, and function interconnect.
1.7 Maturation influences β-cell heterogeneity

How β-cells acquire specialized functionality has been characterized in depth. In mice, initial β-cell maturation proceeds in two developmental phases\(^\text{206-209}\). The first phase lasts two weeks following birth, a period of rapid β-cell proliferation and simultaneous waves β-cell apoptosis, which coordinate to set the foundation for β-cell mass\(^\text{210,211}\). Surviving cells begin to express transcription factors required for β-cell function and are considered immature\(^\text{172,206,212}\). The second developmental phase starts at weaning, characterized by changes in metabolic pathways and functional maturation\(^\text{209,213}\). Comparing β-cells from 4-week old mice and 16-week old mice reveals differential expression of thousands of genes, while only 193 genes were differentially expressed between 3- and 26-month old mice, suggesting β-cells change substantially early in life and little in adulthood\(^\text{96,214}\). Pseudotime analysis of individual β-cells reveals differential expression of genes associated with amino acid uptake, ROS production, and cellular respiration during functional maturation\(^\text{101,178}\). Markers of mature β-cells include Glut2, Ins2, Mafa, Nkx6.1, NeuroD, and Ucn3 while immature β-cells express Hkl, PaxA, Pax6, and Mafb\(^\text{3,215-218}\). In humans, MAFB is expressed in mature β-cells and knockout of MAFB results in β-cell dysfunction, suggesting it plays a nonredundant role from MAFA\(^\text{219-221}\). A set of “disallowed” genes including Hkl, Ldha, Mct1, Rest, and Pdgfra, have been identified as being prohibited from expression in mature β-cells\(^\text{213,222,223}\). Many of these genes are involved in glucose metabolism and exocytosis and would interfere with glucose-stimulated insulin release\(^\text{224}\). The molecular machinery driving insulin release is similar in both mature and immature β-cells, with a few key differences\(^\text{218}\). Neonatal islets have elevated basal insulin secretion due high glucose sensitivity\(^\text{218,225}\). Interestingly, when immature and mature β-cells are
depolarized independent of glucose sensing, both release similar levels of insulin, suggesting their differences lie in glucose sensing and metabolism^{218}. Immature β-cells express Hkl1, which has a higher affinity for glucose than Gck, which is expressed in mature β-cells^{226–228}. Thus, it takes higher concentrations of glucose to trigger glycolysis and downstream oxidative phosphorylation in mature β-cells, which may explain differences in insulin secretion between these two classes of cells. Other components of the insulin secretion pathway, including genes involved in glycolysis, the TCA cycle, oxidative phosphorylation, and exocytosis are also differentially expressed between mature and immature β-cells^{209,213,229}. An overview of immature β-cell characteristics is shown in Figure 1.2A.

Maturation state appears to be a heterogeneous trait in adult β-cells and is associated with replicative capacity^{214,230,231}. Szabat et al. used a Pdx1/Insulin dual reporter to discover that 25% of β-cells in humans and mice are Pdx1 positive but have low insulin expression^{74}. These cells have an immature gene expression profile, including elevated Mafb expression, and low Gck and Glut2 expression. Immature cells have high proliferative capacity and poor insulin secretion, and functionally mature without proliferation in vitro. Bader et al. reinforced these findings by identifying a subpopulation of immature β-cells based on lack of Fltp expression (discussed below)^{45} These cells have high replicative capacity at the expense of mature β-cell function. The replication/maturation dichotomy was illustrated by Klochendler et al., who used a fluorescent reporter mouse for cyclin B1, which is active only during mitosis^{230}. This allowed sorting of cells based on a mitotic state. Performing RNAseq on these populations revealed a global increase in cell cycle genes, but a distinct downregulation of genes associated with β-cell function including Glut2 and Ucn3 as well as enrichment for genes regulated by mature β-cell transcription factors.
In human β-cells, a clustering approach to simultaneously assess intracellular Ca²⁺ dynamics in ~300 islet cells revealed two distinct populations based on Ca²⁺ flux frequency and amplitude, suggesting mature and immature states. The dichotomy between replication and maturation is associated with ER stress response. These observations have implications for understanding post-natal β-cell expansion; individual β-cells cannot simultaneously replicate and be functional.

Observations that β-cell replication and maturation are at odds were coalesced into a unified theory by Bader et al., who separated β-cells into mature and immature populations based on expression of Flattop (FLTP). FLTP is expressed in tissues where the Wnt/Planar cell polarity pathway is active and is necessary for epithelial organization and architecture. Bader et al. used an islet specific FLTP reporter and found expression increases throughout β-cell maturation, resulting in 80% of cells positive for FLTP in adult mice. Transcriptional analysis revealed 997 genes differentially expressed between populations, with positive cells expressing markers of maturation including Ucn3, Mafa, mitochondrial oxidative phosphorylation genes, and insulin secretion genes, while negative cells expressed genes associated with MAP kinases, Gcprs, and had low Glut2 expression. FLTP(-) cells are more proliferative, with 8% of negative cells staining Ki67(+) at postnatal day 11, compared to 2% of FLTP(+) cells. In high fat diet-induced islet hypertrophy, the FLTP(+) population expanded in line with heightened metabolic demand, suggesting that the progeny of FLTP(-) cells can become FLTP(+). Morphologically, FLTP(+) cells are polarized and found in rosette structures. Re-aggregated FLTP(-) cells in vitro have lower glucose stimulated insulin secretion than positive cells, suggesting they are functionally immature. Knockout of FLTP resulted in very mild functional changes, suggesting FLTP is a passive marker for maturation. In humans, islet FLTP expression correlates with
glucose tolerance, with healthy donors expressing higher levels of FLTP than prediabetic and diabetic donors. Thus, FLTP distinguishes proliferative and metabolically active β-cells and suggests β-cell maturation could be driven by the polarization process. The specific mechanism through which the wnt/PCP pathway effects β-cell function is unknown, but it may involve cytoskeleton components and gene expression changes. These findings raise questions about β-cell heterogeneity. Are FLTP(-) cells part of the 25% of β-cells that do not increase metabolic activity in high glucose56? Could these be the Glut2-low cells reported in PSA-NCAM low cells or E-cadherin low cells73,81–83? Or the poor functioning β3 and β4 cells identified by Dorrell et al.110? It is possible that many of these studies are converging on overlapping populations of cells. The differences between immature and mature β-cells are highlighted in Figure 1.1B and Figure 1.2B.

Dedifferentiation of mature β-cell identity is a central component of β-cell dysfunction in type 2 diabetes215,235–239. This is attributed to many factors, including oxidative stress, glucose and lipid toxicity, and inflammation235,240–242. Dedifferentiated β-cells have decreased insulin expression and decreased insulin secretion, thus contributing to loss of glycemic control3,93,240. Dedifferentiation is characterized by decreased expression of β-cell-specific transcription factors Pdx1, Nkx6.1, and MafA, increased expression of developmental genes, Ngn3, Oct4, Nanog, and L-myc, and expression of disallowed genes, Ldha, Mct1, G6pc, and Hk126,222,242–244. These changes are linked to decreased expression of Foxo1, which mediates Pdx1 activity3,26,245–247. An overview of the characteristics of dedifferentiated β-cells is shown in Figure 1.2C. Moving hyperglycemia-induced dedifferentiated β-cells into a healthy environment can reverse the dedifferentiation phenotype215,236,248. Insulin administration can also return immature cells to a
functional state, suggesting therapeutic intervention is possible. We recently published findings on obese SM/J mice, which spontaneously transition from hyperglycemic-obese to normoglycemic-obese with age. Underlying this transition is improved β-cell function, including increased insulin content and glucose-stimulated insulin secretion. This suggests SM/J β-cells are likely undergoing maturation and show that β-cell functionality can be improved in vivo in the context of obesity. Evidence for dedifferentiated β-cells in diabetic humans based on single cell RNA sequencing data has been established. The parallels between nascent β-cell maturation and diabetes-induced dedifferentiation have not been fully explored. Greater understanding of the β-cell maturation process may provide insight into how dedifferentiated β-cells may be rescued, opening exciting new avenues for therapeutic interventions.

1.8 Stress response influences β-cell heterogeneity

An emerging paradigm suggests that β-cells undergo cycles of insulin production and stress response, due to the unique physiological demands they face. Insulin synthesis accounts for 10% of total protein during basal conditions, compared to 50% in stimulated conditions. Insulin is highly prone to misfolding. Once misfolded, insulin can be refolded or degraded. Under demanding conditions, misfolded proteins accumulate, and the UPR machinery is activated to clear the misfolded proteins. The high rate of oxidative phosphorylation in β-cells also generates ROS, which can damage nascent proteins. β-cells have been characterized as having low antioxidant defense, and thus are more susceptible to stress. This combination of factors make the insulin secretion responsibilities of β-cells a unique cellular challenge.

Many single cell studies identified subpopulations of β-cells based on an ER stress response signature, at the expense of genes related to mature β-cell function. Xin et al.
performed pseudotime analysis on human β-cells and identified the transcriptional trajectory between the three β-cell states: High Insulin/Low UPR, Low Insulin/High UPR, and Low Insulin/Low UPR. This suggests mature β-cells undergo a cycle: Elevated insulin production, followed by a period of low insulin production and UPR activation to clear misfolded insulin protein, then a rest period of low insulin and low UPR activity. Under this hypothesis, only a fraction of β-cells produce insulin at a given time while others are resting/recuperating. This is supported by evidence from isolated human and mouse β-cells, where a small fraction of cells are responsible for the majority of insulin secretion. Xin et al. also found Low Insulin/High UPR cells were more proliferative, suggesting they are given the chance to replicate during recovery. Cells under glycemic stress would produce more insulin, thus would have a great degree of UPR activation. Whether replication is induced by the intensity or length of UPR response is unknown. This theory is supported by Szabat et al., who used a Pdx1/INS dual reporter to show that β-cells transition between periods of high and low insulin in ~27 hour cycles. Activation of UPR in human β-cells also stimulates proliferation. A summary of β-cell stress response cycling is summarized in Figure 1. Could the interplay between these metabolic states explain the morphological, functional, and transcriptional heterogeneity seen in β-cells, particularly the identification of β-cell subpopulations in single cell RNAseq data? Loss of β-cell mass by apoptosis occurs in diabetes, driven in part by failure of the ER stress response. In response to prolonged stress, what drives β-cells to undergo apoptosis, dedifferentiation, or replication? Identifying cells that are prone to a specific fate may help prevent loss of β-cell mass.
1.9 Conclusion

Our understanding of β-cell heterogeneity has increased over decades, revealing several trends. First, there is a morphological and functional dichotomy between peripheral and core β-cells. This is attributed to topology including homotypic contacts, heterotypic contacts, vascularization, and proximity to axon terminals. Further, islet topology changes in diabetes, highlighting the importance of understanding the role of the β-cell microenvironment. The developmental origins of the β-cell, including neogenesis, β-cell replication, and differentiation from another cell may play a role in heterogeneity. These different sources produce adult β-cells that vary in cellular age, which is associated with functional decline. How the distribution of young and old β-cells influences islet function and stress response is unknown, but it clearly influences replicative capacity. The existence and relevance of ductal cell-, α-cell-, and PMP derived-β-cells remains controversial, but they are appealing avenues for diabetic therapy because of their stress resistance characteristics. β-cell function and replication are at odds during the initial maturation process, resulting in decreased mitotic index and improved glucose-stimulated insulin secretion. In adults, a subpopulation of cells retain immature gene expression and functional characteristics, including higher replication rates. Loss of mature β-cell identity is a hallmark of diabetes and disrupts the insulin-glucose axis. Can studying the differences between immature and mature β-cell populations in healthy adults inform diabetic therapy? Variation in β-cell metabolic activity is associated with differences in stress susceptibility, suggesting mature and immature populations likely succumb to different fates in diabetes. The relationship between β-cell maturation, replication, and stress response is
complicated by observations that mature β-cells undergo a stress response cycle, and that UPR activation can stimulate replication\textsuperscript{21,178,233,259}. The relationships among classes of variation, sources of variation, and proposed subtypes is summarized in Figure 1.1. These likely contribute to the variance seen in single cell RNA sequencing studies, many of which cluster cells based on expression of stress response genes\textsuperscript{21,90,92,258}.

Assessment of variation in gene expression, protein levels, morphology, and function has led several groups to identify β-cell subpopulations. Given the frequency of these populations, there is likely substantial overlap. This includes FLTP (-), ST8SIA1 (+), PSA-NCAM-low, E-Cadherin-low, and Glut2-low “virgin” β-cells all possibly identifying adult immature β-cells based on gene expression and functional characteristics\textsuperscript{45,72,81,83,260}. How much of the transcriptional heterogeneity identified in single cell RNA sequencing is driven by the ER stress response cycling\textsuperscript{21,90,258,259}? Further, does ER stress response cycling influence the immature β-cell population? This could explain the elevated, but not dramatic increase in β-cell replication seen in immature cells. These observations raise questions over requirements for subtype classification. Are subtype traits under unique selective pressure, different from the general population? What are the temporal requirements for a subtype? What about cell plasticity, the ability of subtypes to interconvert? Should a subtype require a unique functional and transcriptional profile? Answering these questions will clarify which heterogeneous traits are critical for building subtype identities.

Techniques including FACS and single cell RNA sequencing are mainstays in shaping understanding of β-cell heterogeneity. However, these techniques have limitations, as they fail to capture functional, transcriptional, and topological information simultaneously. Further,
questions about the temporal regulation of β-cell heterogeneity are becoming unavoidable. Lineage tracing is the gold standard for understanding developmental trajectories but has received considerable criticism for its shortcomings including promoter leakage, low labeling efficiency, and inability to recapitulate native expression patterns\textsuperscript{197}. Dual trace techniques may overcome some of these issues\textsuperscript{261}. Several new techniques promise to improve our understanding of β-cell heterogeneity, including preserving live sections using perfluorocarbon\textsuperscript{262,263}. This will allow temporal and topological assessment of β-cells and provide the opportunity to test the effects of various compounds on β-cell growth and function \textit{in vivo}. This will also provide a new platform for lineage tracing. SeqFISH allows one to probe the expression of hundreds of genes simultaneously in intact tissue, which can couple extensive transcriptional information with topology\textsuperscript{264}. Patch-Seq couples β-cell ion-channel activity with single cell RNA sequencing, linking functionality with gene expression\textsuperscript{63}. This is an exciting time to be in the field of β-cell heterogeneity, as many of the discoveries made over decades of research are slowly coalescing into a unified understanding of how and why β-cells differ. Whether these discoveries will make meaningful improvements in the way diabetes is studied and treated remains to be seen.

\textbf{1.10 Scope of Thesis}

Previous work established that obese SM/J mice transition from hyperglycemic at 20 weeks of age to normoglycemic by 30 weeks of age. Given the importance of β-cell function in maintaining glycemic control, we sought to explore how obese SM/J β-cells change during this remarkable period. By contrast β-cells in hyperglycemic-obese and normoglycemic-obese mice, we aim to identify the genes and pathways associated with improved glucose homeostasis, which may reveal novel strategies that improve β-cell function in obesity. Given the increasing
relevance of β-cell heterogeneity in diabetes research, we also sought to characterize β-cell subpopulations in obese SM/J mice, and how they contribute to the hyper- and normoglycemic-obese states. By integrating phenotypic information with subpopulation structure, a more complete picture of β-cell function in obese SM/J mice can be achieved. The following chapters explore how obese SM/J β-cell’s change from a morphological, functional, transcriptional, and single cell perspective.

In chapter 2 we characterize insulin homeostasis, islet morphology, and β-cell function during SM/J’s diabetic remission. Obese SM/J mice dramatically increase circulating and pancreatic insulin levels, improve insulin sensitivity, and increase β-cell mass. Functional assessment reveals obese SM/J mice increase glucose stimulated insulin secretion, decrease basal insulin secretion, and increase islet insulin content. In chapter 3, we integrate islet single-cell and bulk RNA sequencing data. We identify 4 β-cell subpopulations associated with insulin secretion, hypoxia response, cell polarity, and stress response are influenced by age, sex, and diet. Network analysis identifies fatty acid metabolism gene expression modules associated with the hyperglycemic-obese state. We identify Pdyn and Fam151a as candidate regulators of β-cell function in obesity. This study establishes that β-cell mass expansion and improved β-cell function underlie the resolution of hyperglycemia and uses a novel data integration method to explore how β-cells respond to obesity and glycemic stress, helping to define the relationship between β-cell heterogeneity and diabetes.
1.11 Figures and Legends

Figure 1.1. Classes, sources, and subtypes of β-cell heterogeneity. β-cells display variation in gene expression (red), protein level (blue), morphology (green), and function (purple). Heterogeneity is influenced by β-cell source (A), maturation state (B), topology (C), and stress response (D). (A) β-cell sources include neogenesis during development, transdifferentiation of α- and ductal cells, β-cell replication, and differentiation PMPs. (B) Immature β-cells (B1) and mature β-cells (B2) present distinct gene expression, protein, and functional characteristics. (C) Topology influences β-cells through the core-mantel structure, where peripheral β-cells (C1) have more heterotypic contacts, resulting in low glucose sensitivity and insulin release compared to core β-cells (C2). (D) β-cell stress response causes β-cells to cycle between states of high insulin with low UPR, low insulin with high UPR, and a period of low insulin with low UPR. Two subtypes of adult β-cells have been identified: Hub cells (E1) are characterized by very high Gck expression but low insulin expression, and high number of homotypic contacts. Extreme β-cells (E2) are located near the islet core, characterized by very high insulin expression but low mature insulin protein stores. It is unclear if hub cells and extreme β-cells stem from a mature or immature population. PMP – Pancreatic multipotent progenitor, INS – Insulin expression, Insulin – Insulin protein, UPR – Unfolded protein response.
Figure 1.2. Maturation state heterogeneity in pancreatic beta \( \beta \)-cells. (A) Following birth, neonatal \( \beta \)-cells are transcriptionally and functionally immature, characterized by \( \text{Pax6, Hk1, Paxa, and Mafb} \) expression, very high proliferation rates, very high basal insulin secretion, and high glucose sensitivity. Maturation is facilitated by transition to a carbohydrate rich diet at weaning. (B) Mature \( \beta \)-cells are characterized by expression of \( \text{Mafa, Ucn3, and Fltp} \), high expression of insulin, \( \text{Glut2, and Gck} \). These \( \beta \)-cells have low proliferation rates but high glucose-stimulated insulin secretion. A subset of adult \( \beta \)-cells maintain an immature-like profile, including \( \text{Mafb} \) expression, low expression of insulin, \( \text{Glut2, and Gck} \), and lack \( \text{Fltp} \) expression. These cells have elevated proliferation rates but low glucose-stimulated insulin secretion. Whether these cells differ in developmental origin or can interconvert is unknown. (C) In diabetes, glucotoxicity, lipotoxicity, and inflammation induces variable fates in \( \beta \)-cells, including dedifferentiation, apoptosis, and replication. Dedifferentiated \( \beta \)-cells have expression of developmental genes including \( \text{Ngn3, Oc4, and Nanog} \), low expression of genes associated with mature beta cells including \( \text{Pdx1, Mafa, Foxo1} \), and insulin, and low glucose-stimulated insulin secretion. Other fates for \( \beta \)-cells in diabetes include replication and apoptosis. The relationship among adult mature and immature cells and the three fates in diabetes remain to be described.
Chapter 2: Spontaneous restoration of functional β-cell mass in obese SM/J mice

By
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Adapted for dissertation from the published manuscript:

2.1 Abstract

Maintenance of functional β-cell mass is critical to preventing diabetes, but the physiological mechanisms that cause β-cell populations to thrive or fail in the context of obesity are unknown. High fat-fed SM/J mice spontaneously transition from hyperglycemic-obese to normoglycemic-obese with age, providing a unique opportunity to study β-cell adaptation. Here, we characterize insulin homeostasis, islet morphology, and β-cell function during SM/J’s diabetic remission. As they resolve hyperglycemia, obese SM/J mice dramatically increase circulating and pancreatic insulin levels while improving insulin sensitivity. Immunostaining of pancreatic sections reveals that obese SM/J mice selectively increase β-cell mass but not α-cell mass. Obese SM/J mice do not show elevated β-cell mitotic index, but rather elevated α-cell mitotic index. Functional assessment of isolated islets reveals that obese SM/J mice increase glucose stimulated insulin secretion, decrease basal insulin secretion, and increase islet insulin content. These results establish that β-cell mass expansion and improved β-cell function underlie the resolution of hyperglycemia, indicating that obese SM/J mice are a valuable tool for exploring how functional β-cell mass can be recovered in the context of obesity.
2.2 Introduction

Obesity and diabetes are a deadly combination, compounding risk for cardiovascular disease, cancer, and stroke\(^{265-267}\). Obesity raises the risk of developing type 2 diabetes \(^{27-76}\) fold, while approximately 60\% of individuals with diabetes are obese\(^ {268-271}\). Chronic obesity exerts glycemic stress on pancreatic \(\beta\)-cells, causing dysregulation and dysfunction, ultimately resulting in hyperglycemia\(^ {272-275}\). Despite the stress obesity places on \(\beta\)-cells, 10-30\% of obese individuals maintain glycemic control and are at low risk for developing diabetes\(^ {276}\). These low-risk obese individuals have elevated \(\beta\)-cell mass and improved insulin secretion compared to BMI-matched diabetic-obese individuals\(^ {2,13,277,278}\). Understanding the differences in \(\beta\)-cell physiology between these populations may reveal therapeutic strategies for maintaining and improving glycemic control in obese individuals.

Recent work suggests \(\beta\)-cells do not respond uniformly to glycemic stress, rather they experience variable fates including dedifferentiation, replication, and apoptosis\(^ {2,27,279}\). Understanding how these changes mediate diabetic risk is complicated by \(\beta\)-cell heterogeneity. \(\beta\)-cell populations include subtypes that specialize in basal insulin secretion, \(\beta\)-cell replication, coordinating “hub” cells, and \(\beta\)-cells derived from transdifferentiated \(\alpha\)-cells, each of which differ in glycemic stress response\(^ {23,25,280}\). Thus, determining what differentiates nondiabetic-obese and diabetic-obese populations requires connecting \(\beta\)-cell subtypes to their fate in prolonged glycemic stress.

Like in humans, diabetic risk in obese mice depends on genetic background\(^ {281-284}\). Variation in \(\beta\)-cell heterogeneity likely underlies variability in islet stress response, and thus needs to be accounted for when comparing nondiabetic-obese and diabetic-obese populations. Loss of function mutations in leptin (\(ob/ob\)) and leptin receptor (\(db/db\)) provide insight into \(\beta\)-cell
physiology in nondiabetic-obese and diabetic-obese states within individual mouse strains\textsuperscript{2,285--288}, however leptin and its receptor play a critical role in β-cell function independent of obesity, limiting interpretations of these studies\textsuperscript{289}. No current mouse model is well-suited to examine physiological differences in β-cell health between nondiabetic-obese and diabetic-obese states.

The SM/J inbred mouse strain has traditionally been used to study interactions between diet and metabolism, and more recently has uncovered genetic architecture underlying diet-induced obesity and glucose homeostasis\textsuperscript{290--295}. After 20 weeks on a high fat diet, SM/J mice display characteristics of diabetic-obese mice, including elevated adiposity, hyperglycemia, and glucose intolerance\textsuperscript{296}. We have previously shown that by 30 weeks of age, high fat-fed SM/J mice enter diabetic remission, characterized by normalized fasting blood glucose, and greatly improved glucose tolerance and insulin sensitivity\textsuperscript{297}. Importantly, these changes occur in the context of sustained obesity. Given the central role of β-cell health in susceptibility to diabetic-obesity, we hypothesize that obese SM/J mice undergo restoration of functional β-cell mass during the resolution of hyperglycemia. This study focuses on how insulin homeostasis, β-cell morphology, and β-cell function change during this remarkable transition and establishes SM/J mice as a useful model for teasing apart diabetic-obese and nondiabetic-obese states.

**2.3 Methods**

*Animal husbandry and tissue collection.* SM/J mice were obtained from The Jackson Laboratory (Bar Harbor, ME). Experimental animals were generated at the Washington University School of Medicine and all experiments were approved by the Institutional Animal Care and Use Committee in accordance with the National Institutes of Health guidelines for the care and use of laboratory animals. Mice were weaned onto a high fat diet (42% kcal from fat; Envigo Teklad
TD88137) or an isocaloric low fat diet (15% kcal from fat; Research Diets D12284), as previously described\textsuperscript{297}. At 20 or 30 weeks of age, mice were fasted for 4 hours, and blood glucose was measured via glucometer (GLUCOCARD). Mice were then injected with an overdose of sodium pentobarbital, followed by a toe pinch to ensure unconsciousness. Blood was collected via cardiac puncture and pancreas was detached from the spleen and duodenum.

*Serum and pancreatic insulin measurements.* Blood obtained via cardiac puncture was spun at 6000 rpm at 4°C for 20 minutes to separate plasma, which was collected and stored at -80 °C. Whole pancreas was homogenized in acid ethanol and incubated at 4°C for 48 hours, shaking. Homogenate was centrifuged at 2500 rpm for 30 min at 4°C. Supernatant was collected and stored at -20°C. Protein content was measured using Pierce BCA Protein Assay kit (Thermo Scientific) according to manufacturer’s instructions and read at 562 nm on the Synergy H1 Microplate Reader (Biotek). Insulin ELISA (ALPCO 80-INSMR-CH01) was used to measure plasma and pancreatic insulin levels following manufacturer’s instructions.

*Insulin Tolerance Test.* At 19 or 29 weeks of age, mice were fasted for 4 hours prior to procedure. Insulin (humulin) was prepared by mixing 10 ul insulin with 10 ml sterile saline. Mice were injected with 3.75 ul insulin mixture/g bodyweight. Blood glucose levels were assessed from a tail nick at times = 0, 15, 30, 60, and 120 minutes via glucometer (GLUCOCARD).

*Islet Histology and Analyses.* At the time of tissue collection, whole pancreas was placed in 3 mL of neutral buffered formalin. These samples were incubated at 4°C while gently shaking for 24 hours. Immediately afterwards, samples were placed into plastic cages and acclimated to 50% EtOH for 1 hour. Samples were then processed into paraffin blocks using a Leica tissue
processor with the following protocol: 70% EtOH for 1 hour x 2, 85% EtOH for 1 hour, 95% EtOH for 1 hour x 2, 100% EtOH for 1 hour x 2, Xylenes for 1 hour x 2, paraffin wax. Pancreas blocks were sectioned into 4 μm thick sections. Four samples per individual were randomly selected, at least 100 μm apart.

Slides were incubated at 60°C for 1 hour, then placed in xylenes to remove remaining paraffin wax. Slides were then rehydrated using successive decreasing EtOH concentrations (xylenes x 2, 50% EtOH in xylenes, 100% EtOH x 2, 95% EtOH, 70% EtOH, 50% EtOH, H2O). Slides were incubated in sodium citrate (pH 6) at 85°C for 30 minutes, then submerged in running water for 5 minutes. Slides were washed with 0.025% Triton X-100 in TBS and blocked in 10% normal donkey serum for 1 hour (Abcam ab7475), followed by incubation with primary antibody overnight at 4°C. [Primary antibodies: rat anti-insulin (1:100, R&D MAB1417), mouse anti-glucagon (1:100, Abcam ab10988), and rabbit anti-phospho-histone H3 (1:100, Sigma SAB4504429)]. After an additional wash, secondary antibody was applied for 1 hour at room temperature. [Secondary antibodies: donkey anti-rabbit 488 (1:1000, Abcam ab150061), donkey anti-mouse 647 (1:1000, Abcam ab150107), and donkey anti-rat 555 (1:1000, Abcam ab150154)]. Fluoroshield Mounting Medium with DAPI (Abcam) was applied to seal the coverslip and slides were stored at 4°C. Imaging was performed using the Zeiss AxioScan.Z1 at 20X magnification and 94.79% laser intensity.

Background was subtracted from DAPI, insulin, glucagon, and phospho-histone H3 images using ImageJ. DAPI channel was used to identify total nuclei in CellProfiler. Insulin and glucagon channels were combined and overlaid on the DAPI image to identify islet nuclei. Insulin (INS+) staining overlaid with DAPI identified β-cell cells, glucagon (GCG+) staining overlaid with
DAPI identified α-cells, based on the proximity of the signal to a given nuclei. Phosphohistone H3 (PHH3+) staining identified mitotic nuclei. Total nuclei, islet cells, β-cells, α-cells, and mitotic nuclei were summed across 4 slides for each individual. Islet, β-cell, and α-cell mass is reported as fraction of total nuclei. Mitotic islet index is reported as proportion of β-cells and α-cells positive for phosphohistone H3. Mean β-cell area was calculated by dividing the total INS+ area by the number of INS+ cells. Mean β-cell area is reported for each individual. Islets with diameter < 50 µm were discarded.

*Islet isolation.* Pancreas was removed and placed in 8mL HBSS buffer on ice. Pancreas was then thoroughly minced. Collagenase P (Roche) was added to a final concentration of 0.75 mg/ml. Mixture was then shaken in a 37°C water bath for 10-14 minutes. Mixture was spun at 1500 rpm for 2 minutes. The pellet was washed twice with HBSS. The pellet was re-suspended in HBSS and transferred a petri dish. Hand-selected islets were placed in sterile-filtered RPMI with L-glutamine (Gibco) containing 11mM glucose, supplemented with 5% pen/strep and 10% Fetal Bovine Serum (Gibco). Islets were rested overnight in a cell culture incubator set to 37°C with 5% CO₂.

*Glucose Stimulated Insulin Secretion and Islet Insulin Content.* Islets of roughly equal size were equilibrated in KRBH buffer containing 2.8 mM glucose for 30 minutes at 37°C. 5 islets were hand selected and placed in 150 µl KRBH containing either 2.8 or 11 mM glucose. Tubes were placed in a 37°C water bath for 45 min. Islets were then spun at 2000 x g, hand-picked with a pipette, and transferred from the secretion tube and placed in the content tube with acid ethanol. The content and secretion tubes were stored at -20°C overnight. Each condition was performed in duplicate for each individual. Mouse insulin ELISA (ALPCO 80-INSMU-E01) was performed.
according to manufacturer’s instructions, with the secretion tubes diluted 1:5, and content tubes diluted 1:100. Normalized insulin secretion was calculated by dividing the secreted value by the content value. Glucose stimulated insulin secretion was calculated by dividing the normalized insulin secretion at 11mM glucose by the normalized insulin secretion at 2.8 mM glucose. Each sample was measured in duplicate. Total islet protein within each content tube was measured using Pierce BCA Protein Assay kit (Thermo Scientific) according to manufacturer’s instructions and read at 562 nm on the Synergy H1 Microplate Reader (Biotek). Islet insulin content was calculated by dividing the insulin level in the content tubes by the total protein value.

Statistical analyses. Phenotypes were assessed for normality by a Shapiro-Wilk test, and outliers removed. ANOVA revealed that sex-by-diet-by-age interactions did not contribute meaningful variance to any phenotype so males and females were pooled at each time point. A student’s t-test was used to assess significance between two cohorts, while a one-way ANOVA with Tukey’s Post Hoc test was used to assess significance among multiple cohorts. Pearson’s correlation was used to determine strength of correlation among variables. P-values < 0.05 were considered significant.

2.4 Results

Obese SM/J mice increase insulin levels and improve insulin sensitivity. The resiliency of β-cells distinguishes nondiabetic-obese and diabetic-obese individuals. While both groups develop hyperinsulinemia, diabetic-obese individuals become insulin resistant, leading to β-cell dysfunction, hypoinsulinemia, and hyperglycemia. Our previous work shows that obese SM/J mice spontaneously transition from hyperglycemic to normoglycemic with age. Principle to
this is a 40 mg/dl decrease in fasting glucose levels in high fat-fed SM/J mice between 20 and 30-weeks (Figure 2.1A). We first sought to characterize how insulin homeostasis changes during this transition. Interestingly, 20-week high fat-fed SM/J mice have comparable levels of plasma and pancreatic insulin levels compared to age-matched low fat-fed mice (Figure 2.1B-C). By 30 weeks, high fat-fed SM/J mice increase circulating insulin levels 5.3-fold and pancreatic insulin levels 1.9-fold, in line with other models of hyperinsulinimic nondiabetic-obesity. We sought to test for peripheral insulin resistance via an insulin tolerance test (ITT), as insulin resistance is a known mechanism for increasing circulating and pancreatic insulin levels. Surprisingly, 20-week high fat-fed SM/J mice display insulin resistance compared to low fat-fed mice, however, insulin sensitivity is restored by 30 weeks (Figure 2.1D-E). The simultaneous increase in insulin production and improved insulin sensitivity is unprecedented and suggests a novel mechanism beyond insulin resistance for enhancing β-cell insulin secretion.

Obese SM/J mice increase islet mass during resolution of hyperglycemia. In humans and mice, obesity initially increases islet mass, and maintenance of that mass in part differentiates nondiabetic-obese individuals from diabetic-obese individuals. To understand the source of increased insulin production in obese SM/J mice, we examined islet morphology during the resolution of hyperglycemia. To quantify islet mass and number, β-cell mass, α-cell mass, and mitotic index, we randomly selected 4 sections per fixed pancreas and stained with antibodies against insulin, glucagon, and phospho-histone H3. Representative images of immuno-stained pancreatic sections for 30-week high fat-fed mice and 30-week low fat-fed mice are shown in Figure 2.2A-B. Consistent with other mouse models of obesity, 20-week high fat-fed SM/J mice have a 2.75-fold increase in total islet mass compared to low fat-fed mice (Figure
2.2C). This increased mass is driven by an increase in both median islet area and number of islets (Figure 2.2D-E). Islet mass is further elevated 2-fold between 20- and 30-weeks in high fat-fed mice, while the islet population remains unchanged in low fat-fed mice. A full summary of the islet quantification is presented in Table 2.1. Distribution of islet size is shown in Figure 2.5, along with corresponding density plot for each cohort. Islet mass correlates with BMI in obese humans, a similar correlation is seen between islet mass and body weight in high fat-fed SM/J mice (Fig. 2F).

Obese SM/J mice increase β-cell mass and α-cell replication. To identify the source of the increased islet mass in high fat-fed SM/J mice, we quantified the relative representation of β-cells and α-cells within each cohort. Increased islet mass in 20-week high fat-fed mice is driven by a 3.3-fold increase in the number of β-cells and a 2.5-fold increase in the number of α-cells compared to low fat mice, while growth between 20- and 30-week high fat-fed mice is driven by a further 2.2-fold increase in β-cell number (Figure 2.3A-B). In obesity, islet mass expands primarily through β-cell hyperplasia. Mean β-cell area is not different across age and dietary cohorts (Figure 2.6). We quantified mitotic index of β- and α-cells in our model using phosphohistone H3 and assessed how mitotic index relates to β-cell mass during the resolution of hyperglycemia in obese SM/J mice. Surprisingly, calculation of β-cell mitotic index reveals similar rates of β-cell replication across cohorts (Figure 2.3C), while α-cell mitotic index is elevated 6-fold in high fat-fed mice compared to low fat-fed controls (Figure 2.3D). Examining the relationship between β-cell mitotic index and β-cell mass in high fat-fed mice reveals β-cell replication correlates with β-cell mass in 20-week mice, but not 30-week mice (Figure 2.3E-F).
Obese SM/J mice increase islet insulin secretion and insulin content. In conjunction with changing β-cell morphology, diabetic-obesity is associated with altered β-cell function, including diminished first phase insulin secretion, increased basal insulin secretion, and decreased β-cell insulin production(12, 17, 50, 60). We sought to examine if changes in β-cell insulin secretion and content corresponded with the resolution of hyperglycemia and expanded β-cell mass we observe. To test this, we isolated islets from high and low fat-fed 20- and 30-week SM/J mice. After allowing islets to rest overnight, we performed a glucose-stimulated insulin secretion assay by subjecting islets to low (2.8 mM) or high (11 mM) glucose conditions. We find that high fat-fed SM/J mice dramatically improve glucose-stimulated insulin secretion between 20 and 30 weeks of age. This includes transitioning from blunted insulin secretion under high glucose conditions to appropriately elevated secretion (Figure 2.4A), and improvement in the ratio of insulin secreted in response to high vs low glucose conditions (Figure 2.4B). Normalized insulin secreted in response to elevated glucose does not differ between cohorts (Figure 2.6A). 20-week high fat-fed mice have elevated insulin secretion in response to low glucose (Fig. 4C), consistent with other studies of islets in type 2 diabetic humans and mice. Correspondingly, 20-week high fat-fed SM/J mice have decreased islet insulin content (Figure 2.4D), which increases 3-fold by 30 weeks. Consistent with current understanding of the β-cell maturation process³¹¹, there is a positive correlation between islet insulin content and glucose-stimulated insulin secretion in high fat-fed (Figure 2.4E) and low fat-fed (Figure 2.6B) SM/J mice. This suggests that obese SM/J mice experience β-cell maturation between 20 and 30 weeks, characterized by increased insulin content and improved insulin secretion in response to high glucose. This spontaneous
improvement in β-cell health and function in the context of obesity has not been reported in other mouse strains, suggesting a genetic basis unique to SM/J.

2.5 Discussion

The ability to maintain appropriate insulin production and secretion, termed functional β-cell mass, is a central determinant of diabetic risk. In this study, we describe insulin homeostasis, islet morphology, and β-cell function in obese SM/J mice as they transition from hyperglycemic to normoglycemic. We determine that increased insulin production and insulin sensitivity accompany improved glycemic control, driven by expanded β-cell mass and improved glucose-stimulated insulin secretion. Our results show obese SM/J mice undergo restoration of functional β-cell mass, providing an opportunity to explore how compensatory insulin production can be achieved in the context of obesity.

Susceptibility to high fat diet-induced diabetes in mice depends on genetic background. Several strains and sub-strains develop diabetic-obesity, including hyperglycemia, glucose intolerance, and insulin resistance, consistent with the diabetic phenotypes observed in obese SM/J mice at 20 weeks. Remarkably, by 30 weeks, obese SM/J mice have characteristics of diabetic-resistant obese strains, retaining glycemic control by dramatically increasing insulin production and improving insulin sensitivity. To our knowledge, this is the first report of transient hyperglycemia in an inbred strain, although similar phenomena have been reported in mice with the leptin receptor (db/db) mutation. C57Bl/6J (db/db) and 129/J (db/db) mice are obese and initially develop mild hyperglycemia at 8-10 weeks of age, but this resolves by 20-30 weeks, concurrent with increased insulin production and β-cell mass. Unfortunately, leptin and its
receptor play an important role in β-cell growth and function independent of obesity, which confounds understanding of how genetic background mediates diabetic risk in obesity\textsuperscript{289}. Interestingly, low fat fed mice increase circulating insulin levels between 20- and 30-weeks, despite no change in pancreatic insulin levels, insulin sensitivity, or β-cell mass.

High fat diet-induced obesity in mice can result in increased islet mass, no change, or decreased mass\textsuperscript{282,284,313–315}. Across these studies, inability to expand islet mass is associated with hyperglycemia. In humans, islet mass correlates with BMI in nondiabetic obese-individuals, while diabetic-obese individuals have low islet mass compared to nondiabetic individuals\textsuperscript{300,309,310}. High fat-fed SM/J mice are unique because they have expanded islet mass at 20 weeks, yet normal insulin levels and insulin resistance. By 30-weeks, islet mass continues to expand, driven by increased islet area and increased islet number, corresponding with increased insulin production and improved insulin sensitivity. Islet neogenesis may contribute to the increased islet number, and fission of large islets has been reported during development, suggesting islets have mechanisms to maintain an appropriate size\textsuperscript{317,318}.

β-cell hyperplasia is the primary driver of islet expansion in mouse models of obesity\textsuperscript{287,288}. Some nondiabetic obese mice experience increased β-cell number, but do not show evidence for elevated β-cell replication in immunostaining of pancreatic sections\textsuperscript{282,315}. This has been attributed to islets in the tail of the pancreas being substantially more proliferative in response to high fat diet than the body and head regions\textsuperscript{118}, thus technical artifacts in sampling could result in inflated variances which mask biological differences. This could be the case here, given that high fat-fed SM/J’s β-cell number is far above low fat-fed controls, that their β-cell number expands 2-fold during the resolution of hyperglycemia, yet we find no evidence for
increased β-cell replication. However, α-cell number also expands in obesity\textsuperscript{319–321}. While α-cell mass is elevated in high fat-fed SM/J mice compared to low fat-fed controls, we find it does not change between 20 and 30 weeks, despite substantial elevation of α-cell mitotic index.

Retention of β-cell function separates diabetic-obesity and nondiabetic obesity\textsuperscript{322–324}. 20-week high fat-fed SM/J mice have an insulin secretion profile similar to diabetic-obese mice and humans, including blunted glucose-stimulate insulin release, elevated basal insulin secretion, and low islet insulin content, which resolves by 30 weeks. Underscoring this transition is the positive correlation between glucose-stimulated insulin release and islet insulin content. Care was taken to select normal sized islets across all cohorts for functional assessment (~100µm in diameter) indicating this robust improvement in β-cell functional mass is due to changes to β-cell physiology.

Three current, non-mutually exclusive components of β-cell stress response may shed light on the perplexing improvement in glycemic control seen in SM/J mice: β-cell dedifferentiation, nascent β-cell maturation, and changes in β-cell subtype proportions. While early studies concluded overworked β-cells undergo apoptosis\textsuperscript{2,325–327}, recent studies have suggested β-cells dedifferentiate into a dysfunctional, progenitor-like state, potentially as a defense mechanisms against prolonged glycemic stress\textsuperscript{236,279,328,329}. These dedifferentiated β-cells have low insulin content and poor glucose-stimulated insulin secretion. Further, the dedifferentiated state is reversible in cultured conditions, revealing potential for therapeutic intervention\textsuperscript{239}. It is feasible that obese SM/J mice have β-cells in the dedifferentiated state at 20-weeks, which would explain the low insulin content and poor functionality despite the elevated β-cell mass. Improvement in insulin sensitivity could ease glycemic stress, allowing
dedifferentiated β-cells to redifferentiate by 30 weeks, reestablishing insulin production and secretion.

Work from several groups suggests β-cells can be divided into two broad categories: functionally immature and functionally mature cells. Immature β-cells have greater proliferative potential and are resistant to stress, at the expense of functional maturity\textsuperscript{45,218,330}. These immature β-cells have low insulin content, high basal insulin secretion, and a lack of glucose stimulated insulin secretion. The large β-cell expansion seen in obese SM/J mice, suggests nascent β-cells must undergo maturation at some point. We have no evidence of enhanced β-cell replication at 20-weeks, but it is possible these β-cell are still functionally immature and reach maturity by 30-weeks. This could explain why islets from these mice lack glucose stimulated insulin release, show elevated basal insulin secretion, and have low insulin content, despite elevated mass.

Recent advances in single cell technology allows for identification of β-cell subtypes, based on functional characteristics and gene expression. These include β-cells that specializes in basal insulin secretion, characterized by low mature insulin content, and enriched in \textit{db/db} diabetic islets\textsuperscript{23}. While these cells are not equipped to respond to elevated glucose, they are enriched for maturity markers including \textit{Ucn3} and \textit{Glut2}, distinguishing them from immature β-cells. Pancreatic multipotent progenitors (PMPs) are rare insulin positive cells capable of generating endocrine cells \textit{in vivo} including functionally mature β-cells\textsuperscript{25,194}. These cells resemble immature β-cells, with low insulin content and \textit{Glut2} expression, whose proliferation is stimulated by glycemic stress in STZ-treated and NOD mouse models. Lastly, β-cell hub cells coordinate calcium signaling and insulin release of surrounding β-cells\textsuperscript{280}. These cells have markers for both mature and immature β-cells, including expression of \textit{Gck} and \textit{Pdx1}, but low
insulin content, and are especially sensitive to glycemic and inflammatory stress. Ablation of these cells results in loss of coordinated insulin release, suggesting they are necessary for mature islet function. Given the elevated β-cell mass, poor insulin secretion, and low insulin content in 20-week high fat-fed SM/J mice, it is possible islets are enriched for basal insulin secretors and PMP’s, while devoid of hub cells. At 30 weeks, as glycemic stress diminishes, basal insulin secretors and PMP populations decline, while hub cells rise, reestablishing β-cell functionality.

Clearly, the interplay between β-cell dedifferentiation, nascent β-cell maturation, and β-cell subtype identity in diabetic-obesity needs to be clarified. SM/J mice are a useful model because they allow for appropriate comparisons across diabetic-obese, nondiabetic-obese, and nondiabetic-lean populations. The improvement in β-cell function and increase in insulin production in obese SM/J mice could be explained by a combination of these innate and stress response β-cell mechanisms. Future studies interrogating how SM/J β-cells change over time will provide insight into the physiological mechanisms that allow β-cell functionality to be maintained and improved in the context of obesity.
2.6 Figures and Legends

**Figure 2.1.** Insulin homeostasis during the resolution of hyperglycemia in obese SM/J mice. Blood glucose levels measured after 4-hour fast from high and low fat-fed, 20- and 30-week SM/J mice (A). Plasma insulin (B) and pancreatic insulin levels (C) assessed via insulin ELISA, collected after 4-hour fast. Insulin tolerance test performed via intraperitoneal insulin injection following 4-hour fast (D), summarized in the area under the curve (E). N = 38-50 mice per cohort for panel A, C, D. N = 10-24 mice per cohort for panel B-C, equal numbers of males and females. Bar represents group means, error bars represent SEM. *p<0.05, **p<0.01, ***p<0.001, N.S. Not Significant.
Figure 2.2. Changes in islet mass during the resolution of hyperglycemia. Representative pancreatic cross sections from 30-week high fat-fed mice (A) and 30-week low fat-fed mice (B) stained for insulin (green), glucagon (white), and phosphohistone H3 (red). Dashed white box identifies location of image in inset. Solid yellow arrows within inset identify INS⁺:PHH3⁺ cells, dashed yellow arrow identifies GCG⁺:PHH3⁺ cell. Islet mass reported as ratio of islet cells over total cells, summed across 4 pancreatic sections (C). Median islet area calculated for each individual across 4 sections (D). Total number of islets quantified per individual, normalized by total DAPI area (E). Correlation between body weight and β-cell mass in high fat-fed mice (F), open circles – 20-week high fat-fed, filled circles – 30-week high fat-fed. N = 12-16 mice per cohort for panels C-F, equal number of males and females. *p<0.05, **p<0.01, ***p<0.001, N.S. Not Significant.
Figure 2.3. Islet cell mass and mitotic index in obese SM/J mice. β-cell mass reported as ratio of INS\(^+\) cells divided by total cells summed across 4 slides per individual (A). α-cell mass reported as GCG\(^+\) cells divided by total cells summed across 4 slides per individual (B). β-cell mitotic index calculated by dividing INS\(^+\):PHH3\(^+\) cells divided by total INS\(^+\) cells summed across 4 slides per individual (C). α-cell mitotic index calculated by dividing GCG\(^+\):PHH3\(^+\) cells by total GCG\(^+\) cells summed across 4 slides (D). Correlation between β-cell mitotic index and β-cell mass in 20 week high fat-fed mice (E) and 30-week high fat-fed mice (F). Open circles – 20-week high fat-fed, filled circles – 30-week high fat-fed. N = 12-16 mice per cohort for all panels, equal males and females. *p<0.05, **p<0.01, ***p<0.001, N.S. Not Significant.
Figure 2.4. Islet insulin secretion and insulin content. Islet insulin secretion in response to low (2.8 mM) and high (11mM) glucose conditions, normalized by islet insulin content (A), reported as a ratio of high glucose to low glucose insulin secretion (B). Comparison of islet insulin secretion under low glucose conditions in 20- and 30-week, high and low fat-fed mice (C). Islet insulin content normalized by total protein measured via protein BCA (D). Correlation between insulin secretion ratio and islet insulin content (E). Open circles – 20-week high fat-fed, closed circles – 30-week high fat-fed. N = 8-19 mice per cohort for panels A-C, n = 5-11 mice per cohort for panel D, n = 22 mice for panel E. *p<0.05, **p<0.01, ***p<0.001, N.S. Not Significant.
Figure 2.5. Islet area histogram (left) and density plot (right) for each cohort, 20-week high fat (A), 30-week high fat (B), 20-week low fat (C), and 30-week low fat (D). Dashed line shows islet size threshold. N= 2,333-10,380 islets per cohort.
Figure 2.6. Mean β-cell area per individual. Total INS⁺ area divided by total INS⁺ cells, summed across 4 slides per individual. N = 12-16 individuals per cohort. N.S. – Not significant.

Figure 2.7. Normalized insulin secretion in response to elevated (11 mM) glucose (A). Islet insulin content vs glucose-stimulated insulin secretion for low fat-fed mice (B). A, N =5-14 individuals per cohort. B, N = 8. N.S. – Not significant
### 2.7 Tables

**Table 2.1.** Cohort metrics for islet mass quantification.

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Chapter 3: Identifying β-cell subpopulations and genetic networks associated with obesity and glycemic control in SM/J mice

By

Mario A Miranda, Juan F Macias-Velasco, Heather Schmidt, Heather A Lawson.

Adapted for dissertation from the manuscript submitted for publication and available in preprint:

3.1 Abstract

Understanding how heterogeneous β-cell function and stress response impact diabetic etiology is imperative for therapy development. Standard single-cell RNA sequencing analysis illuminates some genetic underpinnings driving heterogeneity, but new strategies are required to capture information lost due to technical limitations. Here, we integrate pancreatic islet single-cell and bulk RNA sequencing data to identify β-cell subpopulations based on gene expression and characterize genetic networks associated with β-cell function in high- and low-fat fed male and female SM/J mice at 20 and 30wks of age. Previous studies have shown that high-fat fed SM/J mice resolve glycemic dysfunction between 20 and 30wks. We identify 4 β-cell subpopulations associated with insulin secretion, hypoxia response, cell polarity, and stress response. Relative proportions of these cells are influenced by age, sex, and diet. Network analysis identifies fatty acid metabolism and β-cell physiology gene expression modules associated with the hyperglycemic-obese state. We identify subtype-specific expression of Pdyn and Fam151a as candidate regulators of genetic pathways associated with β-cell function in obesity. In sum, this study uses a novel data integration method to explore how β-cells respond to obesity and glycemic stress, helping to define the relationship between β-cell heterogeneity and diabetes, and shedding light on novel genetic pathways with therapeutic potential.
3.2 Introduction

Proper insulin secretion from pancreatic β-cells is required to maintain glycemic control. Obesity initially promotes β-cell expansion, but prolonged glycemic stress and inflammation drive β-cell death and dysfunction, resulting in type 2 diabetes\(^2,3,241\). Without sufficient β-cell mass and insulin production, sustained hyperglycemia increases risk for deadly metabolic diseases\(^331–333\). Currently, transplantation of cadaveric islets is the only method for restoring β-cell mass in diabetes but is severely limited by donor availability and requires lifelong immunosuppressant therapy\(^334,335\). Differentiating induced pluripotent stem cells into insulin secreting cells may alleviate this bottleneck, but current methods fail to recapitulate fine-tuned glucose sensing and insulin secretion \textit{in vivo}\(^336,337\). There is urgent need for therapies that improve endogenous β-cell function. This requires understanding how and why β-cells become dysfunctional in obesity.

Improving endogenous β-cell function in diabetes is complicated by cellular heterogeneity, because individual β-cells vary significantly in function, gene expression, protein level, and stress response\(^338\). Single cell technologies permit interrogation of the molecular underpinnings of heterogeneity, addressing two fundamental questions: Do functionally distinct subpopulations of β-cells exist? Can accounting for heterogeneity improve diabetic therapy? Several research groups have proposed subpopulations based on clustering analysis using single cell RNA sequencing (scRNA-seq), however, there is little agreement among studies\(^21,90,91,339\). High rates of gene dropout and low read depth contribute to these problems, necessitating approaches that improve information capture without losing cell type-specific information\(^102,104\).

Integrating sc-RNAseq with bulk RNAseq data leverages bulk sequencing’s high read depth, allowing for capture of lowly expressed genes and robust expression analysis. Several
tools can integrate sc- and bulk RNA-seq data, focusing on deconvoluting bulk RNAseq data from heterogeneous tissue to account for differences in tissue composition\textsuperscript{90,340,341}. These methods estimate and control for cell type abundance but do not identify cell type-specific expression signatures in bulk datasets. Analytical strategies that identify cellular gene expression signatures in bulk RNAseq data allow for robust cell type-specific differential expression analysis and complex network analysis that is currently not feasible in scRNA-seq data.

Here, we characterize gene expression in β-cells from obese SM/J mice, who spontaneously transition from hyperglycemic to normoglycemic with improved β-cell function between 20 and 30 weeks of age\textsuperscript{342,343}. We assign functional identities to 4 subpopulations of β-cells using scRNA-seq. These subpopulations vary in proportion among hyperglycemic-obese, normoglycemic-obese, and normoglycemic-lean mice. We identify 316 genes specifically expressed by β-cells and establish a β-cell gene expression profile for each mouse. We leverage this information to focus our analyses of bulk-islet RNAseq and identify β-cell-specific differential expression and gene networks associated with the hyperglycemic-obese, normoglycemic-obese, and normoglycemic-lean states. Two novel potential regulators of β-cell function, \textit{Pdyn} (Prodynorphin) and \textit{Fam151a} (Family with sequence similarity 151 member A), are differentially expressed, highly connected within genetic networks, and primarily expressed by β-cell subpopulations associated with normoglycemia. This analysis demonstrates that integrating scRNAseq with bulk RNAseq is a powerful approach for exploring β-cell heterogeneity and identifying key genes and subpopulations that strongly associate with glycemic state. The genetic networks and β-cell subpopulation signatures we identify have high potential to lead to further research aimed at improving β-cell function in obesity.
3.3 Methods

Metabolic phenotyping. SM/J mice were obtained from The Jackson Laboratory (Bar Harbor, ME). Mouse colony was maintained at the Washington University School of Medicine and all experiments were approved by the Institutional Animal Care and Use Committee in accordance with the National Institutes of Health guidelines for the care and use of laboratory animals. Mice were weaned onto a high-fat diet (42% kcal from fat; Envigo Teklad TD88137) or isocaloric low-fat diet (15% kcal from fat; Research Diets D12284), as previously described\(^3\). At 20 or 30 weeks of age, mice were fasted for 4 hours, body weight measured, and blood glucose was measured via glucometer (GLUCOCARD). Mice were injected with sodium pentobarbital, followed by a firm toe pinch to ensure unconsciousness. Blood was collected via cardiac puncture and pancreas was collected. Blood was spun at 6000 rpm at 4°C for 20 minutes to collect plasma. Insulin ELISA (ALPCO 80-INSMR-CH01) was used to measure plasma insulin levels following manufacturer’s instructions.

Islet isolation and phenotyping. Islets were isolated from pancreas as previously described\(^3\) and rested overnight. 5 Islets were equilibrated in KRBH buffer with 2.8 mM glucose for 30 minutes at 37°C, then placed in 150 µl KRBH containing 2.8 mM glucose at 37°C for 45 minutes, then 150 µl KRBH containing 11 mM glucose at 37°C for 45 minutes. Islets were then transferred into 150 µl acid ethanol. Islet content and secretion tubes were stored at -20°C overnight. Experiments were performed in duplicate per individual, and measurements are reported as the average of replicates. Mouse insulin ELISA (ALPCO 80-INSMU-E01) was performed, with the secretion tubes diluted 1:5, and content tubes diluted 1:100. Glucose stimulated insulin secretion was calculated by dividing insulin secretion at 11mM glucose by insulin secretion at 2.8 mM.
glucose. Basal insulin secretion was calculated by dividing insulin secretion at 2.8 mM glucose by islet insulin content. Total islet protein was measured using Pierce BCA Protein Assay kit (Thermo Scientific) according to manufacturer’s instructions and read at 562 nm on the Synergy H1 Microplate Reader (Biotek). Islet insulin content was calculated by dividing the islet insulin level in the content tubes by total islet protein. All measurements were taken in duplicate, values reported are the average of replicates.

Single cell RNA sequencing. Single cell RNA sequencing (scRNA-seq) was performed on islets isolated from 15 SM/J mice representing 6 cohorts: 20wk high-fat females (n=3), 20wk high-fat males (n=3), 30wk high-fat females (n=3), 30wk high-fat males (n=2), 20wk low-fat females (n=2), and 20wk low-fat males (n=2). Isolated islets were dissociated into single cell suspensions using Accumax cell/tissue dissociation solution (Innovative Cell Technologies). Libraries were prepped using the Chromium Single Cell 3’ GEM, Library & Gel Bead Kit v3 (10xGenomics) and sequenced at 2x150 paired end reads using a NovaSeq S4. After sequencing, reads were demultiplexed and assigned to individual samples. Reads were aligned using 10x Genomics CellRanger (3.1.0) against our custom SM/J reference. Samples that were prepped together were aggregated into batches using CellRanger aggregate. In the R environment (4.0.0), each aggregated batch was run through SoupX (1.5.0) to estimate and correct for ambient RNA contamination. A contamination fraction of 0.05 was chosen. Removal of Ins2 ambient RNA shown in Figure 3.8D-E. Adjusted counts were imported into Seurat (3.2.2), where cells were filtered for number of features detected (500-3000), total counts detected (1000-30000), percent mitochondrial genes (0-30), visualized in Figure 3.8A. For additional quality control, we excluded cells where nCount was not predictive of nFeature; the predictive error (residual) of a
cell had to be within 3 standard deviations of the mean predictive error (~0). Cell counts for samples from one batch shown before and after filtering step shown in Figure 3.8B-C. Expression was then normalized in Seurat (normalization.method = LogNormalize), batches were integrated, and clustered using a shared nearest neighbor approach. Using the top 10 principal components of the filtered expression data and a resolution of 0.14, we identified 9 clusters of cells using Clustree (0.4.3)\textsuperscript{346}. Cell types were assigned by identifying top over-expressed genes for each cluster relative to all other clusters using a Wilcoxon rank sum test, with an average log-fold-change threshold of $\geq 0.25$ and requiring at least 25% of cells express the gene. Identities were assigned by comparing top over-expressed genes for each cluster with known cell-type specific markers for islet cells.

*Identification of β-cell subtypes, differential expression, and composition.* All β-cells were subset for further analysis. Using the top 10 principal components of the filtered expression data and a resolution of 0.09 (Figure 3.8F), we identified 4 populations of β-cells, labeled Beta 1-4. Top over-expressed genes for each population were identified using a Wilcoxon rank sum test, with an average log-fold-change threshold $\geq 0.1$ compared to all other β-cells and an adjusted p-value $< 0.01$ (Bonferroni). Subtype-identifier genes were tested for gene set enrichment (see below) to determine presumed function of each population. Using these thresholds (average log-fold-change $\geq 0.1$ and adjusted p-value $< 0.01$), differential expression was calculated between pairs of β-cell subpopulations, across all β-cells between 20wk high-fat males and 30wk high-fat males, across all β-cells between 20wk high-fat males and 20wk low-fat males, and across Beta 1 cells between 20wk high-fat males and 20wk low-fat males using the “MAST” hurdle-model test\textsuperscript{347}. Relative proportions of β-cell subpopulations in each cohort were estimated using
bootstrapping to calculate 95% confidence intervals by randomly sampling 1,000 cells from each cohort for 100 iterations.

Identification of β-cell-specific genes. To identify β-cell-specific gene expression signatures in the bulk sequencing data, we assume that for a given gene, the sum of expression in the scRNA-seq data, \( Y_{Total} \), approximates the expression in bulk RNA sequencing data, \( Y_{Bulk} \).

\[
Y_{Bulk} \approx Y_{Total}
\]

The \( Y_{Total} \) value can be re-written as the sum of expression from all the contributing cell types, where \( Y \) is the expression from a given cell type, and \( N \) is the total number of cells of that type.

\[
E[Y_{Total}] = E[Y_{\beta-cell}] * N_{\beta-cell} + E[Y_{\alpha-cell}] * N_{\alpha-cell} + \ldots + E[Y_{CellType}] * N_{CellType}
\]

Therefore, the expected relative contribution of β-cells (\( Q_{\beta-cell} \)) to total expression (\( Y_{Total} \)) can be written as:

\[
Q_{\beta-cell} \sim \frac{E[Y_{\beta-cell}] * N_{\beta-cell}}{E[Y_{Total}]}
\]

When \( Q_{\beta-cell} \) is high in all 6 cohorts, we are confident that gene expression in the bulk data is nearly exclusively coming from β-cells. To determine the contribution of β-cells and all other cell types to gene expression, the distribution of normalized expression was assessed using a Cullen and Frey graph using the fitditrplus package (1.1-3)\(^{348}\) in R and identified as beta distributed (Figure 3.9A-B).

scRNA-seq data is characterized by a buildup of expression values at 0 due to cells that do not express a gene or due to gene-dropout. This distribution required us to employ a beta-
hurdle model described by three parameters: \( Pr(Dropout) \), \( \alpha \), and \( \beta \). We treat the probability of attaining an expression value of 0 ( \( Pr(Dropout) \) ) and the distribution of non-zero values separately for a given gene. We fit the expression of each gene in each cell type using a beta-hurdle model optimized by maximum goodness of fit estimation. To fit the first part of the beta-hurdle mode, the Kolmogorov-Smirnov statistic using the ks.test package (4.05)\(^{349} \) in R was used to select the optimal \( \alpha \) and \( \beta \) shape parameters that best fit the expression distribution of non-zero-expressing cells by minimizing the distance between the cumulative distribution of the real data and theoretical beta model data (Ks) (Figure 3.9C). To fit the second part of the beta-hurdle mode, \( Pr(Dropout) \) was iterated between 0 and 1, selecting the \( Pr(Dropout) \) that minimized Ks (Figure 3.9D). In most cases, assuming 100% of cells not expressing the gene was due to gene drop out provided the best fit (Figure 3.9E). From the best fit beta-hurdle model, the expected value for each gene within a cell type \( E[Y_{cell\text{type}}] \) can be calculated using the fit \( \alpha \) and \( \beta \) shape parameters as:

\[
E[Y_{cell\text{type}}] = \frac{1}{1 + \left(\frac{\beta}{\alpha}\right)}
\]

Multiplying the expected value by the total number of cells of that cell type provides the total expression contribution of that cell type. Summing this value across all cell types provides total expression and allows assessment of the contribution of \( \beta \)-cell gene expression to the genes’ total expression. We required \( \beta \)-cells to account for \( \geq 80\% \) of total gene expression in each of the six cohorts analyzed, resulting in 316 “\( \beta \)-cell-specific genes”, shown in Table 3.2.

**Bulk RNA sequencing.** Islets from 32 mice were sequenced: 4 males and 4 females from each diet (high-fat and low-fat) and each age (20wk and 30wk), \( n=32 \). Islet RNA was extracted using
the RNeasy MinElute Cleanup kit (Qiagen), RNA concentration was measured via Nanodrop and RNA quality/integrity was assessed with a BioAnalyzer (Agilent). Libraries were prepped using the SMARTer cDNA synthesis kit (Takara Bio) and sequenced at 2x150 paired end reads using a NovaSeq S4. After sequencing, reads were de-multiplexed and assigned to individual samples. FASTQ files were trimmed and filtered to remove low quality reads and aligned against a SM/J custom genome using STAR\textsuperscript{295,350,351}. Read counts for β-cell-specific genes were normalized via TMM normalization and pairwise differential expression between cohorts was performed using edgeR\textsuperscript{352}.

**Co-expression network analysis.** Weighted Gene Co-Expression Network Analysis (WGCNA) identifies co-expression modules and correlates them with phenotypic traits\textsuperscript{353}. Briefly, edgeR-normalized counts for β-cell-specific genes were converted to standard normal, and an adjacency matrix was created from bi-weight mid-correlations calculated between all genes in all individuals and raised to a power β of 8, chosen based on a scale-free topology index above 0.9, to emphasize high correlations\textsuperscript{354}. The blockwiseModules function created an unsigned Topological Overlap Measure using the adjacency matrix to identify modules of highly interconnected genes. Eigengenes were calculated as the first principal component for each module, and Pearson’s correlations were calculated between eigengene expression and phenotype to estimate module-trait relationships. Module-trait correlations were considered significant at an FDR-corrected p-value < 0.05. The adjacency matrix was then used to calculate the connectivity of each gene with other genes within its module (k\textsubscript{within}). Adjacency matrices were subset for each age x diet x sex cohort, used to calculate cohort-specific connectivity for
each gene, then used to calculate differential connectivity between cohorts. We report differential connectivity between 20wk high-fat males and 30wk high-fat males, \( HM \), calculated as

\[
k_{Diff}(HM) = \frac{HM_{20} - HM_{30}}{HM_{20} + HM_{30}}
\]

and differential connectivity between 20wk high-fat males and 20wk low-fat males, \( M_{20} \), calculated as

\[
k_{Diff}(M_{20}) = \frac{HM_{20} - LM_{20}}{HM_{20} + LM_{20}}
\]

where \( HM_{20}, \ HM_{30}, \) and \( LM_{20} \) are cohort-specific \( k_{within} \) values for each gene. This provides each gene in the comparison with a value between -1 and 1, where \( |k_{Diff}| > 0.5 \) is considered differentially connected. Genes with positive differential connectivity are more highly connected in 20wk high-fat males than 30wk high-fat males or 20wk low-fat males, depending on comparison.

\textit{Gene set enrichment analysis.} Over-representation analysis (ORA) was performed using the WEB-based Gene Set Analysis Toolkit v2019\textsuperscript{355} on genes overexpressed in individual \( \beta \)-cell subtypes (\textbf{Table 3.1}), \( \beta \)-cell-specific genes (\textbf{Table 3.3}), and genes within blue and brown expression modules (\textbf{Table 3.4}), using all genes expressed in the sc-RNAsseq data set as a reference set. Analysis included gene ontologies (biological process, cellular component, molecular function), pathway (KEGG), and phenotype (Mammalian Phenotype Ontology).
3.4 Results

*SM/J islets contain four β-cell subpopulations.* We developed an analysis pipeline that integrates sc- and bulk RNA-seq data to characterize the transcriptional changes in β-cells from obese SM/J mice as they improve glycemic control with age (Figure 3.1A). We identified islet cells (α, β, and δ), exocrine cells (acinar and ductal), vascular cells (smooth muscle and endothelial), and immune cells (B cells and macrophages) (Figure 3.1B). Subsequent clustering of β-cells revealed 4 subpopulations, labeled Beta 1-4 (Figure 3.1C, Figure 3.8F). 20wk hyperglycemic high-fat males have a significantly larger Beta 1 population compared to 30wk normoglycemic high-fat males and 20wk low-fat males, at the expense of a diminished Beta 2 population (Figure 3.1 D-E).

*β-cell subpopulations have unique expression signatures.* To identify genes that are overexpressed in each subpopulation, we performed differential expression analysis between each subpopulation and all other β-cells. The top ten highest differentially expressed genes within each subtype are visualized in Figure 3.2. Gene enrichment analysis on the overexpressed genes in each subpopulation reveals potential specialization: Beta 1 - insulin secretion, Beta 2 – hypoxia response, Beta 3 – cell polarity, Beta 4 – stress response (Table 3.1).

*SM/J β-cells uniquely express 316 genes.* To identify genes primarily expressed by β-cells in scRNA-seq data, we employed a beta hurdle model, which allowed us to estimate the relative contribution of each cell type to total gene expression (Figure 3.9). To be considered a β-cell-specific gene, we required β-cells to account for at least 80% of total expression within each cohort (Figure 3.3A). We identified 316 β-cell-specific genes (Table 3.2), comprised of genes canonically associated with β-cell identity, including *Ucn3*, *Gcgr*, and *Slc2a2* (Figure 3.3B-E).
and genes with unknown function in β-cells (Figure 3.3F-G). Overrepresentation analysis revealed enrichment for terms associated with β-cell function, including mature onset diabetes of the young (MODY) and carbohydrate homeostasis, along with terms related to neuron function including neuroactive ligand-receptor interaction, neuron projection terminus, and axon part (Table 3.3). We then sought to discover if β-cell-specific genes were overexpressed within any of the β-cell subpopulations. We identified 20 β-cell-specific genes overexpressed in Beta 1 cells, far more than would be expected by chance (Figure 3.3H).

β-cell genes are differentially expressed by age, diet, and sex. To robustly characterize β-cell gene expression in SM/J mice, we normalized bulk RNA-seq data from 20- and 30-week high- and low-fat males and females (4 per cohort, n=32) using only the 316 β-cell-specific genes. Principal components analysis on the normalized expression data revealed high-fat males separated from other cohorts (Figure 3.4A). This is consistent with our previous studies showing that high-fat fed SM/J males show a more extreme glycemic response than other cohorts. Pairwise comparison revealed 8 differentially expressed genes between 20- and 30wk high-fat males and 2 differentially expressed females between 20- and 30wk high-fat females (Figure 3.4B). 20wk male mice differentially express 104 genes between diets, while 30wk male mice differentially expressed 17. Females differentially expressed a largely consistent set of genes between diets in 20- and 30wk cohorts. We focused subsequent analyses between 20wk and 30wk high-fat males (Figure 3.4C), and 20wk high and low-fat males (Figure 3.4E). These analyses allow comparison between hyper- and normoglycemic-obese mice, and between hyperglycemic-obese and normoglycemic-lean mice. We highlight two genes with unknown
roles in β-cell function, Pdyn and Fam151a, as differentially expressed between 20- and 30wk high-fat males (Figure 3.4D), and 20wk high and low-fat males (Figure 3.4F), respectively.

**β-cell gene expression networks correlate with metabolic traits.** We performed network analysis to characterize how groups of genes behaved across age and dietary cohorts. We performed weighted gene co-expression network analysis (WGCNA), which groups similarly co-expressed genes into discreet modules, then correlates these modules with phenotypes\(^\text{353}\). We collected metabolic phenotypes in the bulk RNA-seq mice including body weight, blood glucose level, serum insulin, and islet-specific phenotypes including glucose-stimulated insulin secretion, basal insulin secretion, and islet insulin content (Figure 3.10). Performing co-expression analysis identified 9 discreet modules of genes, 6 of which correlated significantly with at least one phenotype (Figure 3.11). We highlight the blue and brown modules for their correlation with phenotypes relevant to hyperglycemic obese 20wk high-fat mice.

**Blue module associated with fatty acid metabolism correlates with blood glucose level and is altered by age in obese mice.** The blue module contains 42 genes, and over-representation analysis reveals enrichment for genes related to fatty acid metabolism (Table 3.4). Blue network eigengene expression correlates with blood glucose level across all mice (Figure 3.5A, Figure 3.12F). This correlation is driven by the resolution of hyperglycemia in high-fat mice (Figure 3.5A, Figure 3.12B). Strength of individual gene membership within the blue module correlates with strength of correlation to blood glucose levels (Figure 3.5B). The blue network is visualized as the sum of gene-pair correlations (Figure 3.12D). This network was then subset into 20wk high-fat males (Figure 3.5D) and 30wk high-fat males (Figure 3.5E). Four genes
were identified as differentially connected between networks, although none were individually differentially expressed between 20- and 30wk high-fat males (Figure 3.5C).

Brown module associated with β-cell function correlates with insulin levels and is influenced by diet. The brown module contains 39 genes, and over-representation analysis reveals enrichment for genes related to abnormal β-cell physiology (Table 3.4). Brown network eigengene expression negatively correlates with serum insulin levels across all mice (Figure 3.6A, Figure 3.12E). This correlation is driven in part by diet, where high-fat mice have high levels of circulating insulin (Figure 3.6A, Figure 3.12A). Strength of gene membership in the brown module negatively correlates with strength of correlation to serum insulin level, including Fam151a, which is highly connected in the brown network (Figure 3.6B). Further, expression of Fam151a negatively correlates with serum insulin levels across all cohorts, independent of network construction (Figure 3.6C). The brown network is visualized as the sum of gene-pair correlations (Figure 3.12C). The network was subset into 20wk high-fat males (Figure 3.6D) and 20wk low-fat males (Figure 3.5E), revealing strong network structure in 20wk low-fat males. Differential connectivity was calculated between 20wk high and low-fat males to identify genetic network connectivity that was altered between cohorts (Figure 3.6F). Differential connectivity was contrasted with differential expression between 20wk high and low-fat males, revealing Fam151a to be both differentially connected and differentially expressed (Figure 3.6G).

Single cell expression of Pdyn and Fam151a are influenced by age and diet. We sought to determine if the differential expression of Pdyn and Fam151a seen in the bulk analysis is recapitulated at the single cell level. We compared expression of Pdyn in all β-cells between
20wk high-fat males and 30wk high-fat males (Figure 3.7A) and found significant differential expression, driven by differences in the proportion of β-cells expressing Pdyn (Figure 3.7B). Likewise, Fam151a is differentially expressed across all β-cells between 20wk high-fat males and 20wk low-fat males (Figure 3.7C), driven by differences in the proportion of β-cells expressing Fam151a (Figure 3.7D). To determine if differential expression could be attributed to changes in β-cell subpopulation proportions, we calculated differential expression between the two most prominent subtypes, Beta 1 and Beta 2, revealing significant differential expression of Pdyn and Fam151a (Figure 3.7E). While Pdyn is overexpressed in Beta 2 cells, which are prominent in 30wk high-fat males, Fam151a is overexpressed in Beta 1 cells, which are prominent in 20wk high-fat males. This suggests differential expression of Pdyn can be attributed to differences in subpopulation proportions, but not for differential expression of Fam151a. Assessing differential expression in Beta 1 cells between 20wk high-fat males and 20wk low-fat males revealed Fam151a was very significantly differently expressed (Figure 3.7F), driven by an increase in the proportion of β-cells expressing Fam151a (Figure 3.7G), which could contribute to the differential expression of Fam151a between 20wk high-fat males and 20wk low-fat males seen in the bulk data.

3.5 Discussion

Pancreatic β-cell heterogeneity has been studied extensively using single cell technology because of the cell type’s unique insulin-secreting capabilities and central role in diabetes etiology. Several groups have proposed the existence of functionally distinct β-cell subpopulations24,25,45,356, but their existence and relevance to insulin homeostasis remain controversial. We identified 4 β-cell populations (Figure 3.1-3.2, Figure 3.8), Beta 1-4, across
hyperglycemic-obese, normoglycemic-obese, and normoglycemic-lean mice. While the functional roles assigned to each population are based on gene enrichment analysis and open to interpretation (Table 3.1), the relative proportion of each population changes in conjunction with islet function across cohorts, suggesting a functional relevance to overall β-cell population structure.

Beta 1 cells are most prevalent in hyperglycemic obese mice and are associated with elevated basal insulin secretion and low insulin content. Beta 1 cells overexpress mature markers including Ucn3, Pdx1, and Acly, and negative regulators of glucose-stimulated insulin secretion including Abcc8, G6pc2, and Ucp2. Terms associated with Beta 1 cells include signal release and hormone transport. Beta 1 cells appear to be primed for insulin release, but not to perform glucose-stimulated insulin secretion. Farack et al identified a subpopulation of “extreme” β-cells that specialize in basal insulin secretion, with high insulin expression and low mature insulin content. These cells have high expression of markers Ucn3, Acly, and Pdx1, and increase in proportion in obese mice. The Beta 1 cells we identified are similar to “extreme” β-cells based on their mature profile, potentially limited glucose response, and abundance in diabetic obese mice. Validation is required to determine if these β-cells function by over-expressing insulin secretion pathway components while suppressing glucose response mechanisms.

Beta 2 cells are prevalent in normoglycemic-obese and normoglycemic-lean mice and are associated with high GSIS and high islet insulin content. Beta 2 cells are enriched for response to oxygen levels, pyruvate metabolism, and nucleotide phosphorylation, each associated with protection from hypoxia. These cells are equally prevalent in obese 30wk high-fat males.
and lean 20wk low-fat males, which differ greatly in islet mass, suggesting Beta 2 cells are not a hypoxic population. Further, 20wk high-fat males have similar islet mass to 30wk high-fat males, but a much smaller Beta 2 population. Importantly, 30wk high-fat males have healthier glycemic parameters than 20wk high-fat males\textsuperscript{342,343}. Several groups have identified a subset of heavily vascularized islets that have elevated oxygen consumption and superior GSIS at the cost of susceptibility to hypoxia. We suspect Beta 2 cells represent a highly functional β-cell population that confer protection against hypoxia\textsuperscript{154,156}. Given their abundance in functional islets from both obese and lean normoglycemic mice, we hypothesize Beta 2 cells represent a mature, and possibly stress-resistant β-cell population.

Beta 3 cells are prevalent in normoglycemic obese mice which have elevated β-cell mass, high GSIS, and high insulin content. Beta 3 cells overexpress plasma membrane components, including several claudin family members, and ribosome components. Claudins provide structural integrity to tight junctions, which maintain cell polarity\textsuperscript{364}. Polarity in β-cells serves as both a driver and a characteristic of mature, highly functional β-cells\textsuperscript{43,45,365}. Cldn4, Cldn3, and Cldn7 are all upregulated in Beta 3 cells and associated with mature β-cell function\textsuperscript{366}. Beta 3 cells upregulate 7 ribosome genes and elevated ribosome biogenesis is associated with increased β-cell apoptosis\textsuperscript{367}, β-cell replication\textsuperscript{45}, and mature β-cell function\textsuperscript{368}, making the functional relevance of overexpression unclear. One gene, Rpl7, is associated with mature β-cell function\textsuperscript{369}, while Rpl23 protects against apoptosis\textsuperscript{370}. From this we conclude that Beta 3 cells represent a polarized and mature β-cell population.

Beta 4 cells are the lowest in abundance and elevated in lean males compared to obese males. Terms associated with Beta 4 cells include response to stress and response to
topologically incorrect protein. In addition to overexpression of UPR components, Beta 4 cells have significant down regulation of \textit{Ins1} and \textit{Ucn3}. Several groups have identified β-cell subpopulations based on a stress response signature\cite{91,92,371}, which have coalesced into a theory about stress response cycling, where β-cells go through periods of UPR activation and low insulin production to clear misfolded proteins\cite{21,259}. Interestingly, 20wk low-fat mice have a similar proportion of stress response cycling cells compared to other groups (~7%), however this population is significantly smaller in high-fat males, suggesting that stress response cycling may be suppressed by obesity.

Efforts to describe β-cell transcriptional heterogeneity are marred by lack of consensus across scRNA-seq studies\cite{372}. This is attributed to low read depth, which results in differences in gene capture driven by technical artifacts and random chance rather than biological variation\cite{102}. Further, it is unclear what level of gene expression fold change is meaningful between individual cells, rendering single cell data poorly suited to assess differential expression across different cohorts. To address these problems, we developed a technique to integrate single cell data with bulk RNA-seq data (\textbf{Figure 3.3, Figure 3.9}), which is not inhibited by these technical limitations, to assess β-cell-specific differential expression across cohorts of high- and low-fat fed male and female SM/J mice. While the list of genes identified as β-cell-specific is only 316 genes (\textbf{Table 3.2}), we are highly confident of their specificity to β-cells, allowing for robust assessment of differential expression and network analysis. Sub-setting the bulk RNA-seq data by β-cell-specific genes provided a small but highly-focused search space to identify genes that influence β-cell function in hyperglycemic-obesity, normoglycemic-obesity, and healthy mice.
We identified Pdyn as a novel candidate gene associated with improved glycemic control in males. Pdyn encodes Proenkephalin-b, which is cleaved into dynorphin A and dynorphin B, and exert effects through the κ-opioid receptor. While Pdyn’s role in β-cell function is unknown, activation of the κ-opioid receptor reduces hyperglycemia in diabetic mice and β-cells secrete dynorphin A in a glucose-dependent manner. Further, Pdyn expression is associated with regulation by Pax6 and Lkb1, hallmarks of mature β-cells. Pdyn is over expressed in Beta 2 cells, which are increased in normoglycemic-obese mice, and may link improved β-cell function with hypoxia response, as Pdyn provides protection from hypoxia in lung and neuronal tissue.

We used weighted gene co-expression network analysis (WGCNA) to identify networks of co-expressed β-cell genes and to assess how networks correlate with metabolic and islet phenotypes (Figure 3.10). Previous efforts to construct networks in islet RNA-seq data failed to account for cell-type specific gene expression, making it difficult to determine if these networks operate within an individual cell. Our analysis revealed 6 β-cell-specific modules that correlate with phenotypes, two of which were enriched for intriguing ontology terms (Figure 3.11). We chose to explore these networks in depth to assess the context in which they were relevant to β-cell function in obesity.

The blue module is highly expressed in 20wk high-fat males, strongly correlating with blood glucose concentration (Figure 3.5, Figure 3.12). The blue module is enriched with fatty acid metabolism genes, which are heavily implicated in obesity and diabetes (Table 3.4). In β-cells, fatty acid metabolism provides a glucose-independent stimulus for insulin release. While short term fatty acid exposure enhances GSIS, prolonged exposure decreases GSIS and
reduces insulin content\textsuperscript{383,385}. Fatty acid metabolism protects against glucotoxicity-induced damage, allowing cells to convert excess glucose into glycerol, which bypasses ROS generation\textsuperscript{386}. How this network connects to the β-cell dysfunction seen in obese, hyperglycemic 20wk high-fat mice is not immediately clear. Since fatty acid metabolism can stimulate insulin release, it is possible that prolonged activation of this network promotes elevated basal insulin secretion but inhibits GSIS. Expression of this network may be related to the abundance of Beta 1 cells in 20k high-fat males, which are geared toward basal insulin release. One gene in the blue module, \textit{Entpd3}, confers susceptibility to diet-induced obesity and hyperglycemia\textsuperscript{387}. This fatty acid metabolism gene network may provide a link between the increased proportion of Beta 1 cells and the β-cell dysfunction observed in 20wk high-fat males.

The brown module is highly expressed in lean mice and negatively correlates with serum insulin levels (\textbf{Figure 3.6, Figure 3.12}). Unsurprisingly, this module is enriched for genes associated with β-cell physiology (\textbf{Table 3.4}). We focused on how the network behaved in 20wk high and low-fat males, as they provided strong contrast for serum insulin levels and β-cell function. Core members of this network include known markers of β-cell maturation including \textit{Ucn3}, \textit{Slc2a2}, and \textit{Pcsk1}. This network is suppressed in 20wk high-fat males, who have dysfunctional β-cells. We homed in on \textit{Fam151a}, a gene highly connected within the network, that independently correlates with serum insulin levels. \textit{Fam151a} is both differentially expressed and differentially connected within the brown module between 20wk high-fat and 20wk low-fat mice, suggesting an important role within the network and a potential link between serum insulin levels and β-cell function. \textit{Fam151a} is a membrane embedded protein of unknown function, but it has been associated with dysregulated insulin secretion in \textit{Isl1} knockout mice, and falls within
a QTL associated with diabetes. Further work will explore how Fam151a influences β-cell function in the context of obesity.

We explored if subpopulation composition contributes to differential expression of Pdyn and Fam151a (Figure 3.7). Total Pdyn expression increased across all β-cells between 20 and 30wk high-fat fed males, in agreement with the bulk RNA-seq data. Pdyn is overexpressed in Beta 2 cells, which increase in proportion between 20 and 30 weeks in high-fat mice, suggesting differential expression of Pdyn is driven by a change in subpopulation proportions. Investigation into how Pdyn influences β-cell function must consider that knockout/overexpression may affect subpopulations differently, and that subpopulation structure changes based on metabolic context. Likewise, total Fam151a increased across all β-cells between 20wk high and low-fat males, in agreement with the bulk RNA-seq data. However, Fam151a is strongly overexpressed in Beta 1 cells, which are most abundant in 20wk high-fat males. To explore this discrepancy, we performed differential expression analysis in Beta 1 cells between 20wk high and 20wk low-fat males and found Fam151a expression is significantly higher in 20wk low-fat male Beta 1 cells. Thus, while the proportion of Beta 1 cells is lower in 20wk low-fat mice, the expression of Fam151a is significantly increased in their Beta 1 cells. These findings underscore the complexity of gene expression and suggest it is important to consider both subpopulation composition and expression within subpopulations when exploring β-cell heterogeneity in diabetes and obesity.

In summary, we identified 4 β-cell subpopulations whose relative proportions change depending on metabolic state. Beta 1 cells are primed for basal insulin secretion and proportionally high in hyperglycemic obese mice. Beta 2 cells are primed for protection from
hypoxia associated with enhanced function and are abundant in normoglycemic obese and normoglycemic lean mice at the expense of Beta 1 cells. In conjunction, hyperglycemic obese mice express a highly connected genetic network associated with fatty acid metabolism, which is lost as glycemic control improves. The interplay between changing β-cell subpopulations and decreased fatty acid metabolism likely contributes to the improved β-cell function and subsequent restoration of glycemic control seen in obese SM/J mice. This study provides a road map for exploring cellular heterogeneity by integrating sc- and bulk RNA-seq data, allowing for robust characterization of subpopulation structure, differential expression, and network analysis associated with obesity and glycemic stress.
**3.6 Figures and Legends**

**Figure 3.1.** SM/J islets contain four subpopulations of β-cells. (A) Data analysis pipeline identifies subpopulations of β-cells and integrates bulk and single cell RNA sequencing data. (B) Single cell RNA sequencing UMAP plot of islet tissue. VSMC – vascular smooth muscle cell, VEC – vascular endothelial cell. (C) Single cell RNA sequencing UMAP of 4 subpopulations of β-cells. (D) Bootstrap analysis quantifies relative proportions of β-cell subpopulations across cohorts. Black dot represents estimated proportion, colored bars illustrate 95% confidence interval. Cell type composition estimates with non-overlapping confidence intervals are significantly different. (E) Ternary plot illustrates differences in subtype composition of Beta 1-3 in high-fat SM/J mice. HF20 – 20wk high-fat female, HF30 – 30wk high-fat female, HM20 – 20wk high-fat male, HM30 – 30wk high-fat male, LF20 – 20wk low-fat female, LM20 – 20wk low-fat male.
Figure 3.2. β-cell subpopulations have unique gene expression signatures. (A) Heat map for top 10 differentially expressed genes in each β-cell subpopulation. Yellow is highly expressed; purple is lowly expressed.
Figure 3.3. Single cell RNA sequencing identifies β-cell-specific genes. (A) Total gene expression and β-cell-specific contribution to gene expression in 20wk low-fat female β-cells. Gold line indicates threshold for β-cell-specific expression. Red dots identify genes that pass the threshold cutoff in this cohort. Arrows identify highly expressed genes associated with β-cell identity. Genes must pass threshold in all cohorts to be considered β-cell-specific in the rest of the analysis. (B) UMAP plot of all islet cell types. UMAP plots for β-cell-specific expression of known β-cell markers (C) Ucn3, (D) Gcgr, (E) Slc2a2. UMAP plots for β-cell-specific expression of novel β-cell markers (F) Pdyn, and (G) Fam151a. (H) Permutation analysis of β-cell-specific genes in Beta 1 overexpressed genes. Distribution shows expected number of β-cell-specific genes in Beta 1 overexpressed gene set based on chance (n = 1000 permutations), red line indicates number of β-cell-specific genes in Beta 1 overexpressed gene set. P-value indicates probability of number of β-cell-specific genes in Beta 1 overexpressed gene set due to chance.
Figure 3.4. β-cell-specific genes are differentially expressed by age, diet, and sex. (A) Principal component analysis of bulk RNA sequencing data normalized with only β-cell-specific genes. (B) Number of differentially expressed genes in females and males, across diets and age. (C) Differentially expressed genes between 20wk high-fat males and 30wk high-fat males. (D) Pdyn is differentially expressed in high-fat males. (E) Differentially expressed genes between 20wk high-fat males and 20wk low-fat males. (F) Fam151a is differentially expressed in 20wk males. Blue genes are significantly under-expressed in comparison, red genes are significantly over-expressed. Vertical golden lines indicate threshold for significance based on average log fold change, horizontal line indicates threshold for significance based on FDR corrected p-value. **p-value <0.01, **** p-value < 0.0001 in FDR corrected pairwise comparison. HF20 – 20wk high-fat female, HF30 – 30wk high-fat female, HM20 – 20wk high-fat male, HM30 – 30wk high-fat male, LF20 – 20wk low-fat female, LM20 – 20wk low-fat male.
Figure 3.5. Blue modules network structure is altered by age in high-fat male mice. (A) Blue module heatmap, eigengene expression, and blood glucose levels across all individuals. (B) Correlation between strength of module membership and blood glucose levels for blue module. (C) Total and differential connectivity between blue module genes in 20wk and 30wk high-fat male mice. Vertical golden lines indicate threshold for differential connectivity. (D) Blue module network structure in 20wk high-fat males and (E) 30wk high-fat males. Size and color of node indicates overall connectivity within the network, thickness of edges indicates strength of correlation between gene pairs.
Figure 3.6. Brown module network structure is altered by diet in 20wk male mice. (A) Brown module heatmap, eigengene expression, and insulin levels across all individuals. (B) Correlation between strength of module membership and serum insulin levels for brown module. Gene of interest, Fam151a, is highlighted. (C) Expression of Fam151a correlates with serum insulin levels across all individuals independent of module membership. Light red – 20wk high-fat mice, dark red – 30wk high-fat mice, light blue – 20wk low-fat mice, dark blue – 30wk low-fat fed mice. (D) Brown module network in 20wk high-fat males and (E) 20wk low-fat males. Gene of interest, Fam151a, is highlighted in yellow. Size and color of node indicates connectivity within the network, edge thickness indicates strength of correlation between gene pairs. (F) Total and differential connectivity between brown module genes in high and low-fat male SM/J mice. Vertical golden lines indicate threshold for significant differential connectivity. Gene of interest, Fam151a, is highlighted. (G) Differential connectivity and differential expression of brown module genes between 20wk high and 20wk low-fat males. Vertical golden lines indicate threshold for differential connectivity, Horizontal golden line indicates threshold for differential expression. Gene of interest, Fam151a, is highlighted.
Figure 3.7. *Pdyn* and *Fam151a* are differentially expressed in β-cell subtypes. (A) Expression of β-cell-specific genes across all β-cells between 20wk and 30wk high-fat males. (B) Percent of β-cells in 20wk and 30wk high-fat males expressing β-cell-specific genes. (C) Expression of β-cell-specific genes across all β-cells between 20wk high and low-fat males. (D) Percent of β-cells in 20wk high and low-fat males expressing β-cell-specific genes. (E) Comparison of *Pdyn* and *Fam151a* expression across β-cell subtypes. (F) Expression of β-cell-specific genes in beta 1 cells between 20wk high-fat and 20wk low-fat fed mice. (G) Precent of beta 1 cells expressing beta specific genes between 20wk high-fat and 20wk low-fat fed mice. Blue genes are significantly under-expressed in comparison, red genes are significantly over-expressed. Vertical golden lines indicate threshold for significance based on average log fold change, horizontal line indicates threshold for significance based on Bonferroni corrected p-value.
Figure 3.8. Single cell RNA sequencing quality control. (A) Total counts, features, and mitochondrial RNA expression plotted for each cell. Cells within blue box and without mitochondrial gene expression were included in analysis. (B) Number of cells identified in individual mice prior to quality control, (C) and after quality control. (D) Expression of Ins2 across all cells prior to ambient RNA removing using SoupX, (E) and after ambient RNA removal. (F) Resolution analysis to determine the number of β-cell subpopulations. Selected resolution highlighted with red box.
Figure 3.9. Quantifying expected expression for β-cell expression of *Dcd*. (A) Distribution of expression across all β-cells. (B) Cullen and Frey analysis identifies *Dcd* expression to be beta distributed. (C) Kolmogorov-Smirnov test identifies alpha and beta parameters that minimize Ks between real (blue line) and simulated (red line) distribution of cells expressing *Dcd*. (D) Estimating percent of cells not expressing *Dcd* due to gene drop out by iterating alpha and beta parameters that minimize Ks between real and simulated data. (E) Density plot visualizing estimated parameters for distribution of *Dcd* expression. Red line shows distribution of actual data, black line shows distribution of simulated data based on optimal alpha and beta parameters. From these parameters, expected expression is calculated.
Figure 3.10. Metabolic and islet phenotypes from bulk RNA sequencing mice. (A) body weight, (B) blood glucose, (C) serum insulin levels collected after 4-hour fast. (D) Glucose-stimulated insulin secretion (GSIS), (E) Basal insulin secretion, (F) Islet insulin content collected after isolated islets were rested overnight. N = 4 mice per age X sex X diet cohort. N= 10 islets per individual for islet phenotypes. Middle bar represents mean, box represents 25th and 75th quartile, whiskers represent minimum and maximum values.
Figure 3.11. Correlation between phenotype and module eigengene expression. Number of genes within each module reported on y-axis, phenotypic trait reported on x-axis. For each module-trait relationship, the Pearson correlation between eigengene expression and phenotype value is reported (top) along with an FDR-corrected p-value for the correlation (bottom). Color of box indicates strength of correlation. NS = non-significant association based on FRD-corrected p-value.
Figure 3.12. Overview of brown and blue modules. (A) Principal component analysis of gene expression within the brown module across all cohorts, segregated by diet. (B) Principal component analysis of gene expression within the blue module, segregated by cohort. (C) Brown module network structure in across all cohorts. Gene of interest, *Fam151a*, is highlighted in yellow. Size and color of node indicates overall connectivity within the network, thickness of edges indicates strength of correlation between gene pairs. (D) Blue module network structure in across all cohorts. Size and color of node indicates overall connectivity within the network, thickness of edges indicates strength of correlation between gene pairs. (E) Correlation between brown module eigengene expression and serum insulin levels across all cohorts. (F) Correlation between blue module eigengene expression and blood glucose levels across cohorts.
### 3.7 Tables

**Table 3.1.** Top five results for over-representation analysis (ORA) on significantly enriched genes within each β-cell subpopulation, including gene ontology terms.

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Table 3.2. β-cell-specific genes identified in single cell RNA sequencing analysis.

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### Table 3.3. Top five results for over-representation analysis (ORA) for β-cell-specific genes, including gene ontology and mammalian phenotype ontology terms.

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### Table 3.4. Type five results for over-representation analysis (ORA) on genes within brown and blue modules, including gene ontology and KEGG pathway terms.

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Chapter 4: Conclusions and Future Directions

By

Mario A Miranda
4.1 Summary

Pancreatic β-cells are optimized to secrete insulin, a process critical for maintaining appropriate blood glucose concentration. In type 2 diabetes, peripheral insulin resistance promotes initial β-cell expansion, but ultimately results in β-cell death and dysfunction\textsuperscript{2-4}. Obesity raises the risk of developing type 2 diabetes 27–76 fold, while approximately 60% of individuals with diabetes are obese\textsuperscript{268–271}. Despite the stress obesity places on β-cells, 10–30% of obese individuals maintain glycemic control and are at low risk for developing diabetes\textsuperscript{276}. These low-risk obese individuals have elevated β-cell mass and improved insulin secretion compared to BMI-matched diabetic-obese individuals\textsuperscript{2,13,277,278}. Understanding the differences in β-cell physiology between these populations may reveal therapeutic strategies for maintaining and improving glycemic control in obese individuals.

Like in humans, diabetic risk in obese mice depends on genetic background\textsuperscript{281–284}. Variation in β-cell heterogeneity likely underlies variability in islet stress response, and thus needs to be accounted for when comparing nondiabetic-obese and diabetic-obese populations. No current mouse model is well-suited to examine physiological differences in β-cell health between nondiabetic-obese and diabetic-obese states. Cell sorting, imaging, and sequencing technologies have allowed for analysis of multiple β-cell properties, revealing that β-cells vary in morphology, function, and gene expression. The importance of heterogeneity is reinforced by observations that β-cells are subject to variable fates in diabetes including apoptosis, replication, and dedifferentiation\textsuperscript{2,26–28}. Characterization of β-cell heterogeneity has led to questions about its role in diabetic etiology and therapy\textsuperscript{29}. Examining heterogeneity lends itself to questions about the
applications and limitations of β-cell plasticity, which can focus research aimed at improving β-cell function.

The SM/J inbred mouse strain has been used to study interactions between diet and metabolism, and more recently has uncovered genetic architecture underlying diet-induced obesity and glucose homeostasis. After 20 weeks on a high fat diet, SM/J mice display characteristics of diabetic-obese mice, including elevated adiposity, hyperglycemia, and glucose intolerance. We have previously shown that by 30 weeks of age, high fat-fed SM/J mice enter diabetic remission, characterized by normalized fasting blood glucose, and greatly improved glucose tolerance and insulin sensitivity. Given the central role of β-cell health in susceptibility to diabetic-obesity, we hypothesize that obese SM/J mice undergo restoration of functional β-cell mass during the resolution of hyperglycemia. By contrasting β-cells in hyperglycemic-obese and normoglycemic-obese. Given the increasing relevance of β-cell heterogeneity in diabetes research, we also sought to characterize β-cell subpopulations in obese SM/J mice, and how they contribute to the hyper- and normoglycemic-obese states. By integrating phenotypic information with subpopulation structure, a more complete picture of β-cell function in obese SM/J mice can be achieved.

In chapter 2, we determined that increased insulin production and insulin sensitivity accompany improved glycemic control, driven by expanded β-cell mass and improved glucose-stimulated insulin secretion. To our knowledge, this is the first report of improved β-cell function in an obese inbred strain. β-cell hyperplasia is the primary driver of islet expansion in mouse models of obesity. Some nondiabetic obese mice experience increased β-cell number, but do not show evidence for elevated β-cell replication in immunostaining of
pancreatic sections\textsuperscript{282,315}. This has been attributed to technical artifacts in sampling could result in inflated variances which mask biological differences. This could be the case here, given that high fat-fed SM/J’s β-cell number is far above low fat-fed controls, that their β-cell number expands 2-fold during the resolution of hyperglycemia, yet we find no evidence for increased β-cell replication. 20-week high fat-fed SM/J mice have an insulin secretion profile similar to diabetic-obese mice and humans, including blunted glucose-stimulate insulin release, elevated basal insulin secretion, and low islet insulin content, which resolves by 30 weeks.

Three components of β-cell stress response may shed light on the perplexing improvement in glycemic control seen in SM/J mice: β-cell dedifferentiation, nascent β-cell maturation, and changes in β-cell subtype proportions. While early studies concluded overworked β-cells undergo apoptosis\textsuperscript{2,325–327}, recent studies have suggested β-cells dedifferentiate into a dysfunctional, progenitor-like state, potentially as a defense mechanisms against prolonged glycemic stress\textsuperscript{236,279,328,329}. These dedifferentiated β-cells have low insulin content and poor glucose-stimulated insulin secretion. Further, the dedifferentiated state is reversible in cultured conditions, revealing potential for therapeutic intervention\textsuperscript{239}. It is feasible that obese SM/J mice have β-cells in the dedifferentiated state at 20-weeks, which would explain the low insulin content and poor functionality despite the elevated β-cell mass. Improvement in insulin sensitivity could ease glycemic stress, allowing dedifferentiated β-cells to redifferentiate by 30 weeks, reestablishing insulin production and secretion.

Recent advances in single cell technology allows for identification of β-cell subtypes, based on functional characteristics and gene expression. Integrating sc-RNAseq with bulk RNAseq data leverages bulk sequencing’s high read depth, allowing for capture of lowly
expressed genes and robust expression analysis. Several tools can integrate sc- and bulk RNA-seq data, focusing on deconvoluting bulk RNAseq data from heterogeneous tissue to account for differences in tissue composition\textsuperscript{90,340,341}. These methods estimate and control for cell type abundance but do not identify cell type-specific expression signatures in bulk datasets. Analytical strategies that identify cellular gene expression signatures in bulk RNAseq data allow for robust cell type-specific differential expression analysis and complex network analysis that is currently not feasible in scRNA-seq data.

In chapter 3 we characterized gene expression in β-cells from obese SM/J mice, who spontaneously transition from hyperglycemic to normoglycemic with improved β-cell function between 20 and 30 weeks of age\textsuperscript{342,343}. Several groups have proposed the existence of functionally distinct β-cell subpopulations\textsuperscript{24,25,45,356}, but their existence and relevance to insulin homeostasis remain controversial. We identified 4 β-cell populations, Beta 1-4, across hyperglycemic-obese, normoglycemic-obese, and normoglycemic-lean mice. While the functional roles assigned to each population are based on gene enrichment analysis and open to interpretation, the relative proportion of each population changes in conjunction with islet function across cohorts, suggesting a functional relevance to overall β-cell population structure.

Beta 1 cells appear to be primed for insulin release, but not to perform glucose-stimulated insulin secretion. Farack et al\textsuperscript{356} identified a subpopulation of “extreme” β-cells that specialize in basal insulin secretion, with high insulin expression and low mature insulin content. The Beta 1 cells we identified are similar to “extreme” β-cells based on their mature profile, potentially limited glucose response, and abundance in diabetic obese mice. Beta 2 cells are enriched for response to oxygen levels, pyruvate metabolism, and nucleotide phosphorylation, each
associated with protection from hypoxia\textsuperscript{362,363}. Several groups have identified a subset of heavily vascularized islets that have elevated oxygen consumption and superior GSIS at the cost of susceptibility to hypoxia. We suspect Beta 2 cells represent a highly functional β-cell population that confer protection against hypoxia\textsuperscript{154,156}. Beta 3 cells overexpress plasma membrane components, including several claudin family members, and ribosome components. Polarity in β-cells serves as both a driver and a characteristic of mature, highly functional β-cells\textsuperscript{43,45,365}. \textit{Cldn4}, \textit{Cldn3}, and \textit{Cldn7} are all upregulated in Beta 3 cells and associated with mature β-cell function\textsuperscript{366}. From this we conclude that Beta 3 cells represent a polarized and mature β-cell population. Terms associated with Beta 4 cells include response to stress and response to topologically incorrect protein. Several groups have identified β-cell subpopulations based on a stress response signature\textsuperscript{91,92,371}, which have coalesced into a theory about stress response cycling, where β-cells go through periods of UPR activation and low insulin production to clear misfolded proteins\textsuperscript{21,259}. Interestingly, 20wk low-fat mice have a similar proportion of stress response cycling cells compared to other groups (~7%), however this population is significantly smaller in high-fat males, suggesting that stress response cycling may be suppressed by obesity.

We developed a technique to integrate single cell data with bulk RNA-seq data to assess β-cell-specific differential expression. While the list of genes identified as β-cell-specific is only 316 genes, sub-setting the bulk RNA-seq data by β-cell-specific genes provided a small but highly-focused search space to identify genes that influence β-cell function in hyperglycemic-obesity, normoglycemic-obesity, and healthy mice. We identified \textit{Pdyn} as a novel candidate gene associated with improved glycemic control in males. While \textit{Pdyn}’s role in β-cell function is unknown, many opioids modulate insulin section\textsuperscript{391}. \textit{Pdyn} is over expressed in Beta 2 cells,
which are increased in normoglycemic-obese mice, and may link improved β-cell function with hypoxia response, as Pdyn provides protection from hypoxia in lung and neuronal tissue\textsuperscript{378,379}.

Weighted gene co-expression network analysis (WGCNA) identified β-cell-specific modules that correlate with phenotypes. The blue module is highly expressed in 20wk high-fat males, strongly correlating with blood glucose concentration, and enriched with fatty acid metabolism genes, which are heavily implicated in obesity and diabetes. Since fatty acid metabolism can stimulate insulin release, it is possible that prolonged activation of this network promotes elevated basal insulin secretion but inhibits GSIS. Expression of this network may be related to the abundance of Beta 1 cells in 20k high-fat males, which are geared toward basal insulin release. The brown module is highly expressed in lean mice and negatively correlates with serum insulin levels and enriched for genes associated with β-cell physiology. This network is suppressed in 20wk high-fat males, who have dysfunctional β-cells. We homed in on Fam151a, a gene highly connected within the network, that independently correlates with serum insulin levels. Fam151a is both differentially expressed and differentially connected within the brown module between 20wk high-fat and 20wk low-fat mice, suggesting an important role within the network and a potential link between serum insulin levels and β-cell function.

We explored if subpopulation composition contributes to differential expression of Pdyn and Fam151a. Total Pdyn expression increased across all β-cells between 20 and 30wk high-fat fed males, in agreement with the bulk RNA-seq data. Pdyn is overexpressed in Beta 2 cells, which increase in proportion between 20 and 30 weeks in high-fat mice, suggesting differential expression of Pdyn is driven by a change in subpopulation proportions. Likewise, total Fam151a increased across all β-cells between 20wk high and low-fat males, in agreement with the bulk
RNA-seq data. However, *Fam151a* is strongly overexpressed in Beta 1 cells, which are most abundant in 20wk high-fat males. To explore this discrepancy, we performed differential expression analysis in Beta 1 cells between 20wk high and 20wk low-fat males and found *Fam151a* expression is significantly higher in 20wk low-fat male Beta 1 cells. Thus, while the proportion of Beta 1 cells is lower in 20wk low-fat mice, the expression of *Fam151a* is significantly increased in their Beta 1 cells.

In conclusion, obese SM/J mice experience improved beta cell function, characterized by enhanced GSIS, decreased basal insulin secretion, and increased islet insulin content. Underlying this improvement is a decrease in β-cells associated with basal insulin secretion, and an increase in β-cells associated with hypoxia response. Concurrently, obese SM/J mice decrease expression of a gene network associated with fatty acid metabolism. Expression of key genes, including *Pdyn* and *Fam151a*, provide strong candidates for exploring differential gene expression between and within β-cell subpopulations, and how changes in subpopulation structure influence glycemic control in obesity.

### 4.2 Future Directions

My thesis work characterized how obese SM/J β-cells change at the morphological, functional, and transcriptional level. I established that improved β-cell function underlies the restoration of glycemic control and identified genetic pathways associated with the hyperglycemic-obese state. While other strains, including LG/J and C57Bl/J6, do not improve glycemic control with age on a high fat diet, this phenomenon has not been rigorously assessed in most inbred mouse strains. Performing this experiment on other inbred strains, particularly those that are susceptible to diet-induced obesity, would reveal if reestablishing glycemic control
is truly unique to SM/J mice. A genomic analysis between strains would reveal the genetic variants that are unique to the SM/J strain, providing candidates that contribute to reestablishing glycemic control in obesity. This would pave the way for identifying the genetic mechanisms underlying the resolution of hyperglycemia in SM/J mice, providing candidate genes and pathways.

The obese SM/J mouse model could be explored in greater depth to characterize the physiological changes underlying the reestablished glycemic control. Insulin production and insulin sensitivity are tightly intertwined. While insulin tolerance tests provide a snapshot of insulin sensitivity, they do not provide a dynamic perspective on the glucose-insulin axis. A hyperglycemic clamp would assess insulin secretion in vivo by measuring insulin levels when hyperglycemia is induced by continuous glucose infusion. This would confirm if the improvement in β-cell function is maintained outside of cultured conditions. Likewise, a hyperinsulinemic-euglycemic clamp would assess insulin sensitivity in real time. By infusing a constant level of insulin while infusing variable levels of glucose to maintain equilibrium, true insulin sensitivity could be assessed. This would reveal the degree to which obese SM/J mice improve insulin sensitivity over time. Similarly, a time course experiment could reveal the precise order of events, addressing if improved insulin sensitivity precedes the increase in insulin production, or vise versa. Examining insulin sensitivity and insulin levels every two weeks over the 20–30 week range would establish if the improved beta cell function can be attributed to improvement in insulin sensitivity of metabolic tissues (extrinsic factors) or improved insulin secretion (intrinsic factors).

Performing islet transplants on obese SM/J mice would also provide insight into the nature of the improved β-cell function. After treatment, ablation of existing β-cells using STZ or
a genetic knockout can address if an islet removed from 30wk high fat mice is sufficient to reestablish glycemic control in 20wk high fat mice. Conversely, transplant experiments can address if islets from 20wk high fat mice spontaneously improve when transplanted into 30wk mice, or will if render the mice diabetic. Further, we can ask if islets from 20wk high fat mice render lean, normoglycemic mice diabetic. If islets from 30wk obese mice improve glycemic control in younger obese mice, then we can conclude that the improved β-cell function is intrinsic, and not primarily driven by the islet microenvironment. Likewise, if islets transplanted from hyperglycemic obese mice improve in 30wk obese mice, we can conclude that the improvement in β-cell function can be attributed to decreased glycemic stress, suggesting the improvement is driven by extrinsic factors. If the improvement is driven by extrinsic factors, focus should shift to other metabolic tissues in the obese SM/J mouse.

Using immunohistochemistry to stain pancreatic sections for subpopulation overexpressed genes may verify the existence of the subpopulations at the protein level. Other gene quantification techniques, such as seqFISH, can confirm differential expression between subpopulations. If the β-cell subpopulations can be confirmed using imaging techniques, the effects of genetic knockouts and islet transplantation on subtype composition could be assessed, helping confirm or reject a role for subpopulation structure in the resolution of hyperglycemia. Topographical analysis would assess if β-cell subpopulations localize to the islet core or periphery, or are in proximity to neurons, blood vessels, or other islet cell types, providing insight into how microenvironment may contribute to the improved β-cell function. If there are meaningful differences in protein level among subpopulations, cell sorting would allow for subpopulations to be isolated and reaggregated for analysis, including functional assessment and gene expression profiling.
Generating β-cell-specific knockouts of *Pdyn* and *Fam151a* in the SM/J genetic background would allow for examination of each gene in the context of improved glycemic control in obesity. I hypothesize that ablation of *Pdyn* will decrease glucose-stimulated insulin secretion *in vivo* due to decreased vasodilation but will not affect insulin secretion *ex vivo*. Further, I hypothesize treatment with a fatty acid oxidation inhibitor may improve insulin secretion in 20wk obese islets, including improving GSIS and decreasing basal insulin secretion. The effects of fatty acid oxidation inhibitor treatment *in vivo* will be difficult to untangle among the changes associated with inhibited fatty acid metabolism in other tissues, but it would be interesting to see if this could alter the proportion of basal insulin secreting β-cells. These experiments would confirm the importance of the gene expression changes seen in obese SM/J β-cells and would warrant exploration in human β-cell models.

The data integration strategy developed here can be applied to other cell types, including α-cells. Dysfunction of α-cells has been associated with diabetes and obesity and may also provide potential avenues for treatment. Identification of α-cell-specific genes would allow for similar assessment of differential expression and genetic networks associated with the hyper- and normoglycemic obese states. Although analyzing α-cells was outside the scope of this thesis, I hypothesize α-cell function and gene expression changes during the resolution of hyperglycemia, and further analysis is likely to uncover candidate genes associated with improved glycemic control. This data integration strategy could also be applied to healthy and diabetic human islet datasets, as bulk islet RNA-seq data is in much greater abundance than β-cell scRNA-seq data. Knockdowns of candidate genes could be performed in human iPSC’s to assess how the gene contributes to differentiation into a β-cell-like cells, which could improve the efficiency or function of generating β-cells *in vitro*.
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