Regulated Glucagon Secretion from Islet and Pseudo-Islet Alpha Cells

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Dissertation

Submitted to the Faculty of the

Graduate School of Vanderbilt University

in partial fulfillment of the requirements

for the degree of

DOCTOR OF PHILOSOPHY

In

Molecular Physiology and Biophysics

May, 2017

Nashville, Tennessee

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For Lexie, Tess, Arya, and Sheldon, my loves outside of science

ACKNOWLEDGEMENTS

In science, having a great idea is only half the battle. The other half is being able to communicate that idea efficiently in both verbal and written form. I will be the first to admit I had a rocky start to PhD research due to my inability to write well. While many things come naturally to me, scientific writing does not. Without the thoughtful guidance of my PI, David Piston, and my dissertation committee members, I might not have survived certain steps of this graduate program. Moreover, the comments, questions, and suggestions from committee members resulted in a high quality publication in *Diabetes,* with the entire review process taking only 4 months. As Dave says, "it is better be lucky than good." Without a doubt, I've been lucky to have such great support over the years.

This support was also at home. My wife, Lexie, has never once questioned my ability as a scientist despite the missteps over the years. She has always known what is best for me, even when I didn't know myself. I constantly channel her unflinching confidence when tackling any obstacle, including ones outside of the lab. Having a baby has put many things, including science, into a new perspective.

I would also like to thank current and past members of the Piston lab. The eclectic, quirky bunch that Dave recruited have come together to produce top tier research, with humor that is not far behind. It's much easier to go into lab when the people you work with make it that much more fun. A specific thank you is in order for Alessandro Ustione. If Dave is the metaphorical head, Ale is the back bone. His scientific knowhow is nearly infinite and his baking skills have kept us all a little happier and fatter.

Graduating is bittersweet. I'm eager to be the first PhD in my family, but I will dearly miss Dave and the Piston lab.

ACKNOWLEDGEMENT OF SUPPORT

PRIOR PUBLICATIONS

Some of the data included in this dissertation has been published in peer-reviewed journals. Chapter 2 includes work published in *PLOS One* (Schwetz AT, Reissaus CA, Piston DW, 2014). Chapter 3 and 4 includes work published in *Diabetes* (Reissaus CA and Piston DW, 2017). Figures are reproduced or adapted with permission and cited in the figure captions.

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Introduction

1.1   Glucose Homeostasis

A variety of organic molecules, including lipids, amino acids, ketones, and sugars, are utilized as energy sources to sustain mammalian life (1). The circulating levels of these molecules in the blood are tightly regulated to avoid reaching extremely low or high levels, which can be detrimental to an organism's survival (2,3). For glucose, this regulation is particularly important. Excess glucose in the blood can lead to tissue damage, while the absence of glucose can lead to tissue starvation and death (2,4). Glucose levels are tightly regulated by multiple organ systems in order to match energy demand. This regulation is referred to as glucose homeostasis (1–3).

Several key tissues involved in the regulation of glucose levels include the liver, brain, and pancreatic islets (Figure 1) (2,3,5–7). The liver acts as a primary depot for glucose in the form of glycogen; it can store or liberate glucose depending upon energy demand and associated signals from other organs (5). Hepatic glucose storage and release capacity directly depends upon both neuronal signals and hormones secreted by the pancreatic islets (2,3,8). Complex signaling within various brain regions influences the regulation of glucose homeostasis by modulating feeding and satiety and via nerve innervations of tissues like the liver and pancreatic islets (2,6,8–11). The pancreatic islets secrete numerous factors, including the hormones insulin and glucagon, primarily to regulate glucose uptake and release from the liver in a glucose dependent manner (3,12–15).
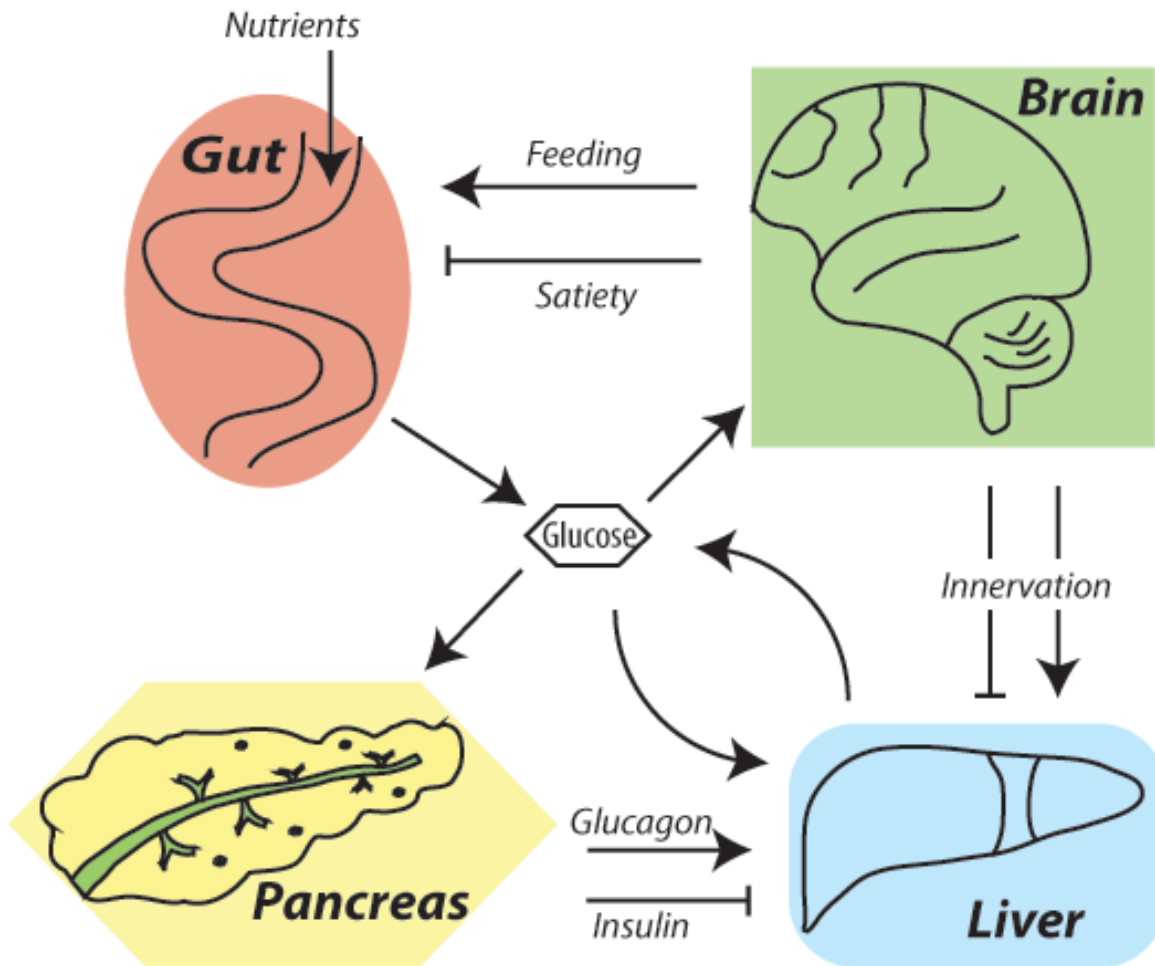
Figure 1: A Simplified Model of the Organ Systems Involved in Glucose Homeostasis. In this model, the brain, liver, and pancreas all aid in the regulation of glucose homeostasis. Hormones secreted by the pancreas fulfill multiple roles, including signaling uptake or release of glucose from the liver. The liver acts a depot for glucose, which can store or liberate glucose based on external signals. The brain can alter feeding and satiety to change nutrient intake, along with glucose uptake and output from the liver.

Each of the organ systems described above has an entire field of research dedicated to its role in glucose homeostasis. Unlike the other organ systems, the pancreatic islets function almost exclusively to regulate glucose homeostasis (2,3). This dissertation will introduce, interrogate, and discuss critical aspects of islet physiology, glucose regulated hormone secretion, and diseases resulting from islet dysfunction.

1.2   The Pancreatic Islet

1.2.1   Islet Overview

The pancreatic islet, also known as the islet of Langerhans, is a multicellular, collagen-encapsulated endocrine micro-organ (2,15). Hundreds to thousands of islets are spaced throughout the mammalian pancreas; however the total endocrine mass makes up only a small fraction of the whole mass of the pancreas (< 5%) and the remaining pancreatic tissue mass is exocrine in nature and functions to aid in digestion (2,16). The islet is highly vascularized and sympathetically innervated, both of which aid in the islet's ability to rapidly sense and respond to changes in blood glucose concentrations (10,17). Depending upon an increase or decrease in glycemia, islets will secrete the applicable hormone to recover euglycemia (2,11). The islet hormones primarily signal to the liver, directing it to store or liberate glucose (2,8,18). The islet hormones also affect other organs, including the brain, muscle, and adipose tissue, to aid in the regulation of glucose homeostasis (2,3,8,19).

1.2.2   The Islet Cell Types and Organization

The islet is composed of several cell types, including insulin-secreting beta cells, glucagon-secreting alpha cells, and somatostatin-secreting delta cells (20). Various other cell types are also present within the islet, such as endothelial cells, ghrelin-secreting epsilon cells, pancreatic polypeptide cells, and immune cells. However, the roles of these cells within the context of normal and disease islet physiology are still being elucidated (20–22). Altogether, the islet averages around 100-150 μm in diameter, which encompasses hundreds to thousands of cells (20,23). The islet is primarily composed of beta cells, with alpha and delta cells making up a smaller fraction of the overall population (Figure 2). Differences in islet cellular composition

ratios, diameter, and organization have been observed between species, and even age groups within a species (20,23,25). For example, murine islets have a large proportion of beta cells to alpha and delta cells (~ 80% beta; ~ 10% alpha; ~ 5% delta) (20,23), while human islets have more of the alpha and delta cell types (~ 65% beta; ~ 25% alpha; ~ 10% delta) (24,25). In the mouse islet, a beta cell core is surrounded by a mantle of the other cell types, including alpha and delta cells (20,23). In humans, similar patterns are observed, but in micro-domains that are created by a more lobular islet architecture (23,26–28). In both species, it appears blood flow originates at beta cells and proceeds to the other cell types (17). It is unclear how islet differences relate to glucose sensing and hormone secretion; however, disorganization of the islet has been observed in several disease states (20,23,29,30). As a result, it is generally accepted that the architectural organization of the islet plays an important role in glucose homeostasis.
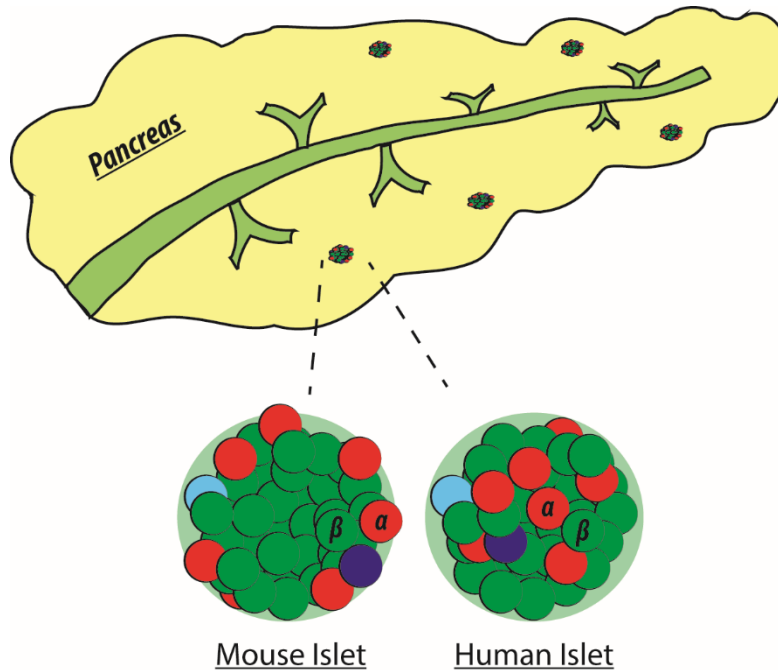
Figure 2: The Pancreatic Islets. The islets of Langerhans are a multicellular, endocrine tissue located within the exocrine pancreas. Though structurally different between mice and humans, both species' islets fulfill a central role in glucose homeostasis by secreting hormones into the bloodstream.

1.2.3   Glucose Dependent Hormone Secretion

In humans, normal blood glucose is maintained at levels around or below 100 mg/dl or 5.5 mM (2,3). To properly maintain euglycemia, insulin secreted by beta cells and glucagon secreted by alpha cells play opposing roles in the response to changes in circulating glucose levels. When glycemia increases (> 5.5 mM), like in response to a meal, glucagon secretion decreases and insulin secretion increases to promote glucose uptake from the blood and decrease liver glucose output (2,31). Conversely, as glycemia decreases (< 5 mM), like in times of fasting, insulin secretion shuts off and glucagon secretion increases to promote glucose liberation from the liver into the blood, preventing tissue starvation (3,11,13) (Figure 3).

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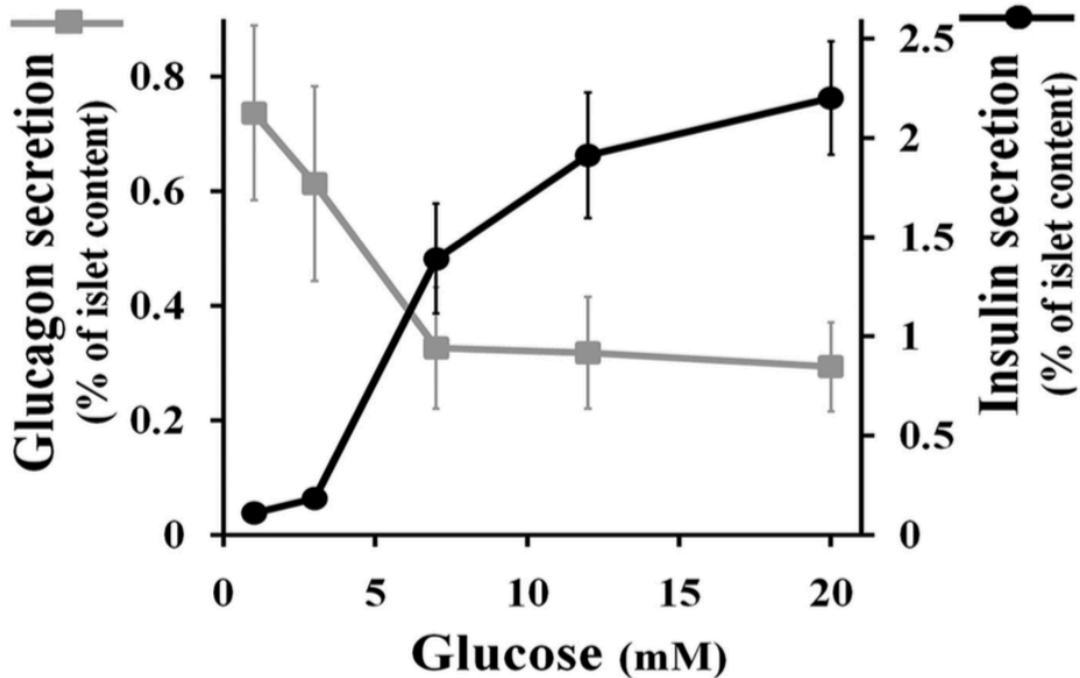
Figure 3: Hormone Secretion from Mouse Islets in Response to Glucose. Data demonstrate the *in vitro* secretion profiles of the islet hormones, glucagon and insulin, in response to glucose. Glucagon is maximally secreted at low glucose levels and inhibited at elevated glucose levels. Inversely, insulin is maximally inhibited at low glucose levels and potently secreted at elevated glucose levels. Reproduced with permission (64).

The islet's ability to respond to changes in blood glucose requires both intra- and intercellular signaling pathways (3,31,32). This includes intracellular calcium and cyclic adenosine mono-phosphate (cAMP) signaling, along with secreted paracrine factors and cell-to-cell contacts between islet cells (14,32–37). Since the majority of the islet cell types share a common cellular progenitor, their signaling pathways were assumed to be similar between the cell types (16,38–41). Research indicates that the mechanisms regulating glucose-dependent hormone secretion differ between beta and alpha cells (14,32,37). These differences are outlined and discussed in the follow sections.

1.2.4 Insulin and Glucose-Stimulated Insulin Secretion

Following the discovery of insulin by Banting and Best in 1922, nearly 100 years of research established a solid foundation of understanding in regard to insulin's role in the body and its glucose-regulated secretion. Insulin, a 51 amino acid 2-chain peptide, binds to its receptor to signal targeted tissues to uptake glucose (42). Insulin signaling results in the translocation of the glucose transporter, GLUT4, to the plasma membrane to transport circulating glucose into tissues like muscle and adipose tissue (3,12,31,42). Additionally, insulin inhibits the production and release of glucose from the liver and suppresses feeding cues within the hypothalamus in the brain (3,9,19). Altogether, these coordinated signaling cascades collaborate to decrease the levels of circulating glucose and maintain euglycemia.

Longstanding research in the beta cell field has resulted in a well-characterized consensus model of how beta cells regulate insulin secretion; a process collectively referred to as glucose-stimulated insulin secretion (GSIS) (12,31,43–50). In this model, circulating glucose is internalized by the glucose transporter GLUT2 and consequently undergoes glycolysis and the Krebs cycle (47,48). These metabolic steps result in an increase in the intracellular concentration of adenosine triphosphate (ATP) and a decrease in adenosine diphosphate (ADP) (47,48). The change in ATP/ADP ratio results in the closure of ATP-dependent potassium channels (K_{ATP}), which impedes the flow of some potassium ions out of the beta cell and depolarizes the membrane (44,51). This depolarization leads to an opening of voltage-gated calcium channels (VGCC) and an influx of extracellular calcium (46). Both extracellular and intracellular stores of calcium contribute to the increase in intracellular calcium that stimulates calcium-dependent exocytotic events (Figure 4) (50). Secondary pathways that modulate GSIS are described in Chapter 2.1.2.
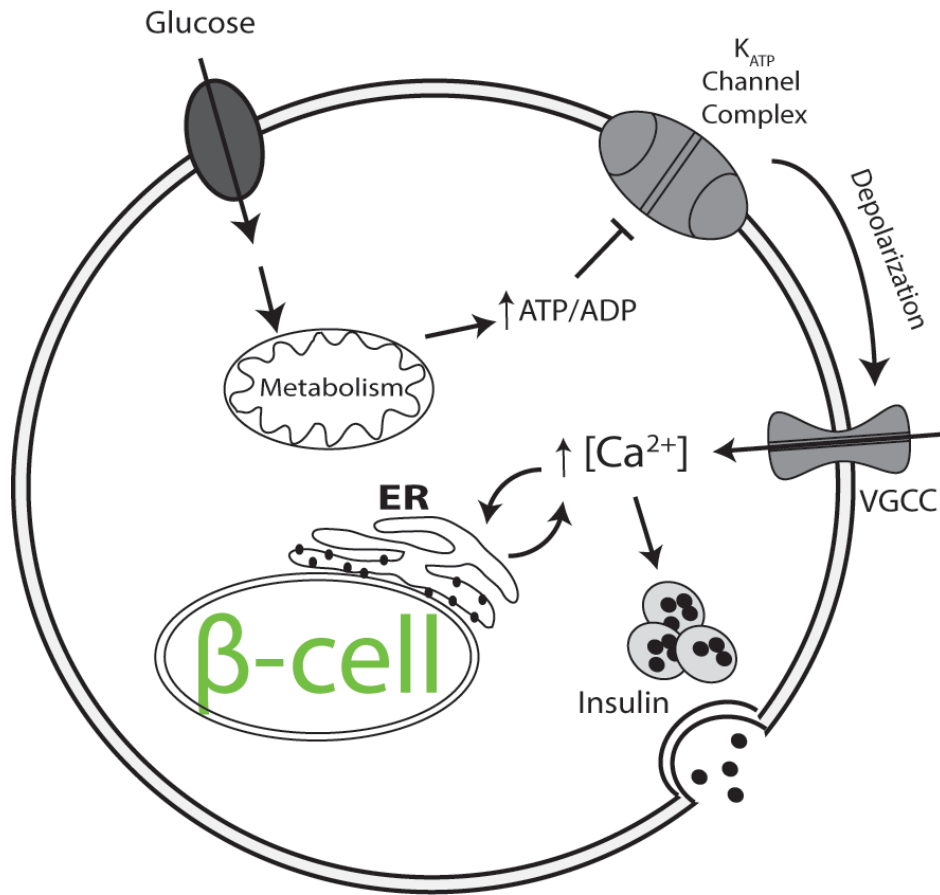
Figure 4: Glucose-Stimulated Insulin Secretion from Beta Cells. Briefly, the uptake and metabolism of glucose results in an increase in the intracellular ATP/ADP ratio. This leads to the closure of the K_{ATP} channel complex, which leads to depolarization of the membrane and opening of the voltage-gated calcium channel, followed by calcium influx. Cytoplasmic levels of calcium also depend upon the storage and release rates to and from the endoplasmic reticulum (ER). An increase in intracellular calcium drives calcium-dependent exocytosis.

The endoplasmic reticulum (ER) plays a key role in the regulation of insulin secretion by controlling the levels of cytosolic calcium through ion liberation under stimulatory conditions or ion sequestration during inhibitory periods (52,53). In beta cells, calcium-dependent factors like protein kinase RNA-like ER kinase (PERK) and the phosphatase Calcineurin directly contribute ER calcium regulation and insulin secretion through the modulation of calcium channels and pumps on the ER membrane (53). While the regulation of ER calcium it not completely understood, it is accepted that dysfunction in beta cell ER calcium levels or handling can result

in a variety of cellular abnormalities, including ER stress, insulin misfolding, and blunted calcium-dependent insulin secretion (52,53).

Calcium-dependent exocytosis broadly refers to a detailed mechanism in which an array of vesicle and membrane associated proteins interact in the presence of calcium to actively control the fusion of secretory granules to the plasma membrane. While many of the proteins involved in exocytosis are conserved between tissues, different isoforms of each protein can be preferentially expressed. For example, complexin-1 is enriched in beta cells (54), while islet cell transcriptome analysis suggests complexin-2 is enriched in alpha cells (55). The complexins play a critical role in calcium sensing in hormone exocytosis (56).

Exocytosis is followed by repolarization of the membrane, which results from the activation of voltage- and calcium-dependent rectifying potassium channels (44,46,51,57). This depolarization-repolarization cycle repeats, resulting in calcium pulses and oscillations within the beta cell (33,35). The periodic changes in intracellular calcium are of particular interest to islet biologists for two reasons. First, data indicate that insulin secretion is tightly coupled to calcium influx and pulses (31). Pulsatile insulin secretion is thought to be, in part, a protective mechanism against hepatic insulin resistance (58). Second, these oscillations become synchronized across all beta cells within the islet. The synchronization depends upon calcium permeable gap-junction coupling, which aids in the complete inhibition of insulin secretion at low glucose levels and enhanced insulin secretion at elevated glucose levels (33,59–61).

Chapter 2 will expand on the working model for GSIS and calcium dynamics in beta cells with the introduction and incorporation of novel data from experiments utilizing endogenous ligands that modulate insulin secretion.

1.2.5 Glucagon and Regulated Glucagon Secretion

Research on the regulation of glucagon secretion from alpha cells has been overshadowed by the insulin and beta cell field. Even though glucagon was identified in 1923 (18,62), glucagon's role in glucose homeostasis did not gain recognition until the 1970s (18). Glucagon, a 29 amino acid cleavage product of the larger pro-glucagon peptide, has the potent ability to increase glucose output from the liver and cause changes in heart, brain, and adipose tissue physiology (3,13,18). Glucagon exerts its effect on target tissues by binding to the glucagon receptor, a G-protein-coupled receptor, and stimulating the formation of the downstream second messenger cyclic adenosine mono-phosphate (cAMP) (See Ch. 2.1.2) (13,63). In healthy individuals, glucagon secretion from islet alpha cells decreases in response to elevated glucose. Paradoxically, alpha cells, when dispersed from the islet, increase their glucagon secretion under the same conditions (18,64–66). The mechanistic origin of this paradox is currently unknown, but recent publications suggest that multiple signaling pathways within alpha cells and between islet cells likely simultaneously regulate glucagon secretion (18,32,34,36,64,67–69).

The first theories to explain glucagon secretion utilized known mechanisms present in beta cells (e.g. GSIS), but discrepancies between beta and alpha cell hormone secretion became clear as glucagon research expanded. Specifically, this was observed in the opposing glucose-dependent hormone secretion profiles of insulin and glucagon (14,18,32,37,64,70) (Figure 3). The original model also failed to explain why, in an islet, alpha cells suppress glucagon secretion at high glucose, but increase secretion at high glucose in the dispersed or sorted cell state (64). Currently, data support several models to explain how alpha cells regulate glucagon secretion. These models fall into three classes: alpha cell intrinsic, paracrine signaling, and juxtacrine signaling models (Figure 5) (14,32,34–37,64,70).
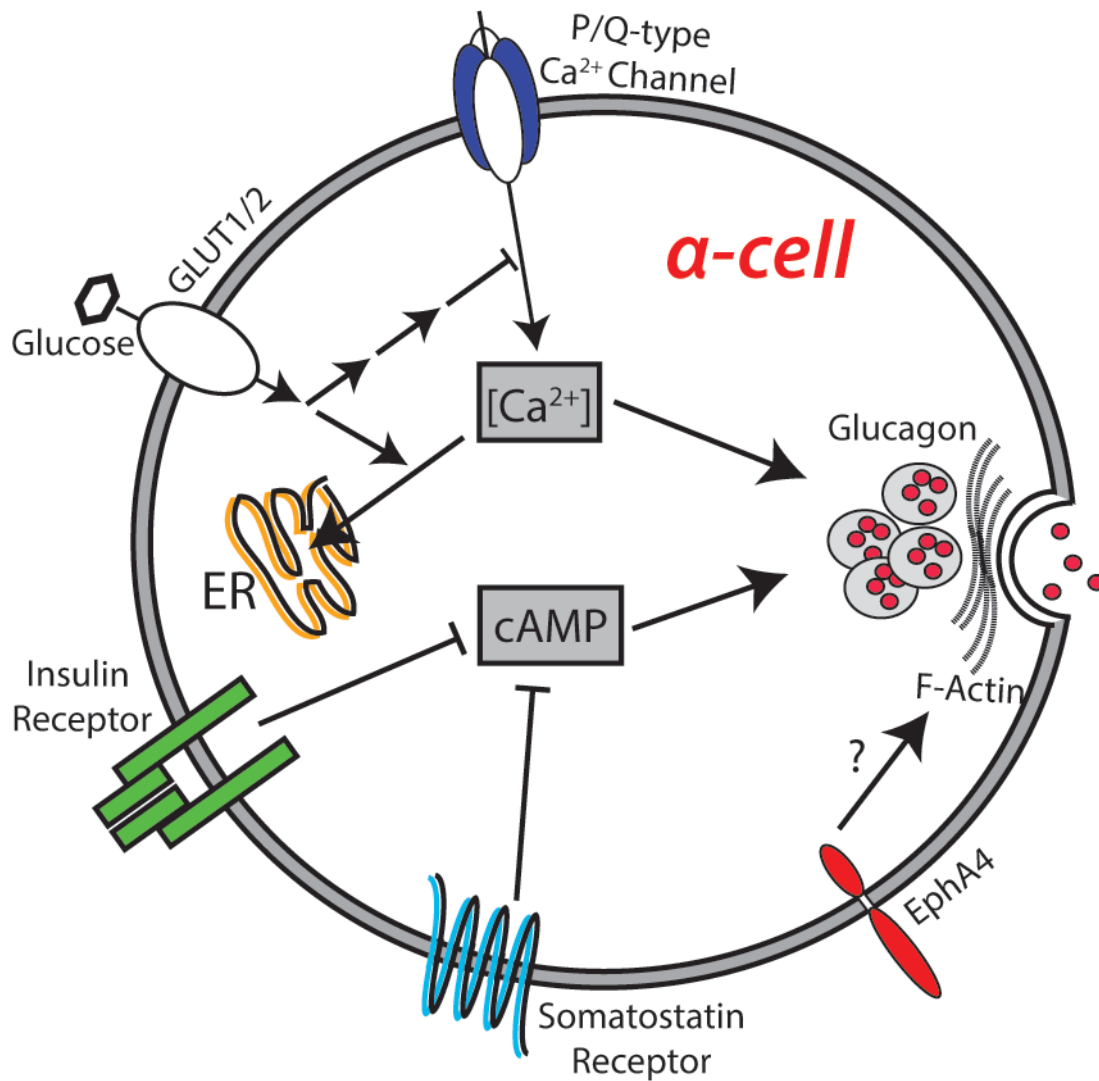
Figure 5: The Models for the Glucose Inhibition of Glucagon Secretion. Insulin and somatostatin receptor signaling, EphA4 juxtacrine signaling, and calcium signaling have each been demonstrated to be involved in the regulation of glucagon secretion. Changes in intracellular calcium, cAMP, and F-actin density have all been implicated as part of the glucose-regulated secretion mechanism.

In the alpha cell intrinsic model, alpha cells directly regulate their own glucagon

secretion through changes in intracellular metabolism and electrical signaling in response to

glucose (14,50,71). In the paracrine signaling model, glucose indirectly inhibits glucagon secretion through factors secreted by the other islet cell types. This includes insulin from beta cells and somatostatin from delta cells, both of which act on receptors present on the alpha cell (35,36,51,64,72). In the juxtacrine signaling model, specific cell-to-cell contacts impinge upon the receptors present on the alpha cell to regulate glucagon secretion. This occurs putatively through ligands on the neighboring beta cells (34). Individually, each of these models has critical deficiencies, yielding an incomplete model of how glucose inhibition of glucagon secretion is established in alpha cells.

In the following chapters, aspects of the models for both GSIS and glucose inhibition glucagon secretion will be discussed in greater detail. A variety of techniques will be utilized to examine the modulation of GSIS in beta cells (Ch. 2) and regulated glucagon secretion in alpha cells (Ch. 4). Together, we aim to build a better understanding of regulated hormone secretion from the islet.

1.3   Pathophysiology of Diabetes

1.3.1   Diabetes: Type 2 and Type 1

Diabetes is a term utilized to define a family of metabolic diseases that is characterized by the body's inability to properly regulate blood glucose, manifesting as fasting hyperglycemia or glucose intolerance in patients (73,74). The loss of glucose homeostasis is severely detrimental and leads to chronic tissue damage from hyperglycemia and the possibility of acute brain starvation and death from hypoglycemia (3,14,18,75). Diabetes is classified into several categories, with Type 2 being most prominent (> 90%) and Type 1 comprising a minor fraction of the overall diagnoses (> 5%) (4,74,76). Other rare forms of diabetes can be caused by

monogenic mutations (~ 1-5%), and share some disease characteristics with Type 2 and Type 1 Diabetes (77).

Type 2 Diabetes (T2D) can result from a variety of mechanisms, including insufficient insulin secretion, insufficient insulin action, beta cell failure/death, and peripheral insulin resistance (73–75). The pathophysiology of T2D has been directly attributed to cellular defects within the beta cell at critical regulatory junctures, including insulin expression, processing, trafficking, or secretion (78–80). Other defects have been linked to ER stress, replication, and mass expansion of beta cells in response to the increased insulin demand (81–83). Furthermore, non-islet tissues defects, including muscle and brain insulin resistance, are known to contribute to the pathophysiology of T2D (84–86).

Many of the defects that can lead to diabetes also range in severity and prevalence and are dependent on diet, age, race, and ethnicity (73,75,79,85,87). Despite underlying defects present in each afflicted individual, fasting hyperglycemia and glucose intolerance are the primary markers for disease progression. Exogenous insulin administration or supplementary medications are currently prescribed to aid in reestablishing and maintaining euglycemia (73). Two classes of prescribed drugs include the insulin action enhancing thiazolidinediones and insulin potentiating glucagon-like peptide-1 agonists (86,88). Exogenous insulin and these drugs provide several different modes of treatment to aid in the management of T2D.

Type 1 Diabetes (T1D) is characterized by autoimmune destruction of beta cells, which results in insulin-insufficiency (74,76). Interestingly, T1D, once referred to as juvenile diabetes, develops primarily in children and adolescents (76). While the underlying trigger for the autoimmune response is unclear, it has been shown that the activated immune cells specifically target antigens that are unique to the beta cell, efficiently destroying insulin-secreting cells

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(21,76,89–91). Without frequent insulin replacement therapy, T1D is fatal (76). With intensive

insulin treatments, insulin-dependent diabetics exhibit near-normal blood glucose and a

glycosylated hemoglobin level in the pre-diabetic range (92). Conversely, insulin-dependent

diabetics not on an intensive insulin regime exhibit chronic hyperglycemia and significantly

elevated glycosylated hemoglobin levels (92). The chronic hyperglycemia in these patients

greatly increased the incidence of hypertension, retinopathy, and other complications (93). In

T1D, several genetic components have been identified that correlate with disease prevalence and

progression (76). Genetically susceptible patients, typically close relatives to known T1

diabetics, are now screened and monitored in the clinic to better define T1D progression (94,95).

While there is no preventive treatment, disease progression data will aid in the future

development of intervention therapies for T1D (90,95).

1.3.2   Changes in Glucagon Secretion in Diabetes

In both T1D and T2D, the loss of insulin or its action play a central role in disease

pathophysiology. Insulin replacement therapies have aided in the correction of hyperglycemia,

however the dynamics of insulin dosing are imperfect and do not fully protect a patient against

long term complications (74,75). Recent work has demonstrated that glucagon secretion also

becomes misregulated and hypersecreted in the diabetic state (Figure 6) (13,18,66,67).

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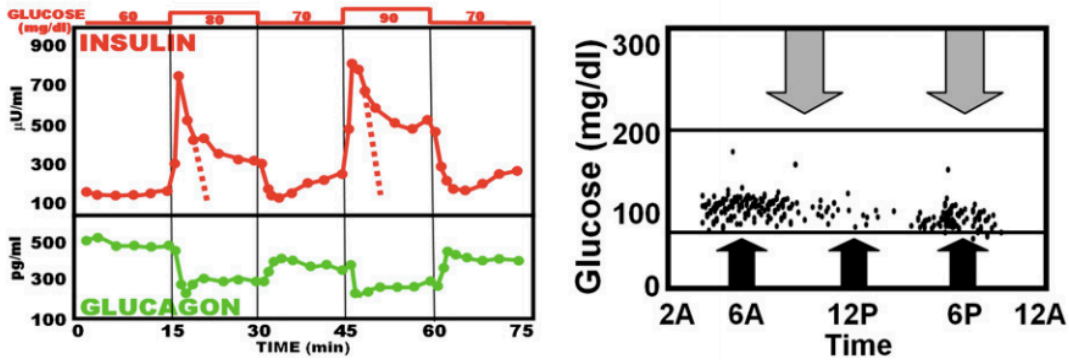
Healthy Adults
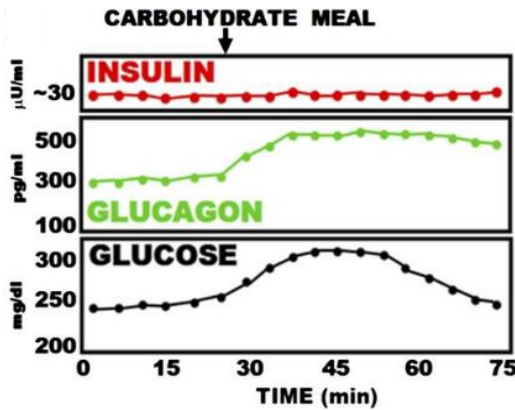
Type 1 Diabetes   **Type 2 Diabetes**
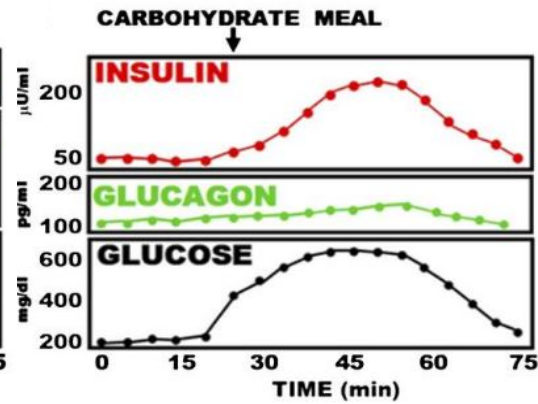
Figure 6: Insulin, Glucagon, and Glucose in Diabetes. Representative traces of insulin, glucagon, and glucose in healthy adults (Top) and T1D/T2D patients (bottom). In healthy adults, insulin and glucagon have opposing secretion profiles, which maintains euglycemia. In T1D, no insulin is secreted and increasing levels of glucagon are secreted in response to a meal. In T2D, insulin is secreted in response to a meal, but glucagon release is not fully suppressed. In both types of diabetes, prolonged hyperglycemia persists. Adapted with permission from (66).

Hyperglucagonemia in some diabetic patients complicates treatment, since glucagon in the disease state continues to signal to the liver to release more glucose, further exacerbating hyperglycemia. Additionally, the dysfunction of alpha cells also increases hypoglycemia susceptibility since the alpha cells fail to increase glucagon output at low glucose levels (18,64,74). These observations stress the importance of islet research, specifically on alpha cell

glucagon secretion regulation. Therapies targeting glucagon secretion or action may provide an alternative avenue, independent of insulin, to ameliorate hyperglycemia seen in both Type 1 and 2 Diabetes.

1.3.3   Modulating Hormone Secretion

The secretion of islet hormones primarily depends on glucose as a master regulator. However, additional layers of regulation exist to fine tune insulin and glucagon secretion. Numerous signals, originating from both outside and within islets, stimulate intracellular signaling cascades that can either dampen or enhance hormone secretion. For example, the hormone glucagon-like peptide 1 (GLP-1), secreted by L-cells in the gut, potentiates insulin secretion in response to a carbohydrate meal (96). Somatostatin, secreted from delta cells within islets, inhibits both insulin and glucagon secretion (36,72,97). These modulators, along with countless others, are being studied extensively to understand their roles in islet physiology. While some modulators have demonstrated clinical efficacy, others need additional investigation before they are utilized as therapies in the treatment of diseases like T1D and T2D (86,96,98,99).

In Chapter 2, two stimulating factors, the peptide hormones GLP-1 and kisspeptin-10, will be introduced (Ch. 2.1.3) and further characterized (Ch. 2.3) due to their ability to potentiate insulin secretion from beta cells. In Chapter 4, several inhibitory modulators, including insulin and somatostatin, will be discussed and utilized to improve our understanding of glucose inhibition of glucagon secretion from alpha cells.

Chapter 2

GPCR Modulation of Insulin Secretion from Beta Cells

2.1 Introduction

2.1.1 Modulating Insulin Release

The consensus model of GSIS acts as a backbone for studies that focus on secondary modulators that regulate insulin secretion from beta cells. Within this model, several specific targets can be modulated in order to increase insulin release as a possible therapy for T2D. For example, a commonly prescribed class of drugs, sulfonylureas, specifically targets and closes the K_{ATP} channel to increase calcium influx and insulin secretion (98). While sulfonylureas alter the electrical properties of the beta cell, other modulators, like GLP-1, target secondary pathways that feed into secretion.

Though calcium influx acts as the primary signal and trigger for insulin exocytosis, a secondary pathway is known to fine tune insulin secretion. This pathway utilizes G protein-coupled receptors (GPCRs), the second messenger cAMP, protein kinase A (PKA), and exchange protein directly activated by cAMP (EPAC) (Figure 7). Specific components of this pathway are described in detail in the following section (Chapter 2.1.2).
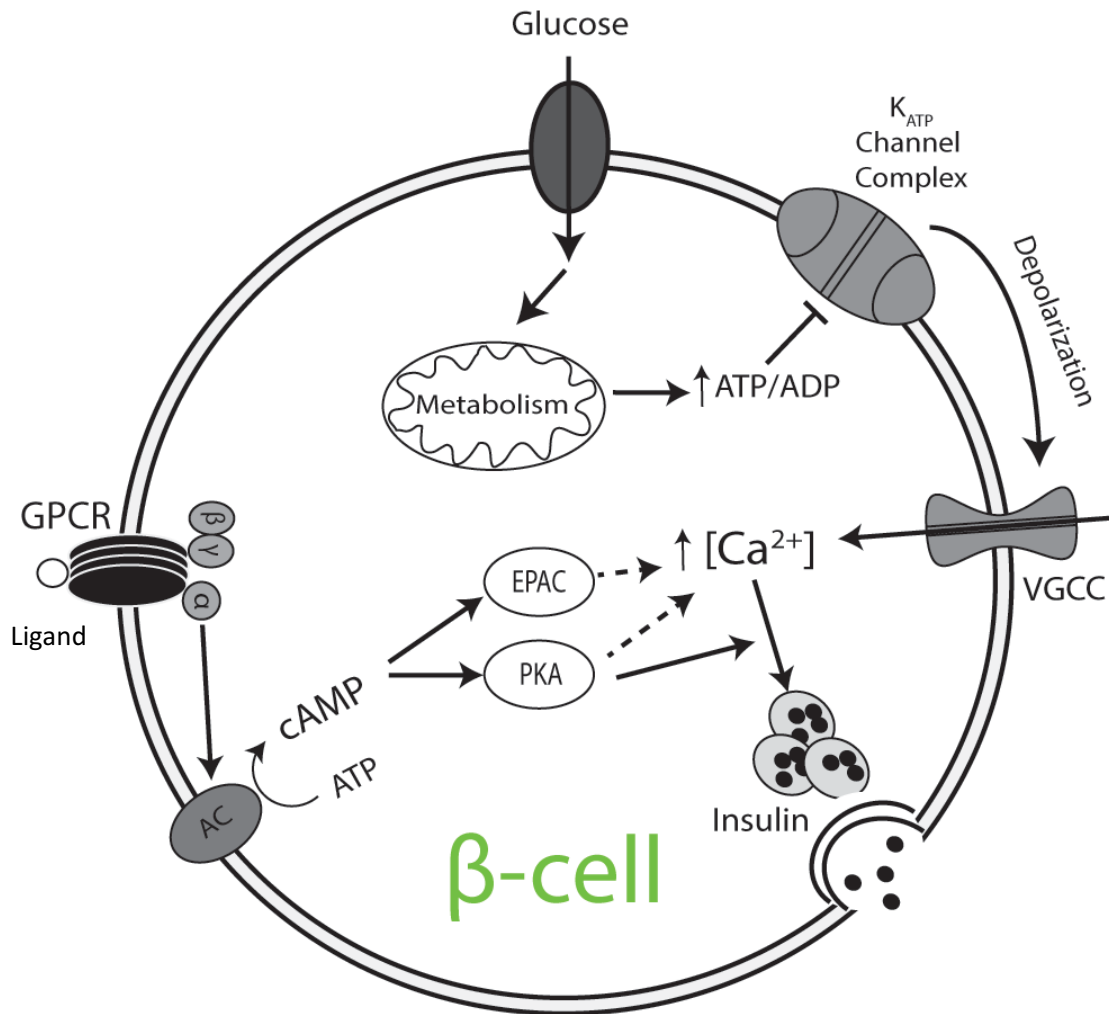
Figure 7: A Model for cAMP Regulation of Insulin Secretion. Ligand binding to the extracellular portion of a GPCR results in the activation of intracellular G proteins. Here, $G_{\alpha s}$ stimulates adenylyl cyclase (AC) to increase the production of cAMP, which in turn, activates exchange proteins directly activated by cAMP (EPAC) and protein kinase A (PKA). EPAC and PKA both contribute to the amplification of calcium-dependent insulin secretion.

Interestingly, the cAMP signaling pathway was first observed in beta cells when the addition of exogenous glucagon stimulated insulin release (100). The specifics of this signaling pathway were determined through several seminal studies that characterized cAMP signaling in muscle and adipose tissue, along with the liver in response to the addition of glucagon (63,101–103). cAMP signaling has been characterized to have diverse effects in different tissues. For

example, this pathway has been demonstrated to play a critical role in amplifying insulin release from beta cells (31,96). Specifically, the levels of cAMP positively correlate with the levels of insulin secreted during GSIS (47,104). Based on this observation, researchers have worked tirelessly to find novel ways to modulate cAMP signaling and further promote insulin secretion.

2.1.2   G Protein-Coupled Receptor Signaling

Eukaryotic cells interact with their extracellular environment by expressing various cell-surface receptors that can be bound by external ligands (1). One class of these receptors is the GPCR, the largest receptor protein family, which is classified by its seven transmembrane structure (1,105). The binding of ligands, including hormones and neurotransmitters, to the extracellular portion of the receptor initiates various signaling cascades via a conformational change in the intracellular portion of the receptor. This in turn, causes GDP-GTP exchange in trimeric GTP-binding G protein subunits bound to the intracellular portion of the receptor (1,105,106). The activation of the G proteins can elicit a variety of downstream effects depending upon which G proteins are coupled to the receptor.

One role of GPCRs is to increase or decrease the levels of intracellular cAMP by utilizing one of two G protein subunits, $G_{\alpha s}$ (stimulatory) or $G_{\alpha i}$ (inhibitory). $G_{\alpha s}$ activates while $G_{\alpha i}$ inhibits the activity of adenylyl cyclase, an enzyme that catalyzes the conversion of ATP to cAMP. The newly synthesized cAMP in turn binds to and activates cAMP-dependent protein kinase A (PKA), which has been demonstrated to phosphorylate targets that are centrally important to beta cell electrical properties (e.g. K_{ATP} channel), calcium-dependent exocytosis (e.g. SNAP25), and cytoskeletal arrangement (e.g. RhoA) (107–113). Like PKA, exchange protein directly activated by cAMP (EPAC) also has a regulatory role in GSIS. Even though

EPAC has a lower affinity for cAMP than PKA, EPAC still has a significant role in the regulation of islet cell cAMP-dependent calcium flux by modulating ER calcium stores (114). Together, the activation of PKA and EPAC potentiate calcium-dependent insulin secretion.

A second role of GPCRs is to modulate the phospholipase-C (PLC) pathway. This occurs through G protein subunit $G_{\alpha q}$ (105,108), which activates PLC and leads to the formation of diacylglycerol (DAG) and the consequent activation of protein kinase C (PKC) (115). Like PKA, PKC has been demonstrated to phosphorylate beta cell-specific targets involved in electrical properties, ER calcium regulation, and calcium-dependent exocytosis (108,115).

Upon ligand binding and G_{α} activation, the $G_{\beta\gamma}$ subunit complex dissociates and can have additional activity. One role for $G_{\beta\gamma}$ is to activate G protein-coupled inward-rectifying potassium (GIRK) channels, which has a profound effect on membrane electrical properties and calcium flux (116).

Since the extracellular portion and ligand binding site can be a drug target and the downstream effects are specific to the receptor, GPCRs are of particularly significance. Many GPCRs and their endogenous ligands have been investigated for their potential to enhance insulin secretion from beta cells by stimulating the formation of cAMP or altering membrane potential (96,99,116).

2.1.3   GPCR Regulation of Insulin Secretion

Beta cells express numerous GPCRs on their plasma membrane (117). One well-characterized GPCR on the beta cell is the glucagon-like peptide-1 receptor (GLP-1R). Secreted by L-cells in the gut, GLP-1, another cleavage product of the pro-glucagon peptide, exerts what is called the incretin effect on beta cell. This effect potentiates insulin secretion through GLP-1

binding to the GLP-1R, a $G_{\alpha s}$ coupled receptor. This binding results in the stimulation of adenylyl cyclase and the formation of cAMP (96,118,119). In turn, the increase in cAMP and activation of PKA potentiates insulin secretion in conjunction with GSIS. While GLP-1 exerts the majority of its effects through the $G_{\alpha s}$ subunit *in vivo*, GLP-1 has also been demonstrated to signaling though $G_{\beta\gamma}$ to alter calcium handling *in vitro* to further potentiate insulin secretion (118,120). Less is known about the effects of GLP-1 on beta cell calcium handling in intact islets or about how GLP-1 might affect the other islet cell types within the islet. Recent research has demonstrated that GLP-1's effects on non-beta cells may also be critical to glucose tolerance and homeostasis (121).

A second receptor postulated as important to beta cell regulation is the kisspeptin (KP) receptor, GPR-54 (122,123). Kisspeptin-10 is a cleavage product from the KISS1 pro-peptide that is released by KP-expressing neurons found in various locations like the hypothalamus, pituitary, and kidneys (124,125). KP contributes to the regulation of reproductive hormones and renal function, and is postulated to be an insulin secretogogue *in vivo* (122,125). GPR-54 has been proposed to signal through the $G_{\alpha q}$ subunit to potentiate insulin secretion (126), but insulin suppressing effects of GPR-54 signaling have also been shown in beta cells (127,128). Additional investigation is required to fully understand the role of KP on insulin secretion.

While the role of the $G_{\alpha s}$ subunit in GLP-1 signaling in beta cells is largely understood, additional experiments are necessary to determine if the $G_{\beta\gamma}$ subunits are involved in GLP-1R signaling. Furthermore, the role of KP and GPR-54 signaling in regulating insulin secretion from beta cells is unclear. For both GPCRs, complete and detailed signaling mechanisms are absent from the literature. To better understand GLP-1R and GPR-54 signaling in the islet, we investigated the effect of GLP-1 and KP on various downstream signaling cascades, including

calcium signaling and hormone secretion, in the presence of specific GPCR-modulating drugs (129).

2.2   Methods

2.2.1   Animal Experiments, Islet Isolation and Culture

All animal studies were completed under approval by the Vanderbilt Institutional Animal Care. Male mice, ages 8 – 16 weeks on the C57BL/6J background, were used. Islets were isolated using a 0.075% collagenase digestion at 34°C and allowed to recover overnight in mouse islet media (RPMI 1640 with 10% FBS, penicillin-streptomycin, and 11 mM glucose) prior to experimentation.

2.2.2   Hormone Secretion Assay and ELISA

Islets were equilibrated in KRBH buffer (128.8 mM NaCl, 4.8 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$ 7H20, 2.5 mM $CaCl_2$, 20 mM HEPES, 5 mM $NaHCO_3$ and 0.1% BSA; pH 7.4) at 2.8 mM glucose for 1 hour at 37°C, then transitioned to fresh 1 mM, 2.8 mM, 10 mM, 11mM or 16.7 mM glucose for 1 hour at 37°C with or without the following conditions: 20 nM GLP-1 (7-36), 1 μM kisspeptin-10, 10 μM gallein, 30 μM mSIRK and 200 nM CYN154806. The supernatant was collected, then the cells were lysed for hormone content using 1.5% 12 N HCl in 70% ethanol. Secretion and content samples were measured by ELISA (Insulin – Alpco; RayBioTech – Glucagon). Secretion data are presented as percent of total hormone content to control for differences in islet size.

2.2.3   Live-cell Calcium Imaging

Islets were equilibrated in KRBH at 2.8 mM glucose with 5 µM of the cell permeant calcium indicator Fluo-4 AM (Invitrogen) for 30 minutes, then transitioned to 10 mM glucose at 37^0C. Samples were allowed to equilibrate in a CO_2-controlled 37^0C stage for 10 minutes prior to imaging. Fluo-4 signal was measured using an LSM (Carl Zeiss) confocal microscope with a 488 nm laser. Fluo-4 intensity, an indicator for free intracellular calcium, was measured in response to GPCR ligands or modulators and changes in intensity over baseline were analyzed for oscillation frequency using MatLab-based computational analysis, as previously described (97,130). To control for unevenness of indicator labeling among cells, the fluorescent signal was normalized to the average basal intensity for each cell. Data were plotted as normalized frequency for each islet.

2.2.4   Data Analysis and Statistical Testing

Data were analyzed with Microsoft Excel, ImageJ, MatLab, or GraphPad Prism software. For all imaging data, the background signal was subtracted prior to image analysis. Student's *t*-tests and ANOVAs were used where applicable and Welch's correction was used in cases where variance was different between groups. $p < 0.05$ was considered statistically significant unless otherwise noted.

2.3   Results

2.3.1   Effects of GLP-1 and KP on Insulin Secretion

While GLP-1 has an agreed upon role in potentiating insulin secretion, the role of KP in regulating insulin secretion is unclear (126,128). We first established an insulin secretion profile for both hormones at increasing glucose concentrations in primary mouse islets (Figure 8). Both KP and GLP-1 potentiated insulin secretion at glucose concentrations at which insulin is normally secreted (>5.5 mM). KP did stimulate a small increase in insulin secretion at 2.8 mM glucose, however further investigation is required to determine if this change is physiologically relevant.
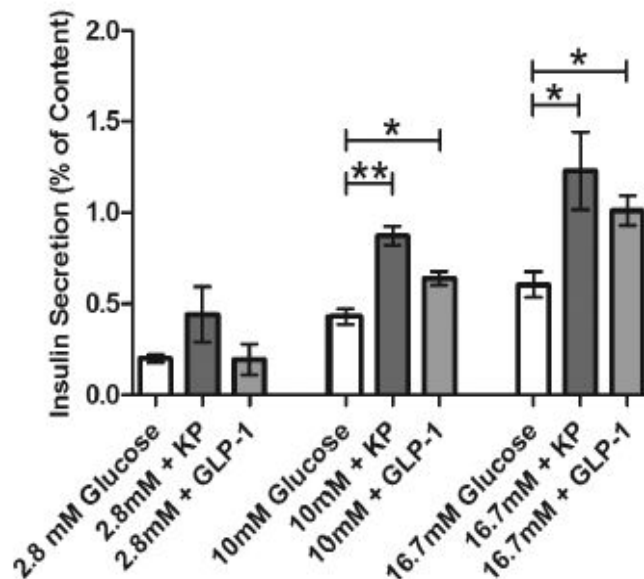
Figure 8: Insulin Secretion in Response to Glucose, KP, and GLP-1. The treatment with 1 μM KP or 20 nM GLP-1 failed to stimulate insulin secretion at 2.8 mM glucose. The addition of either KP or GLP-1 at 10 mM and 16.7 mM glucose further stimulated insulin secretion compared to glucose alone. Data are the mean ± S.E. n = 4-19. *(p<0.05) and **(p<0.001) indicate significance compared to untreated control. Adapted with permission from (129).

2.3.2 Effects of GLP-1 and GPCR Modulators on Calcium Dynamics and Insulin Secretion

The incretin effect of GLP-1 has been demonstrated to signal primarily through the $G_{\alpha s}$ subunit to stimulate the formation of cAMP and activate PKA (96,118–120). Others have proposed that the $G_{\beta\gamma}$ subunits are also involved and stimulate an increase in intracellular calcium (118,120). However, these studies utilized *in vitro* tissue culture models. To investigate the role of GLP-1 $G_{\beta\gamma}$ signaling in intact primary mouse beta cells, islets loaded with Fluo-4 were treated with GLP-1 and the GPCR modulators gallein or mSIRK and changes in Fluo-4 intensity were recorded (Figure 9A). Gallein is a cell-permeable $G_{\beta\gamma}$ inhibitor and mSIRK is a $G_{\beta\gamma}$ activator that acts independently of the G_α subunit. GLP-1 treatment alone resulted in no change in beta cell oscillation frequency within the intact islet compared to glucose alone. The combination of GLP-1 with gallein or mSIRK also had no additional effect (Figure 9A).

Even though there was no measureable difference in calcium oscillation frequency using GLP-1 with Gallein or mSIRK, insulin secretion should be increased in response to GLP-1. To verify this, insulin secretion was measured after GLP-1 treatment in combination with gallein or mSIRK (Figure 9B). The addition of GLP-1 at 10 mM and 16.7 mM glucose had a potent insulin potentiating effect. While gallein alone has a potent inhibitory effect on insulin secretion at 16.7 mM glucose, GLP-1 was able to overcome this inhibition. Treatment with mSIRK potently stimulated insulin secretion at 2.8 mM glucose and the co-treatment of GLP-1 with mSIRK had no additional stimulatory effect compared mSIRK alone at 10 and 16.7 mM glucose.
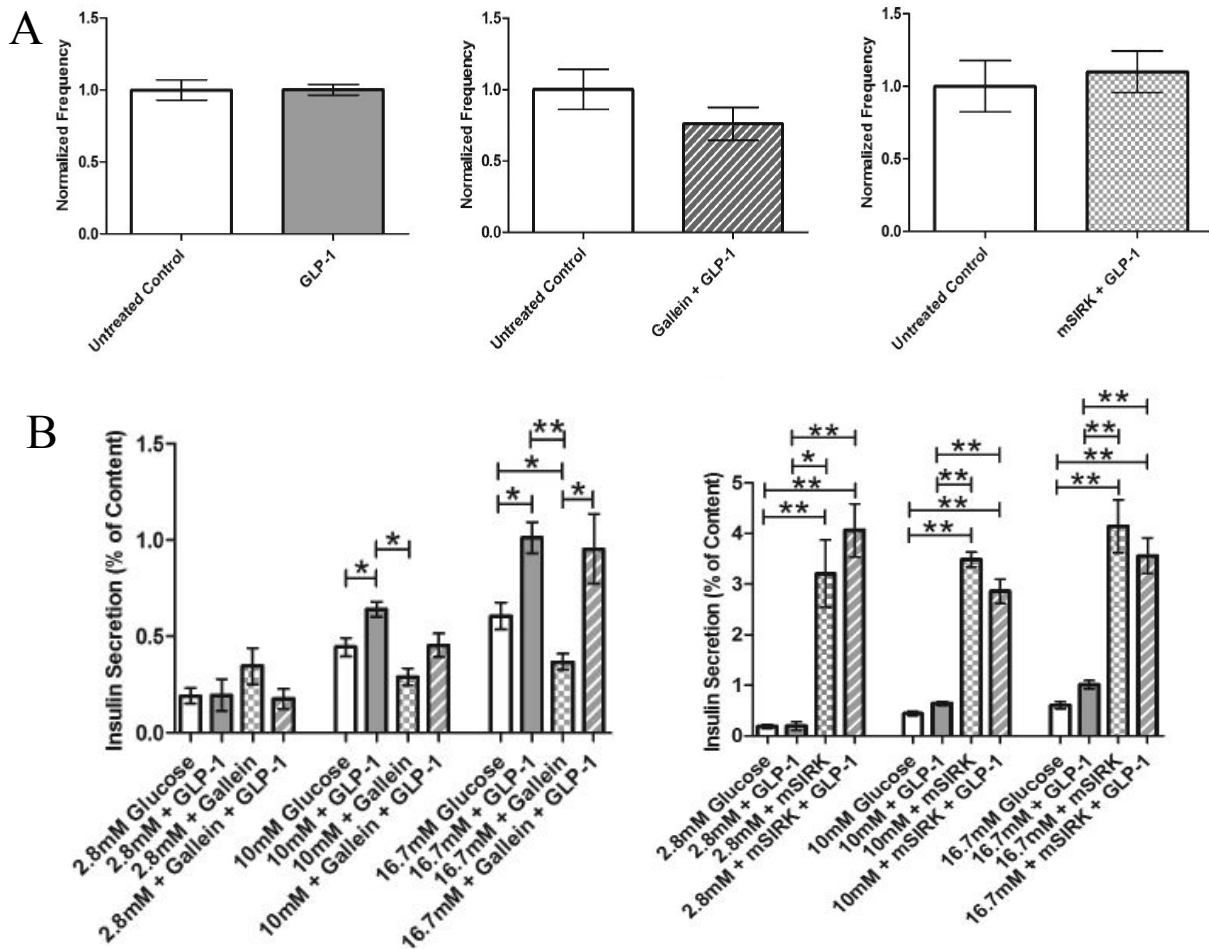
Figure 9: Calcium Dynamics and Insulin Secretion in Response to GLP-1 with Gallein and mSIRK. A) The normalized frequency of calcium dynamics in islet beta cells in response to 20 nM GLP-1 with or without 10 µM Gallein or 30 µM mSIRK at 10 mM glucose. The addition of GLP-1 in combination with either drug failed to alter the frequency of beta cell calcium oscillations. Data are the mean ± S.E. n = 4-5. B) Insulin secretion from intact islets in response to GLP-1 with or without gallein or mSIRK. The stimulatory effect of GLP-1 was blocked by the addition of gallein at 10mM, but not at 16.7 mM. The addition of mSIRK with GLP-1 did not increase insulin secretion over GLP-1 alone. Data are the mean ± S.E. n = 4-19. *(p<0.05) and **(p<0.001) indicate significance compared to untreated or singly treated control. Adapted with permission from (129).

2.3.3 Effects of KP and GPCR Modulators on Calcium Dynamics and Insulin Secretion

The insulin stimulation by KP may signal through either $G_{\alpha q}$ or $G_{\beta \gamma}$ signaling pathways (122). To investigate the role of KP on beta cell calcium activity, mouse islets loaded with Fluo-4 were treated with KP and the GPCR modulators gallein or mSIRK and changes in Fluo-4 intensity were recorded. Like GLP-1, KP had no effect on the frequency of beta cell calcium oscillations. The combination of KP with gallein or mSIRK also had no additional effect (Figure 10A). Concurrently, insulin secretion was measured in response to KP in combination with gallein or mSIRK at 2.8, 10, and 16.7 mM glucose (Figure 10B). The addition of KP failed to overcome the inhibitory effect of gallein at 10 and 16.7 mM glucose. The co-treatment of KP with mSIRK had no additional stimulatory effect compared mSIRK alone at these glucose concentrations.
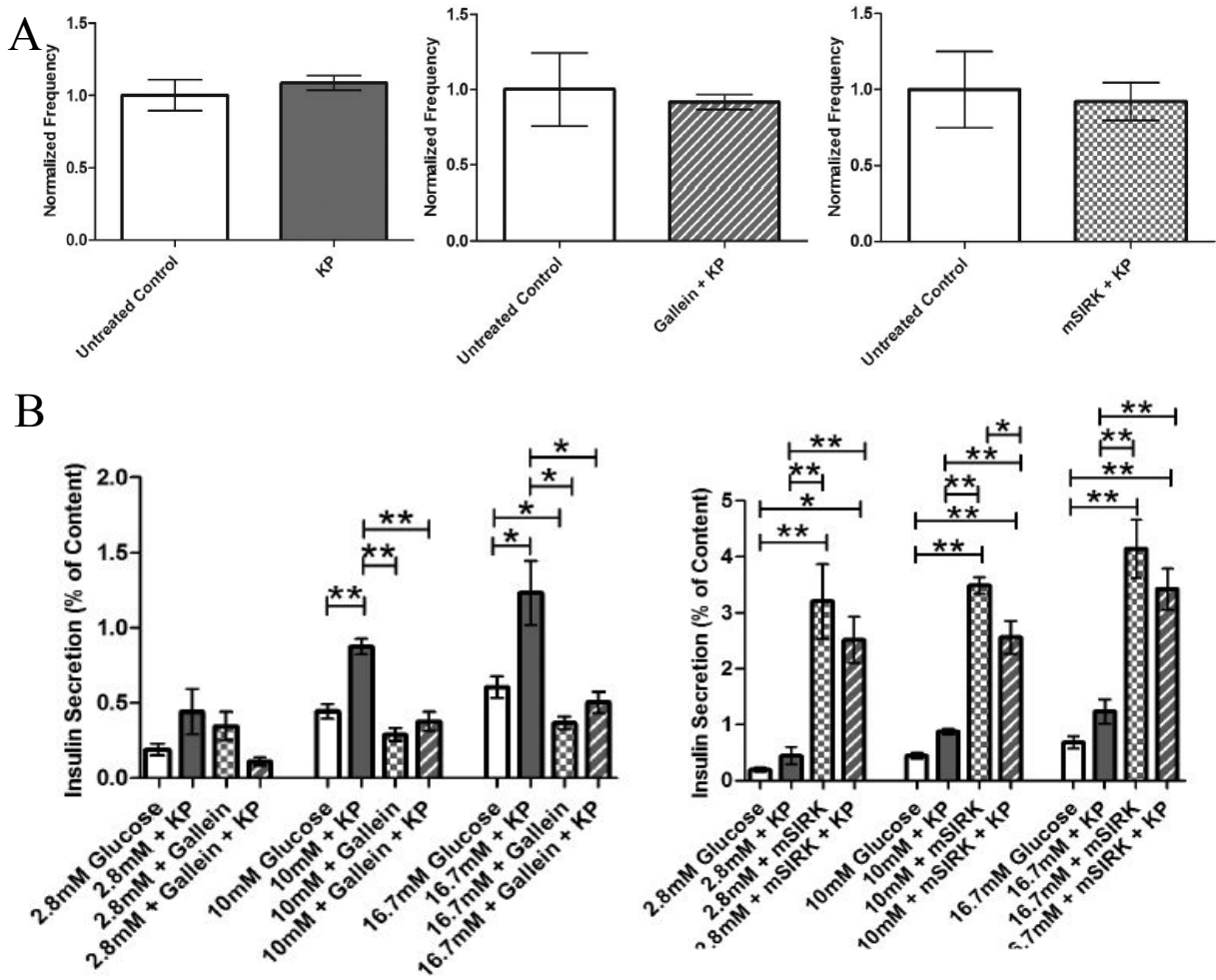
Figure 10: Calcium Dynamics and Insulin Secretion in Response to KP with Gallein and mSIRK. A) Calcium dynamics in islet beta cells in response to 1 µM KP with or without 10 µM Gallein or 30 µM mSIRK. The addition of KP in combination with either drug failed to alter the frequency of beta cell calcium oscillations. Data are the mean ± S.E. n = 4-5. B) Insulin secretion from intact islets in response to KP with or without Gallein or mSIRK. The stimulatory effect of KP was blocked by the addition of Gallein at 10 mM and 16.7 mM. The addition of mSIRK with KP did not increase insulin secretion over KP alone. Data are the mean ± S.E. n = 4-19. *(p<0.05) and **(p<0.001) indicate significance compared to untreated or singly treated control. Adapted with permission from (129).

2.3.4 Effects of GLP-1 and KP on Dispersed Beta Cell Calcium Dynamics

Our data show that neither GLP-1 nor KP alter intact islet calcium oscillation frequency, which contradicts previous publications (120,128). Based on the beta cell models utilized in each study, we hypothesized that these inconsistences stemmed from differences between the intact primary islet and the dispersed or tissue culture beta cell state. To reconcile these differences, the Fluo-4 calcium activity assay described above was completed using dispersed beta cells instead of intact islets. GLP-1, but not KP, increased the oscillation frequency of dispersed beta cells (Figure 11). These data more closely parallel previously published data (120,128).
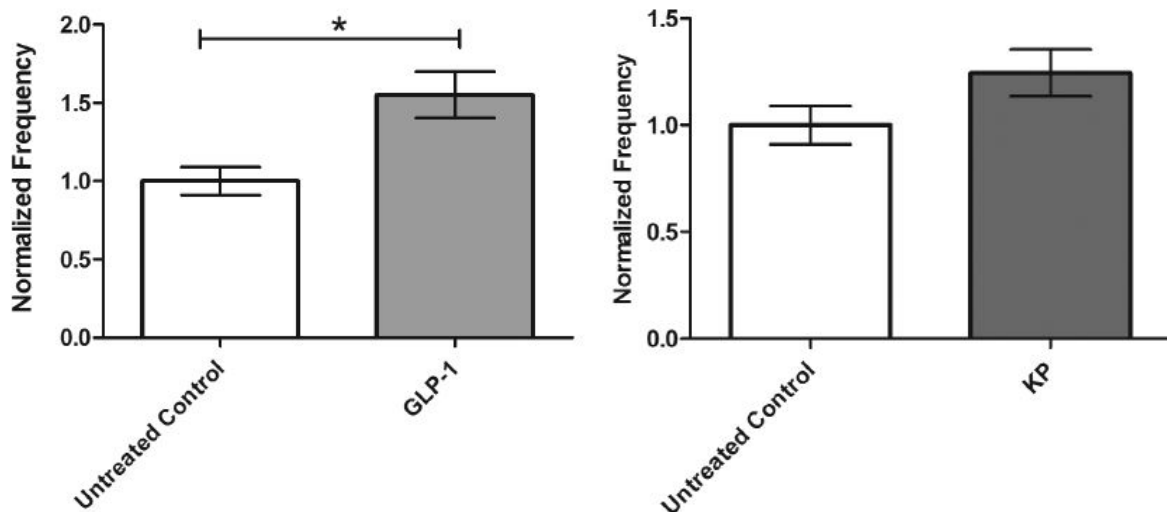
Figure 11: Calcium Dynamics in Dispersed Beta Cells in Response to GLP-1 and KP. The addition of 20 nM GLP-1, but not 1 μM KP, increases the frequency of calcium oscillations in beta cells. *($p<0.05$) indicate significance compared to untreated control. Adapted with permission from (129).

2.3.5 Effects of GLP-1 on Glucagon Secretion

GLP-1 has been well-characterized for its insulin potentiating effects *in vivo* and *in vitro*, which were quickly recognized and utilized as a novel T2D therapy. Treatment with GLP-1, while stimulating insulin secretion, also results in a marked decrease in glucagon secretion *in vivo* and *in vitro*. These observations strongly argued for the use of GLP-1 as an insulin secretogogue, as well as an inhibitor of glucagon secretion. GLP-1 analog treatment in T2D potentiates insulin secretion and lowers blood glucose, however, mixed results were observed on glycemia in T1D patients who did not have endogenous beta cell function (88,131). While GLP-1 did decrease fasting glucose levels in T1D patients, GLP-1 failed to suppress glucagon levels (88,131). Based on these data, we sought to determine if GLP-1 directly modulated hormone secretion from alpha and delta cells. Since GLP-1 failed to suppress glucagon when beta cells were absent in T1D patients, we hypothesized that GLP-1 indirectly lowers glucagon through the direct stimulation of insulin and somatostatin secretion. To test this hypothesis, we treated isolated mouse islets with GLP-1, with and without a somatostatin receptor antagonist (200 nm CYN154806) at 1 mM glucose. Thus, we could minimize the indirect potentiation of insulin secretion by GLP-1 at elevated glucose levels (Figure 12). Treatment with GLP-1 at low glucose had a significant inhibitory effect on glucagon secretion. Interestingly, this effect is completely abolished when somatostatin signaling is blocked with an antagonist of the somatostatin receptor 2, the primary receptor on alpha cells (72). These data support our hypothesis that GLP-1 does not have a direct effect on alpha cells, but rather works indirectly through stimulating insulin from beta cells and somatostatin from delta cells as elevated glucose concentrations.

Glucagon Secretion From Intact Islets
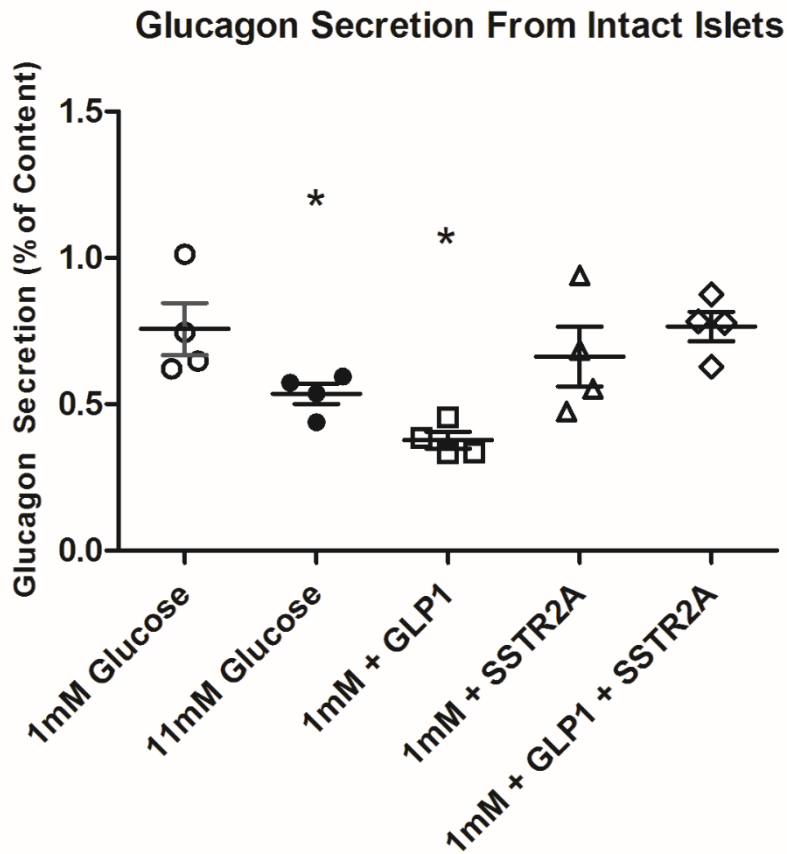
Figure 12: Glucagon Section from Intact Mouse Islets in Response to GLP-1 and Somatostatin Receptor Antagonism. High glucose and treatment with 20 nM GLP-1 both inhibit glucagon secretion. The inhibitory effect of GLP-1 is blocked by the addition of a SSTR2A antagonist (200 nM CYN154806), which has no effect alone. *($p < 0.05$) indicate significance compared to 1 mM glucose.

2.4   Discussion of GPCR Modulation in Islet and Dispersed Beta Cells

2.4.1   GLP-1 Signaling

In beta cells, insulin secretion is regulated by changes in intracellular calcium, and further modulated by changes in cAMP. GLP-1 is able to potentiate insulin secretion independent of measurable changes in calcium oscillation frequency in intact islets. While GLP-1 may have minor, secondary signaling through the $G_{\beta\gamma}$ subunits of the GLP-1R, the primary signaling appears to occur though the $G_{\alpha s}$ subunit and changes in cAMP (120). While it is clear that

31

mSIRK stimulates insulin secretion like GLP-1, it is unclear how $G_{\beta\gamma}$ activation enhances secretion independent of changes in calcium dynamics. The observation that GLP-1 alters calcium handling and oscillation frequency in dispersed beta cells suggests gap-junction coupling might mask some of the $G_{\beta\gamma}$-mediated effects within the islet. It is unclear if the $G_{\beta\gamma}$ mechanism is relevant *in vivo* to an islet that is normally electrically coupled.

From a holistic perspective, the role of GLP-1 on the islet requires more research to fully understand the direct and indirect effects on all islet and non-islet cell types (121). While GLP-1-based treatments have shown clinical efficacy by potentiating insulin secretion (132), a more complete understanding of the mechanism of action in the other islet cell types may lead to novel treatment avenues for diabetics independent of beta cells and insulin.

2.4.2   KP Signaling

It is becoming clear that KP has stimulatory effects on insulin from islet beta cells (122,129). Our data suggest that KP signals through the G_{α} subunit. This is supported by the observations that modulation of $G_{\beta\gamma}$ signaling with gallein and mSIRK in combination with KP fail to change either insulin secretion or calcium dynamics. Current data suggest that KP may signal through a $G_{\alpha q}$ mechanism, which signals through PLC, DAG, and PKC (126). Additional studies are required to understand this signaling pathway in beta cells and how this pathway modulates insulin secretion independent of changes in cytoplasmic calcium. Since KP has been characterized to be critical in other organ systems, it is unclear if KP treatment *in vivo* could be a viable therapeutic option due to the possible off-target effects on other tissues (125). For example, administration of KP could stimulate the release of aberrant reproductive hormones

from the pituitary (124). Despite this complication, KP may still be used to interrogate the role of $G_{\alpha q}$ signaling in the regulation of insulin secretion in isolated islets.

2.4.3   Islet versus Single Cell Measurements

The stark difference between islet and dispersed beta cell calcium dynamics highlight the importance of the intact islet environment when studying hormone secretion and intracellular dynamics. Electrical coupling, paracrine signals, and cell-to-cell contacts are all required for normal cell function and regulated hormone secretion (133–135). When any of these islet functions are lost, glucose regulated hormone secretion can be lost. While it is clear that beta cells become dysfunctional in the dispersed state, less is known about the functional changes between the intact islet and dispersed state for other islet cell types.

In the following chapter, we will introduce a unique model that allows islet researchers to manipulate the islet into new arrangements and sizes, while still leveraging the islet state and overcoming dispersed islet cell dysfunction. We will use this model to improve our understanding of alpha cell physiology and the regulation of glucagon secretion.

Chapter 3

Pseudo-Islets

3.1 Introduction

3.1.1   Pseudo-Islets and Current Applications

The islet can be dispersed or dissociated into single cells by chemical or physical means (136). If these islet cells are kept in culture following dissociation, they will spontaneously reaggregate to form new structures, most commonly referred to as pseudo-islets (136–138). While dispersed islet cells have dysfunctional hormone secretion in response to glucose, the pseudo-islet recapitulates properties of the intact islet, including GSIS and transcriptional regulation (137,139–143). This phenomenon of reformation appears to be consistent across mammalian species (139,144,145). Publications suggest that functional pseudo-islets form in culture over a window of 7 or more days. Earlier time points of function were not assayed (139,140).

One major hurdle in islet biology is controlling for isolated islet heterogeneity observed in metrics like size, composition, organization, and even hormone secretion (133,139,146–148). Pseudo-islets can be utilized to overcome this hurdle. To generate homogenous samples, a heterogeneous population of isolated islets is dispersed and allowed to reaggregate in a controlled fashion, which generates uniform pseudo-islets of a specific size (139,149). Homogeneous populations of pseudo-islets were assayed for beta cell function or transplanted under the kidney capsule to increase beta cell mass (139,140,144,149).

One underlying assumption in the current literature about pseudo-islets is that the final formed pseudo-islet must be a specific size in order to recapitulate islet biology. For this reason,

most work has focused on pseudo-islets with diameters larger than 100 μm (139–141,149). This is particularly interesting since other data support the idea that native islets that are smaller in size (~ 50 μm) function better than larger islets (> 100 μm) in both the *ex vivo* and transplant environments (23,146,150). The improved metrics of small islets has been attributed to increased nutrient and oxygen diffusion (149). A similar rationality might be applicable to small pseudo-islets.

3.1.2 Further Characterization of Pseudo-Islet Biology

Pseudo-islets provide a unique model for studying aspects of islet biology. However, several key concepts regarding pseudo-islet formation are unclear. Current pseudo-islet studies use tissue that has been in culture for days or weeks in order to allow complete pseudo-islet formation prior to experimentation (140–142). It is currently unclear what occurs during the initial window of recovery and reaggregation immediately after dispersion. Based on the observation that islet cells begin to reaggregate within hours after dispersion, we hypothesized that pseudo-islets form and regain function rapidly, on the time scale of hours to days, rather than days to weeks. Furthermore, it has been assumed that pseudo-islets need to reach a specific size to regain islet-like properties. We hypothesized that small pseudo-islets (< 100 μm) are able to recapitulate islet-like properties.

Research on pseudo-islets has thus far focused on aspects of beta cell function. Thus, it remains unclear how alpha cells behave in this pseudo-islet model. Since GSIS is recovered in pseudo-islet beta cells, we predicted that glucose inhibition of glucagon secretion would also be recovered and in a similar temporal manner. It is likely that the underlying cellular processes that regulate hormone secretion undergo changes during pseudo-islet formation. For example,

the regulation of intracellular calcium is critical for the proper release of insulin from islet beta cells (59,151). In the islet, the complete inhibition of insulin secretion at low glucose is due to calcium-dependent gap-junction coupling between beta cells, which is lost in dispersed cells (59,129). In contrast, alpha cells show heterogeneous calcium dynamics at all glucose levels in both the intact islet and dispersed state, suggesting alpha cells are electrically isolated and regulated independently (64,151–154). We predicted that normal calcium dynamics will be reestablished in pseudo-islet beta cells through the recovery of gap-junction coupling. Since each alpha cell has independent calcium dynamics, we predict to see no change in alpha cell behavior between the dispersed and pseudo-islet states.

To test our predictions, intact mouse and human islets were isolated, dispersed, and allowed to form small pseudo-islets containing all cell types *in vitro*. During the various cell states (i.e. intact, dispersed, forming pseudo-islet, formed pseudo-islet), glucagon and insulin secretion were assayed, along with mouse alpha and beta cell calcium dynamics (155).

3.2   Methods

3.2.1   Animal Experiments, Islet Isolation and Culture

Mouse islets were obtained as described in Chapter 2.2.1. Additional mouse research was completed at Washington University under approval by the Division of Comparative Medicine. To generate fluorescently labeled mouse alpha cells, we crossed transgenic mice expressing a CRE-dependent tandem-dimer red fluorescent protein knocked into the ubiquitously expressed ROSA26 locus (ROSA26-tdRFP) with a transgenic mouse expressing CRE recombinase under the control of a fragment of the glucagon promoter (glucagon-CRE) (64,156). In mice expressing both ROSA26-tdRFP and glucagon-CRE, CRE recombinase removes a

transcriptional antisense 'stopper' to allow RFP expression in only glucagon expressing cells (156). Human islets were obtained through the Integrated Islet Distribution Program. Upon arrival, human islets were cultured in human media (RPMI 1640 with 20% FBS, penicillin-streptomycin, and 11 mM glucose) for 2 hours prior to experimentation.

3.2.2   Islet Dispersion and Pseudo-Islet Culture

Islets were washed in Dulbecco's Phosphate-Buffered Saline (No Ca^{2+} or Mg^{2+}, pH 7.4) then dispersed using Accutase (Life Technologies) for 6 minutes at 37°C under gentle pipetting. Cells were resuspended in islet media for further culture. Dispersed islet cells from ~25 islets were plated on 30 µg of Bovine T1 collagen (BD Biosciences) and 20 µg human fibronectin (Thermo) coated MatTek glass bottom dishes with islet media. Islet media was refreshed every 24 hours.

3.2.3   Pseudo-islet Static Hormone Secretion Assays

Islets, dispersed cells, or pseudo-islets were equilibrated in KRBH buffer at 2.8 mM glucose for 1 hour at 37°C, then transitioned to 1 mM or 11 mM glucose for 1 hour at 37°C. The supernatant was collected, then the cells were lysed for hormone content using 1.5% 12N HCl in 70% ethanol. Insulin and glucagon secretion and content was measured using ELISA (Alpco – insulin; glucagon - Ray-biotech). Secretion data are presented as percent of total hormone content to control for viability of islet or pseudo-islet size.

3.2.4   Pseudo-islet Calcium Dynamics Imaging

Calcium imaging was completed as described in Chapter 2.2.3, but with the following changes: Islets, dispersed cells, and pseudo-islets were equilibrated in KRBH at 2.8 mM glucose

with 5 μM of the cell permeant calcium indicator Fluo-4 AM for 30 minutes, then transitioned to 1 mM or 11 mM glucose at $37^{\circ}C$. RFP labeled cells were imaged using 561 nm excitation. Fluorescence was also collected from non-alpha cells. This population encompasses mostly (>90%) beta cells. Data are reported as the percentage of cells actively oscillating under each condition, determined by the presence of significant increase in Fluo-4 intensity compared to baseline fluorescence during a 6-minute time-course.

3.2.5   Data Analysis and Statistical Testing

Data were analyzed and prepared using ImageJ, MatLab, Microsoft Excel, or GraphPad Prism 5. Error bars represent standard error of the mean with $p < 0.05$ considered statistical significant, as determined by one-way ANOVA's. To designate comparisons, an asterisk (*) labels a difference between 1 mM and 11 mM glucose, while a hash mark (#) labels a difference between control (islet or pseudo-islet sample used) and experimental conditions at the same glucose concentration.

3.3   Results

3.3.1   Mouse Pseudo-Islets and Recovery of Regulated Hormone Secretion

We began this set of experiments by imaging the behavior of dispersed mouse islet cells, and observed how they reassociate to form pseudo-islets *in vitro*. In samples containing all islet cell types, cell aggregates began forming within an hour post-dispersion and continued forming larger aggregates over a period of 72 hours (Figure 13A). The resulting pseudo-islets were smaller than native islets. Using the protocol outlined above (3.2.2), the pseudo-islets averaged approximately 40 µm in diameter (Figure 13B), and typically contained fewer than 30 cells.
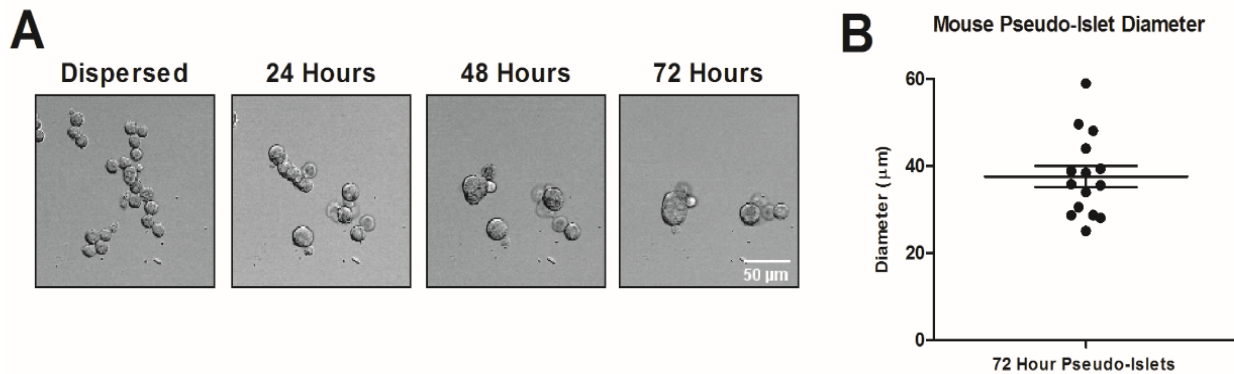
Figure 13: Representative Images of Pseudo-Islets Forming and Diameter Analysis. A) Representative images of mouse pseudo-islets forming *in vitro* over 72 hours after dispersion. Scale bar = 50 µm. B) Islet cells, under these culture conditions, formed pseudo islets that averaged ~40 µm at 72 hours. Adapted with permission from (155).

Next, we measured glucagon, which is normally secreted at 1 mM glucose and inhibited in response to 11 mM glucose from intact islets. Glucagon secretion was significantly increased in the dispersed state at both 1 and 11 mM glucose, but as the pseudo-islets recapitulated islet-like morphology, the levels of glucagon secreted in response to glucose became comparable to intact islets by 72 hours (Figure 14A). Concurrently, we measured insulin secretion from beta

cells throughout pseudo-islet formation. GSIS, which is lost in dispersed beta cells, was

reestablished in pseudo-islets by the 72 hour time point (Figure 14B).

Figure 14: Glucagon and Insulin Secretion from Mouse Pseudo-Islets. Glucagon (A) and insulin (B) secretion in response to 1 mM and 11 mM glucose was measured from mouse intact islets, dispersed islets, and pseudo-islets at 24, 48, and 72 hours (n = 6 mice). Error bars represent standard error of the mean. Asterisks (*) label differences between 1 mM and 11 mM glucose; hash marks (#) label differences between control and conditions at the same glucose concentration ($p < 0.05$ by one-way ANOVA). Adapted with permission from (155).

We tested whether these observed changes in regulated hormone secretion were due to

extracellular damage to surface receptors or proteins from Accutase dispersion. To examine this

possibility, we used exogenous stimulation with 1 μM epinephrine, a well characterized

glucagon-stimulating factor, during pseudo-islet formation (157). The treatment with

epinephrine potently stimulates similar levels of glucagon release from alpha cell in the islet,

dispersed, and pseudo-islet states. This result suggests that the adrenergic receptors responsible

for epinephrine signaling on alpha cells are intact on the alpha cell surface after dispersion and

do not change or recover during pseudo-islet formation (Figure 15).
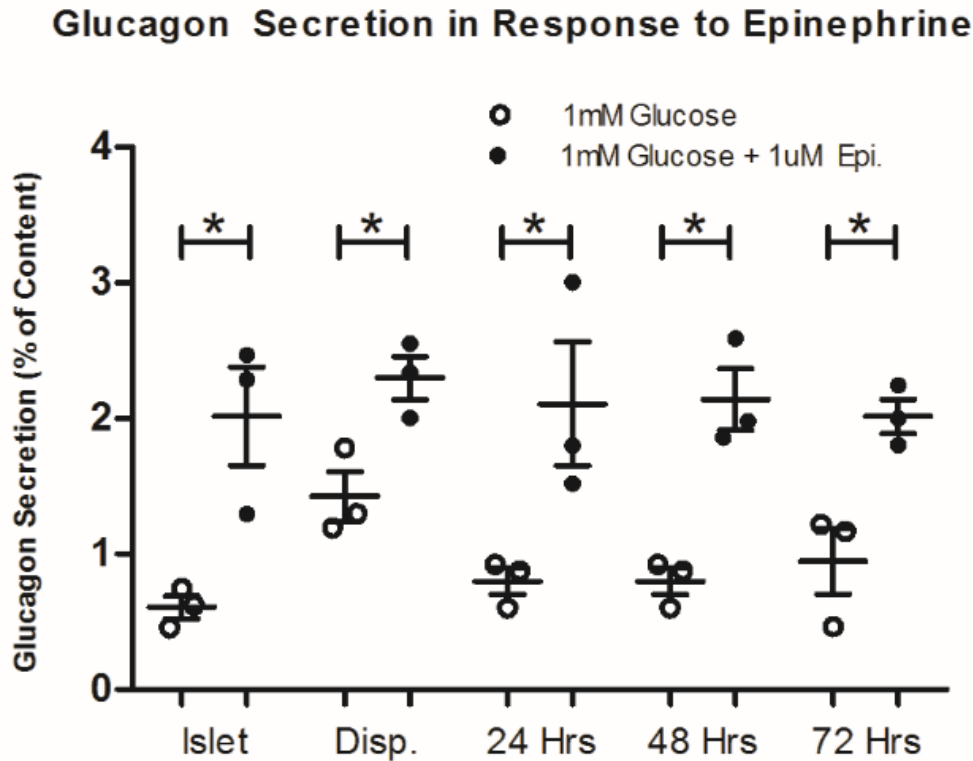
Figure 15: Glucagon Secretion in Response to Epinephrine. Glucagon secretion was measured from intact, dispersed, and pseudo-islets treated with 1 mM glucose or 1 mM glucose with 1 μM epinephrine. Data represent samples collected from 3 mice. Error bars represent standard error of the mean. Asterisks (*) label differences between sample pairs ($p < 0.05$ by one-way ANOVA). Adapted with permission from (155).

We also ran a parallel control experiment with sparsely plated islet cells to rule out time-dependent recovery of individual cell function as a confounding variable to pseudo-islet formation. We wanted to determine if the recovery in regulated glucagon secretion was specifically due to time and not complicated by changes in insulin or cell-to-cell contacts. In this experiment, we plated islet cells at ~ $1/10^{th}$ the density of the normal pseudo-islet protocol and

measured glucagon secretion over time. We found these cells remained in a single cell suspension and did not become pseudo-islets. These single cells failed to recover regulated glucagon secretion over time (Figure 16). Successful dispersion into single cells was verified empirically by FACS scatter data (>95% single cells).
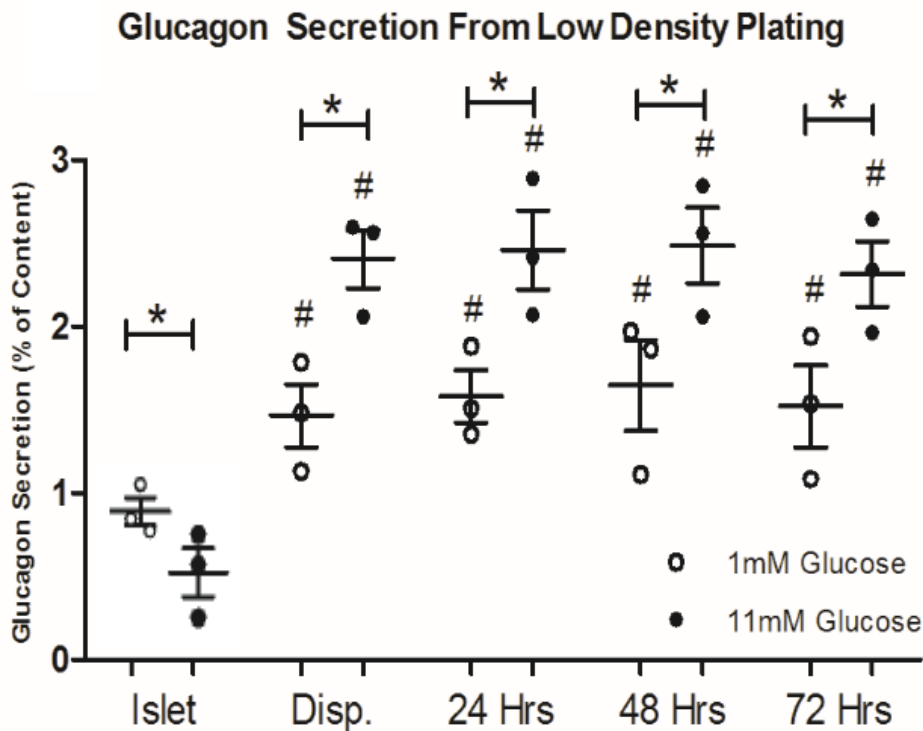
Figure 16: Glucagon Secretion from Low Density Plating. Glucagon secretion was measured from intact islets and dispersed islet cells plated at low density at 24, 48, and 72 hours after dispersion (~ 1/10 density described in methods). Data represent samples collected from 3 mice. Error bars represent standard error of the mean. Asterisks (*) label differences between 1 mM and 11 mM glucose; hash marks (#) label differences between control and conditions at the same glucose concentration (p < 0.05 by one-way ANOVA). Adapted with permission from (155).

The size range that permits functional pseudo-islets is highly debated within the pseudo-islet field (139,140,142,143). Until now, the majority of studies in the field have focused on

generating pseudo-islets that mimic the size of isolated islets (>100 μm). The difference in size between our pseudo-islets and those published, coupled with the shared similarity of regulated secretion, raises the question as to what extent the size component influences glucose regulated glucagon secretion. Furthermore, it is unclear if islet cells recover more quickly if forced into close proximity. To address this, we amended our formation protocol to increase the initial dispersed cell density and thus generate very large pseudo-islets (>250 μm). To do this, we allowed dispersed islet cells to collect at the bottom of a rounded Eppendorf tube instead of on flat, coated plates. We measured glucagon secretion during the established time-course of pseudo-islet formation (Figure 17). As observed for the small pseudo-islets, the larger forced pseudo-islets recovered regulated glucagon secretion over a similar time-course. There was a slight decrease in the sample-sample variabilty in large forming and formed pseudo-islets, suggesting the large pseudo-islet protocol increased pseudo-islet uniformity compared to plated pseudo-islets.

Forced Creation of Large Pseudo-Islets
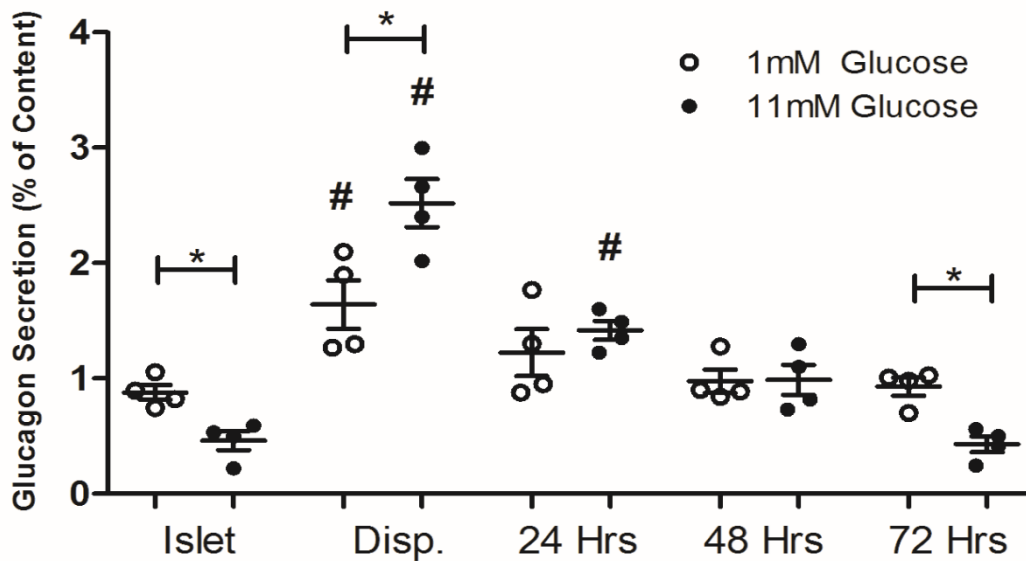
Figure 17: Glucagon Secretion from Large Forced Creation of Pseudo-Islets. Glucagon secretion was measured from intact, dispersed, and forced, large pseudo-islets treated with 1 mM glucose or 11 mM glucose. Data represent samples collected from 4 mice. Error bars represent standard error of the mean. Asterisks (*) label differences between 1 mM and 11 mM glucose; hash marks (#) label differences between control and conditions at the same glucose concentration ($p < 0.05$ by one-way ANOVA).

3.3.2 Human Pseudo-Islets and Recovery of Regulated Hormone Secretion

We examined whether dispersed human islets reassociate to form pseudo-islets in a manner similar to mouse islet cells. Glucagon secretion, which is normally inhibited in the islet in response to 11 mM glucose, was significantly increased in the dispersed state for human alpha cells (Figure 18A). This can be seen similarly in murine pseudo-islets, though the time-course of formation with human cells appears to be somewhat slower. Human pseudo-islets secreted glucagon at near islet-like levels trending towards normal inhibition of glucagon secretion in response to glucose after approximately 14 days ($p = 0.18$). Additionally, GSIS, which is lost in dispersed beta cells, is reestablished in human pseudo-islets after approximately 14 days (Figure

18B). The difference in recovery time between mouse and human pseudo-islets could be influenced by a variety of factors, including donor age, culture time, and species. Human donor samples had an average age of 42.7 years old and BMI of 28.9 (Table 1).
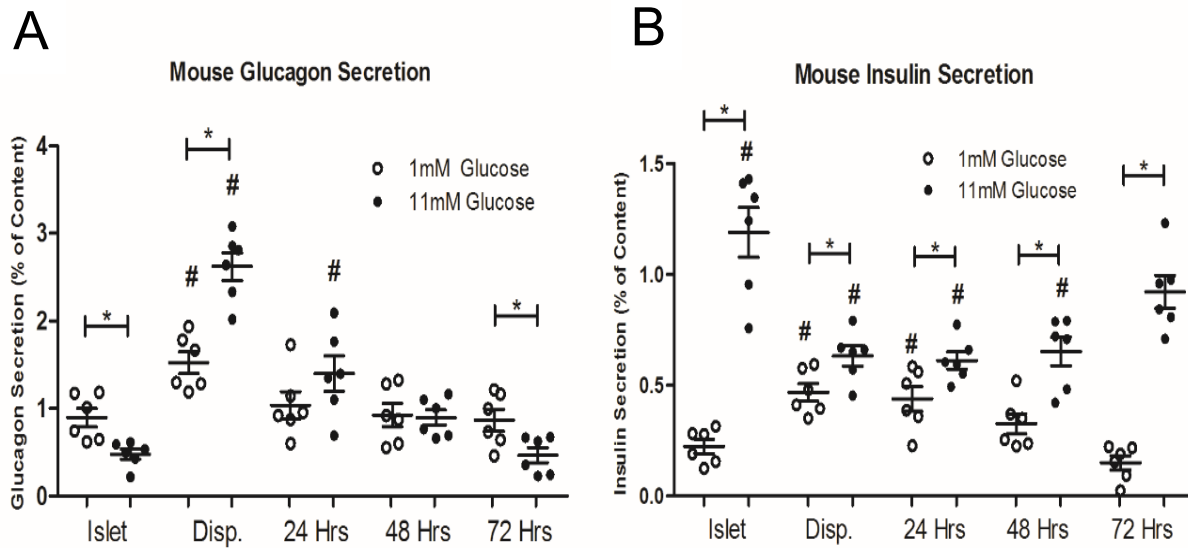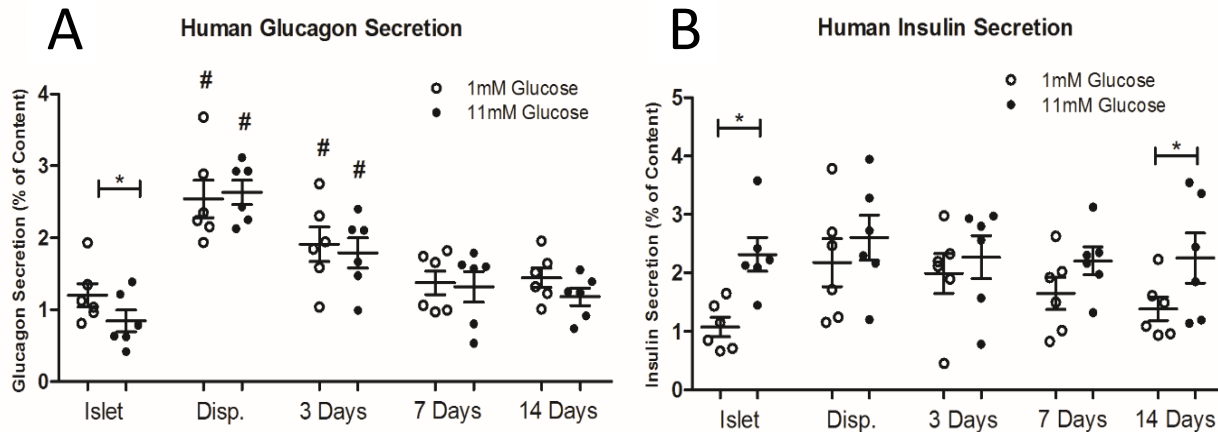

Figure 18: Glucagon and Insulin Secretion from Human Pseudo-Islets. Glucagon (A) and insulin (B) secretion in response to 1 mM and 11 mM glucose was measured from human donor islets, dispersed islets, and pseudo-islets at 1, 7, and 14 days (n = 6 donors). Donor information can be found in Table 1. Error bars represent standard error of the mean. Asterisks (*) label differences between 1 mM and 11 mM glucose; hash marks (#) label differences between control and conditions at the same glucose concentration ($p < 0.05$ by one-way ANOVA). Adapted with permission from (155).

Donor	Age	BMI	Gender
A	38	34.4	F
B	35	24.2	M
C	45	30.5	M
D	47	31	M
E	59	28.3	M
F	32	24.9	F
	42.7	**28.9**	

Table 1: Human Islet Donor Information. Age, Body Mass Index (BMI), and Gender. Averages are listed in bold. Adapted with permission from (155).

3.3.3 Recovery of Calcium Dynamics in Pseudo-Islets

To assess the role of intracellular calcium dynamics in the recovery of islet-like properties and hormone secretion in pseudo-islets, we labeled islets, dispersed cells, and pseudo-islets with Fluo-4, an intracellular free calcium indicator dye. We used the presence of RFP to identify the minority population of alpha cells (see Chapter 3.2.1), so these experiments could only be completed using transgenic murine islets or cells (Figure 19A/B) (151). Changes in Fluo-4 fluorescence from both beta and alpha cells were recorded over time in response to glucose. Data are reported as the percentage of active cells, determined by the presence of significant increases in Fluo-4 intensity over baseline.

In islets, beta cells are electrically coupled, which led to no measurable calcium activity at low glucose and coordinated oscillatory activity at high glucose (Figure 19C) (59,133). This electrical coupling is lost in the dispersed state, and resulted in aberrant calcium activity in some beta cells at low glucose, as well as decreased glucose stimulated calcium oscillations. As the beta cells in murine pseudo-islets recover normal insulin secretion over a 72 hour period, we also

observed decreased calcium activity at low glucose and increased calcium activity at high glucose. In contrast, the percentage of active alpha cells does not appear to change between the islet, dispersed, and pseudo-islet states (Figure 19D).
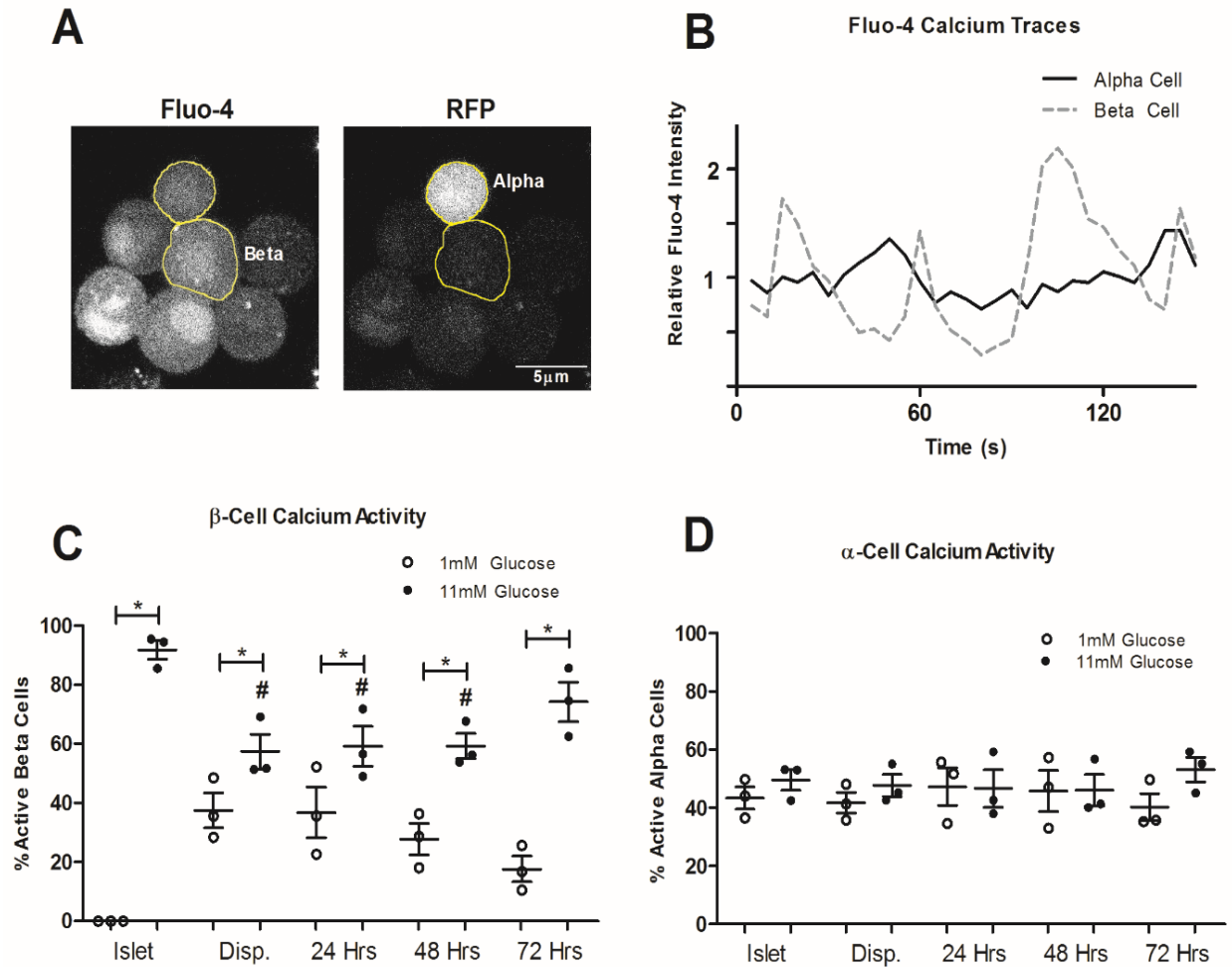
Figure 19: Calcium Dynamics in Beta and Alpha Cells in Pseudo-Islets. A) Representative image of a Fluo-4 labeled pseudo-islet. Alpha cells were identified by the presence of transgenic RFP expression. B) Representative traces of Fluo-4 intensity changes in an alpha cell and one beta cell within a pseudo-islet at 11 mM glucose. The percent of oscillating beta cells (C) or alpha cells (D) represent data from 3 mice (>25 beta cells/mouse or >10 alpha cells/mouse). Error bars represent standard error of the mean. Asterisks (*) label differences between 1 mM and 11 mM glucose; hash marks (#) label differences between control and conditions at the same glucose concentration ($p < 0.05$ by one-way ANOVA). Adapted with permission from (155).

3.4 Discussion of Pseudo-Islet Characterization

3.4.1 Recovery of Regulated Hormone Secretion and Calcium Dynamics in Pseudo-Islets

Both beta cells and alpha cells recover near-normal regulated hormone secretion upon the formation of small pseudo-islets from the dispersed state. Our data show that pseudo-islet formation occurs more slowly for human cells than for mouse cells, but in both cases, insulin and glucagon recover near-normal behaviors in parallel.

These data highlight the importance of pseudo-islet culture time with regard to function. Pseudo-islets, prior to full recovery, appear to remain in a dysfunctional dispersed state rather than an islet-like state for ~48 hours with mouse and several days with human cells (139,158). This dysfunction, however, is not due to extracellular damage from dispersion, since islet cells remain sensitive to exogenous stimulation throughout the process, and fail to recover near-normal secretion in the single cell state after 72 hours. Furthermore, the rate of recovery is not sped up or altered by increasing the proximity of islet cells or the size the pseudo-islet generated.

Pseudo-islet beta cells recover normal electrical coupling, which leads to regulated calcium oscillations that potentiate GSIS, while glucose concentrations or cell state have little to no effect on the calcium dynamics of alpha cells. The end result is that pseudo-islet behave similarly to isolated islets, and is consistent with previous reports on recovered pseudo-islet beta cell function (36,64,151). Although the role of alpha cell calcium dynamics in glucagon release remains unclear (Chapter 4.1.2), the restoration of tightly regulated calcium dynamics in beta cells quantitatively parallels the recovery of insulin secretion in pseudo-islets. Moreover, the recovery of regulated glucagon secretion appears to follow a time-course similar to what is seen for the restoration of GSIS from beta cells.

3.4.2   Minimizing Indirect Effects in Islet and Pseudo-Islet Studies

Current studies of intact islets are complicated by a myriad of indirect effects present within the multiple cell-type system of the islet. Likewise, perturbations to alpha cells may be masked by effects specific to beta cells since the islet is predominately beta cells and insulin is a potent regulator of alpha cell function. Specific pharmacological targeting of alpha cells within the islet is difficult because of the shared similarities in receptors and intracellular proteins between the islet cell types (159–161). This presents a difficult hurdle for islet biologists to overcome when studying minor populations of cells within the beta cell-rich islet.

As demonstrated in Figures 11 and 14, beta and alpha cells separated from the islet environment for direct interrogation in the single cell state demonstrate abnormal function. However, using a separation technique like flow-cytometry, coupled to a pseudo-islet formation protocol, allows for the creation of unique pseudo-islets containing specific cell types. In Chapter 4, we will describe the use of fluorescence active cell sorting (FACS) to isolate beta, alpha, and delta cells and reconstitute them in combinations to form selective pseudo-islets for targeted questions in regards to alpha cell function and regulated glucagon secretion.

Chapter 4

Glucose Regulated Glucagon Secretion from Islets and Pseudo-Islets

4.1 Introduction

4.1.1   Glucose Inhibition of Glucagon Secretion

The first hypotheses proposed to explain glucose-regulated glucagon secretion utilized aspects of GSIS from beta cells since alpha cells shared similar neuro-endocrine properties (32,37). Later, it became apparent that the models explaining glucose-dependent hormone secretion were different between beta and alpha cells. Alpha cells inhibit glucagon secretion in response to glucose in the islet, but a paradoxical glucose-dependent increase in glucagon secretion is observed in the dispersed state (18,64). These data led the inclusion of new models to explain glucose regulated glucagon secretion from alpha cells in both the intact islet and dispersed state.

Recent research has moved the alpha cell field toward more specific models. Currently, three models exist to define the regulation of glucagon secretion by glucose (32,37,162). First, the intrinsic model, which encompasses several similar theories, speculates that the alpha cell regulates its glucagon secretion through changes in intracellular metabolism and electrical signaling in response to glucose. Second, the paracrine signaling model asserts that glucose indirectly inhibiting glucagon secretion through factors secreted by the other islet cell types, including insulin from beta cells and somatostatin from delta cells. Third, the juxtacrine signaling model proposes that specific cell-to-cell contacts impinge upon receptors on the alpha cells, putatively by ligands on the neighboring beta cells, to regulate glucagon secretion. Other cell-to-cell connections, including pannexins and cell adhesion molecules, have been proposed to

be important to alpha cell function through the modulation of gap junctional coupling (163,164), however the proposed mechanisms are based upon the intrinsic model of regulation. The following sections will expand on each model. Data that support each model, along with possible flaws in each model, will also be discussed.

4.1.2   Alpha Cell Intrinsic Model

In the intrinsic model, alpha cells inhibit their own glucagon secretion through changes in intracellular metabolism and electrical signaling in response to glucose. Like beta cells, alpha cells are able to sense, uptake, and metabolize glucose, which results in an increased ATP/ADP ratio, membrane depolarization, and calcium influx (32,37,152,162,165–169). As glucose levels increase, the inhibition of secretion is dependent upon membrane depolarization which reduces P/Q-type calcium channel activity in close proximity to glucagon granules waiting to be secreted. This local decrease in calcium suppresses calcium-dependent glucagon secretion (Figure 20) (50,152,162,165,168,169).
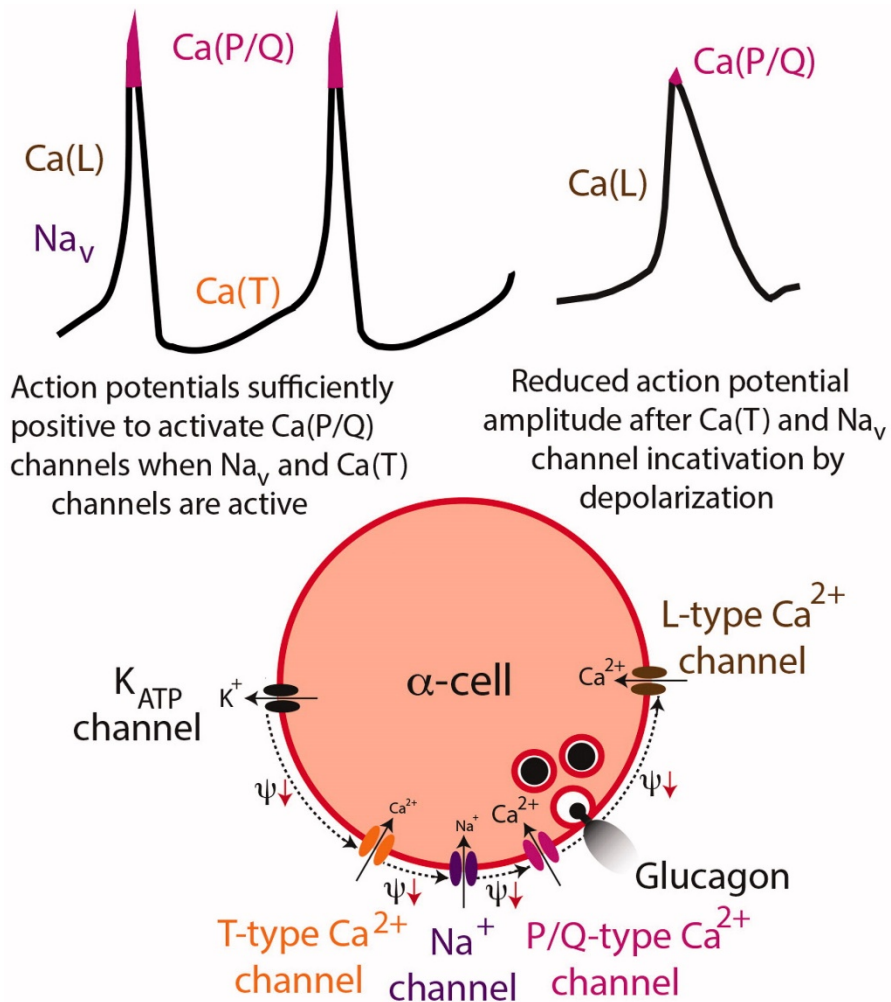
Figure 20: A Model for Intrinsic Regulation of Alpha Cell Electrical Properties. In this model, the characteristics of the action potential are altered by the presence and activity of several different ion channels, including L, T, and P/Q-type calcium and sodium channels. Together, these channels decrease action potential amplitude, inhibit calcium flux and decrease glucagon secretion. Reproduced with permission from (32).

The role of ER calcium has also been proposed to be central to the intrinsic regulation of glucagon secretion from alpha cells. While less is known about the regulation of ER calcium in alpha cells compared to beta cells, this model suggests that the inhibition of glucagon occurs through the uptake and metabolism of glucose to ATP, activation of the ATP-dependent Sarco/Endoplasmic Reticulum Calcium-ATPase (SERCA), and sequestration of cytosolic

calcium into the ER (170,171). SERCA, normally inactive at low glucose, becomes active at high glucose and pumps cytosolic calcium in to the ER, thus suppressing calcium-dependent exocytosis.

The intrinsic model is complicated by observations that membrane potential changes may occur in narrow ranges, localized regions of the membrane may depolarize while others hyperpolarize, and the alpha cell population within an islet has heterogeneous electrical properties in response to glucose and perturbations (32,50,64,151,169). While the regulation of alpha cell electrical properties is undoubtedly complex, the theories that comprise the intrinsic model assume and require intracellular calcium levels to decrease in response to glucose to inhibit glucagon secretion. This central point remains highly debated, since both increases and decreases in cytoplasmic calcium have been observed in both intact islet and dispersed alpha cells in response to glucose (64,151,153,165).

Furthermore, this model fails to explain the differences in glucagon secretion data from islet and dispersed alpha cells. The switch from glucose-inhibition of glucagon secretion within the islet to glucose stimulated glucagon secretion in dispersed alpha cells suggests that the regulatory mechanisms underlying secretion are not fully intrinsic, rendering this model incomplete (32,34,36,64,65,167). If we consider what is disrupted or lost upon dispersion, paracrine regulators and cell-to-cell contacts all become possible candidates as regulators of glucagon secretion.

4.1.3 Alpha Cell Paracrine Model

While circulating endocrine factors have measurable effects on hormone secretion, critical modulators of glucagon secretion are postulated to originate from within the islet

(32,37,134). In the paracrine model, glucagon secretion is inhibited by factors released by other neighboring islet cells. The abundant beta cell secretes factors, including insulin, GABA, and zinc, that are putative regulators of glucagon secretion (32,37,134). Additionally, somatostatin, secreted by neighboring delta cells, has a putative role in the modulation of glucagon secretion (36,72,172).

Insulin, GABA and zinc have each been proposed to regulate alpha cell exocytosis in a similar manner. These factors are expected to regulate glucagon secretion by causing the alpha cell to repolarize and hyperpolarize, which inhibits calcium influx (Figure 21).
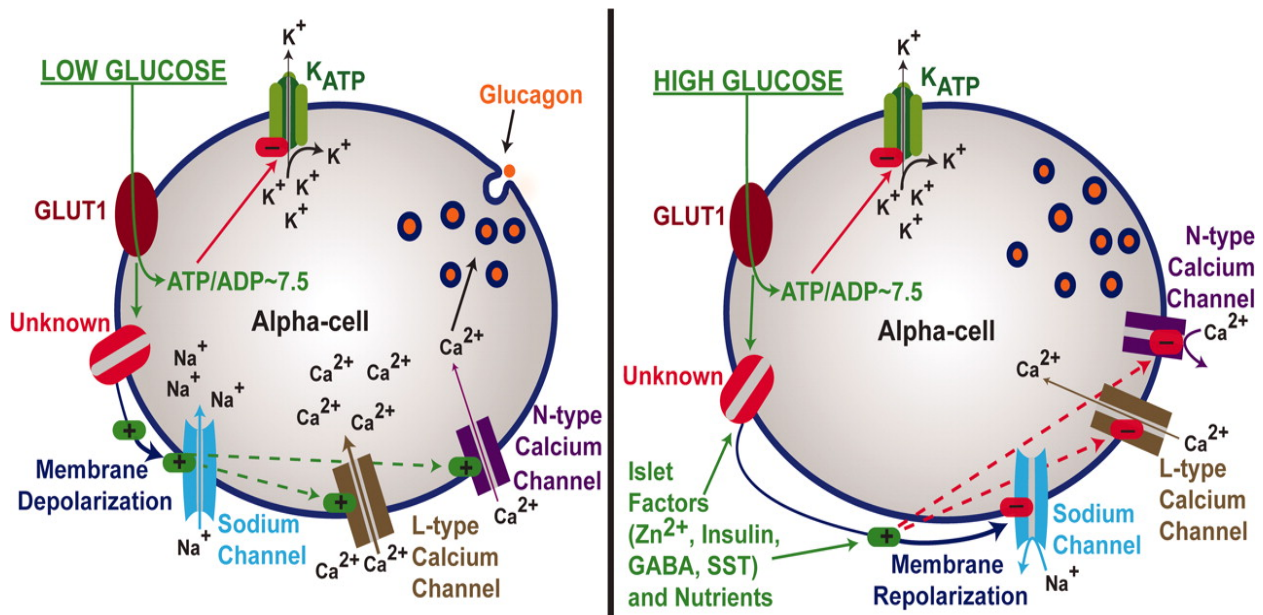
Figure 21: A Model for Paracrine Regulation of Alpha Cell Electrical Properties. In this model, secreted factors from neighboring cells are able to modulate alpha cell electrical properties. Paracrine factors promote the repolarization of the membrane, which inhibits calcium influx and inhibits glucagon secretion. Reproduced with permission from (162).

As with the alpha cell intrinsic model, this mechanism requires a decrease in calcium for proper glucagon inhibition. While the full, detailed mechanism is unknown, it is postulated that this signaling cascade may act independently of the K_{ATP} channel or by increasing the activity of

GABA-sensitive chloride channels on the cell membrane to alter membrane potential (51,173–177).

Recent work has shown that insulin may have a secondary mode of action in alpha cells. This aspect of insulin signaling works in conjunction with somatostatin secreted from delta cells (36). It is postulated that insulin signaling, through the insulin receptor and protein kinase B (a.k.a. Akt), activates phosphodiesterase 3B, which results in the degradation of cAMP. Furthermore, somatostatin signaling through the somatostatin receptor 2, a $G_{\alpha i}$ coupled GPCR, inhibits the formation of cAMP. Together, the binding of insulin and somatostatin to their receptors on alpha cells results in a potent decrease in the levels of cAMP. The levels of cAMP within the alpha cell are critical in the regulation of hormone secretion and appear to positively correlate with the levels of glucagon secreted (Figure 22) (35,36,153).
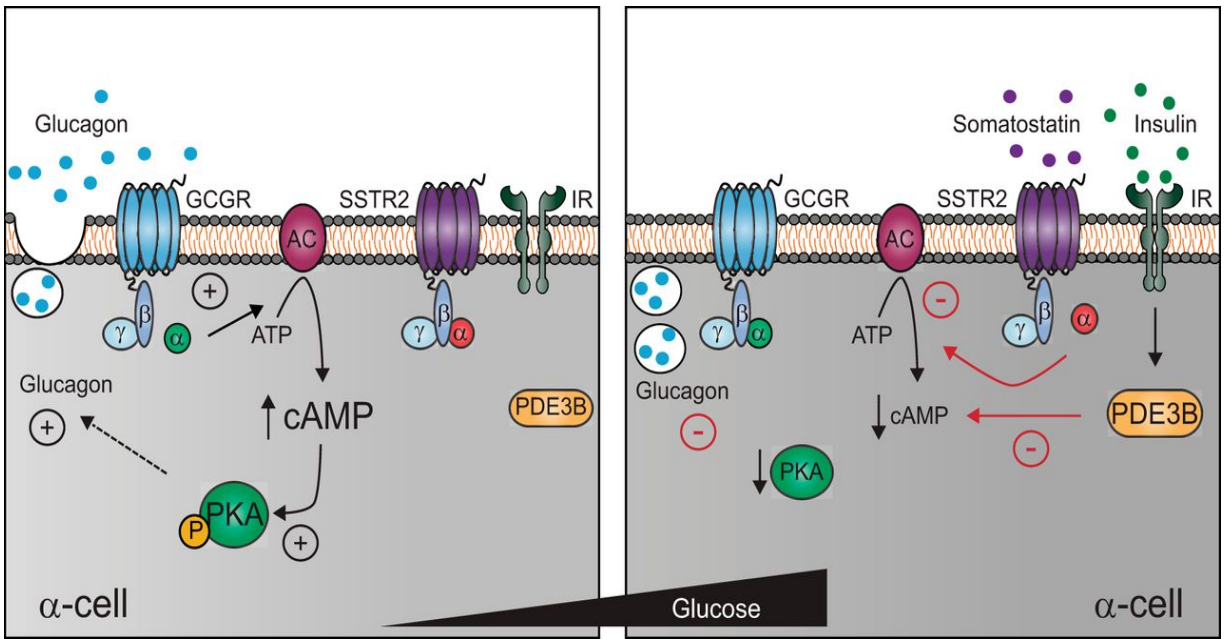
Figure 22: A Model for Paracrine Regulation of Alpha Cell cAMP. In this model, secreted factors from neighboring cells are able to modulate the levels of cAMP within the alpha cell. Insulin, which activates cAMP degradation, and somatostatin, which inhibits cAMP formation, cooperatively interact to decrease cAMP to inhibit glucagon secretion at elevated glucose levels. Reproduced with permission from (36).

It is widely accepted that paracrine factors are required for the proper regulation of glucagon secretion (32,37,162). However, aspects of the paracrine model fail to explain the glucose-dependent secretion profiles of glucagon observed *in vivo* and *in vitro*. For example, glucagon secretion begins to decrease prior to the secretion of factors from neighboring cells (2-3.5mM glucose range) (32,37,134,178). Furthermore, proposed modulators of glucagon secretion lose their inhibitory effect on dispersed or sorted alpha cells (64). These data suggest that the islet milieu is somehow required for the proper regulation of glucagon secretion.

4.1.4   Alpha Cell Juxtacrine Signaling Model

In the juxtacrine model, cell-to-cell contacts that signal bi-directionally between islet cells contribute to the regulation of glucagon secretion from alpha cells. Ephrin ligands on beta cells bind Eph receptors on alpha cells to establish juxtacrine signaling. While the importance of this signaling pathway in hormone secretion was initially established in beta cells (135), consequent work has demonstrated that this pathway is present in alpha cells (34). Specifically, EphA4 and EphA7 receptors on alpha cells are stimulated by ephrinAs on beta cells to initiate forward signaling and inhibit glucagon secretion (Figure 23) (34).
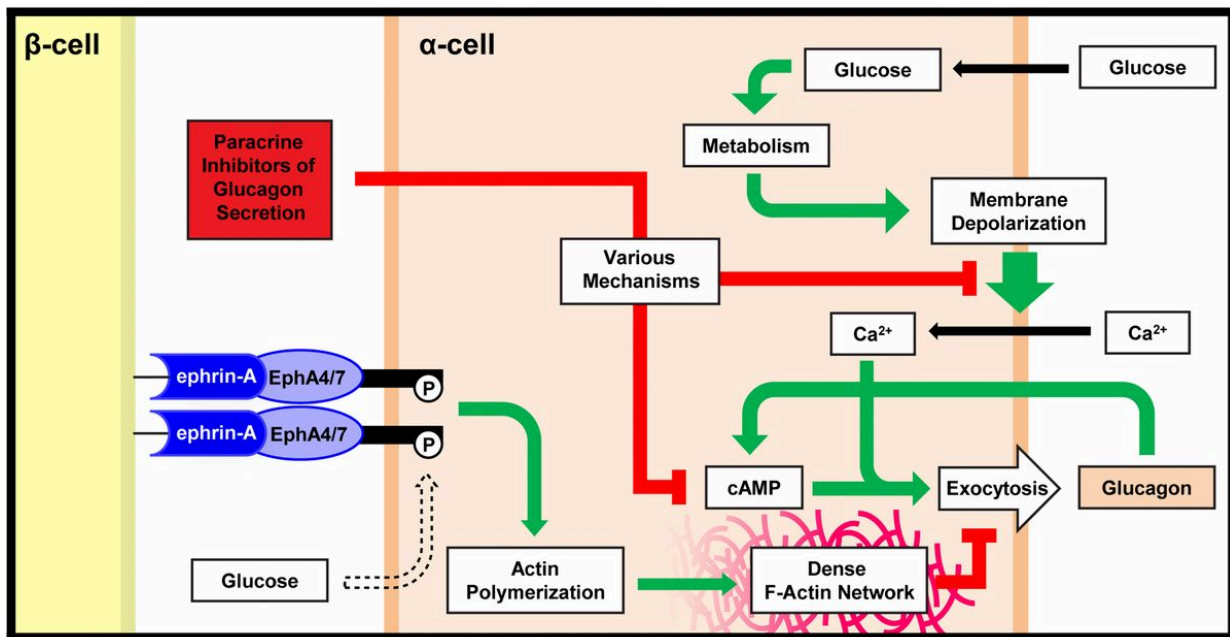
Figure 23: A Model for Juxtacrine Regulation of Glucagon Secretion. In this model, ephrinA on beta cells stimulates forward signaling on alpha cells via EphA4/7. This results in the formation of a dense F-actin network at the membrane which inhibits glucagon secretion. This pathway, in conjunction with other signaling pathways, results in glucose-dependent glucagon secretion. Adapted with permission from (34).

While this signaling pathway is normally dominated by EphA4, EphA7 is also expressed in alpha cells, and it has been shown to compensate partially for the genetic deletion of EphA4 (34). The inhibition of glucagon secretion following EphA stimulation appears to be due to the formation of F-actin density at the cell membrane, which putatively impedes granule trafficking and exocytosis (34). F-actin density and remodeling has been shown to be critical for insulin secretion from beta cells (179,180). The juxtacrine model helps explain the observed increase in glucagon secretion in response to glucose when beta cells are lost in T1D or the dispersed state (18,66,67). However, it is unclear if EphA4 signaling simply provides an unchanging F-actin density that impedes secretion or if this pathway has regulatory elements that change F-actin levels in response to stimuli.

4.1.5   Elucidating the Multiple Influences on Glucose Regulated Glucagon Secretion

Alone, each of the proposed models fails to explain regulated glucagon secretion in response to glucose from islet and dispersed alpha cells. In Chapter 3, we showed that the alpha cell undergoes changes between the dispersed and pseudo-islet states that result in the recovery of regulated glucagon secretion. While important to GSIS in beta cells, calcium appears to play a lesser role in the regulation of glucagon secretion in alpha cells (Figure 19) (34,36). Calcium, while required for secretion, does not correlate with the amount of glucagon released at increasing glucose concentrations. This discrepancy leaves the possibility of other factors that might be critical to the glucose-dependent regulation of glucagon secretion.

The levels of cAMP in alpha cells are decreased by paracrine factors like insulin and somatostatin, and have been proposed to play a key role in the inhibition of glucagon secretion in response to glucose (35,36,153). Juxtacrine signaling between EphA4 on alpha cells and

ephrinAs on beta cells has also been proposed to reduce glucagon secretion by modulating F-actin density (34). While insulin secretion from beta cells can be simplified into the GSIS consensus model, alpha cells appear to be under multiple layers of regulation.

The layered complexity led to the hypothesis that a combination of signaling pathways, both intracellular and intercellular, are required to inhibit glucagon secretion from alpha cells in response to glucose. Specifically, we hypothesize that proper regulation of glucagon secretion depends on the paracrine actions of insulin and somatostatin, combined with juxtacrine signaling between beta cell ephrinAs and alpha cell EphA4/7, independent of changes in alpha cell calcium (Figure 24). We also hypothesized that both alpha cell cAMP and F-actin levels are misregulated in the dispersed state, but recover in formed pseudo-islets.
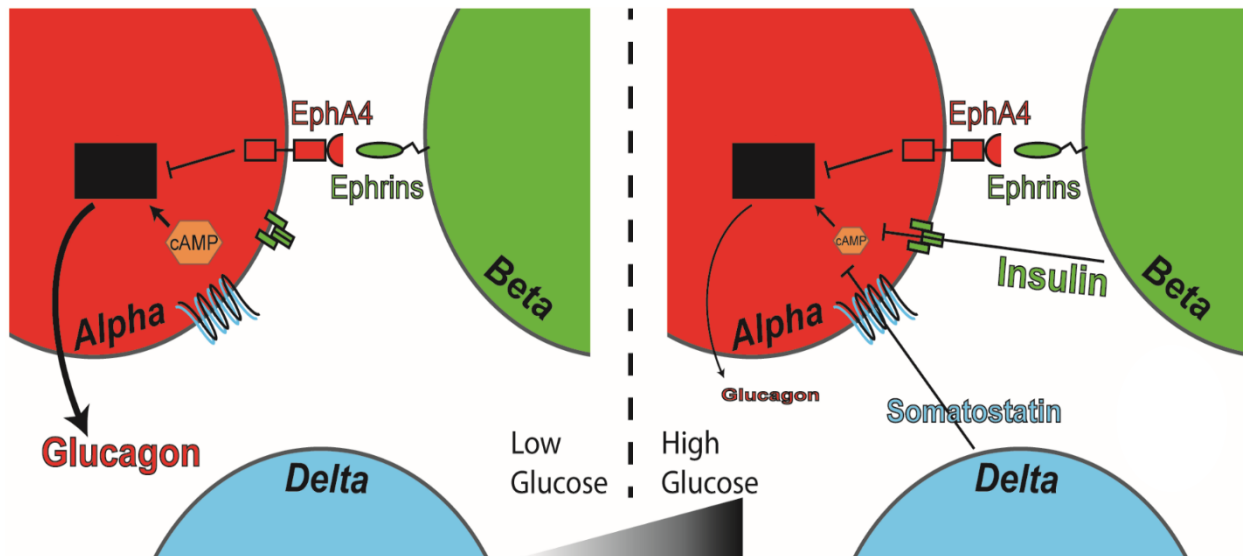
Figure 24: The Proposed Mechanistic Model for Glucagon Secretion. In this model, the proper inhibition of glucagon secretion depends upon the presence of ephrinA on beta cells to stimulate EphA4/7 on alpha cells, along with the secretion of insulin and somatostatin from beta and delta cells, respectively.

When generating pseudo-islets, we utilized an additional method to limit the indirect influences from the other islet cell types during pseudo-islet formation. Using FACS, it is possible to generate pseudo-islets that contain or lack specific islet cell types (Figure 25). For example, it might be possible to generate alpha/beta pseudo-islets independent of delta cells or alpha/delta pseudo-islet independent of beta cells. These selective pseudo-islets can be designed to have similar cell-type ratios as intact mouse islets. Without paracrine factors or cell-to-cell contacts from islet cell types absent from culture, it is possible to measure glucagon secretion in response to glucose and other putative modulators with a more direct approach in functional, selective pseudo-islets.
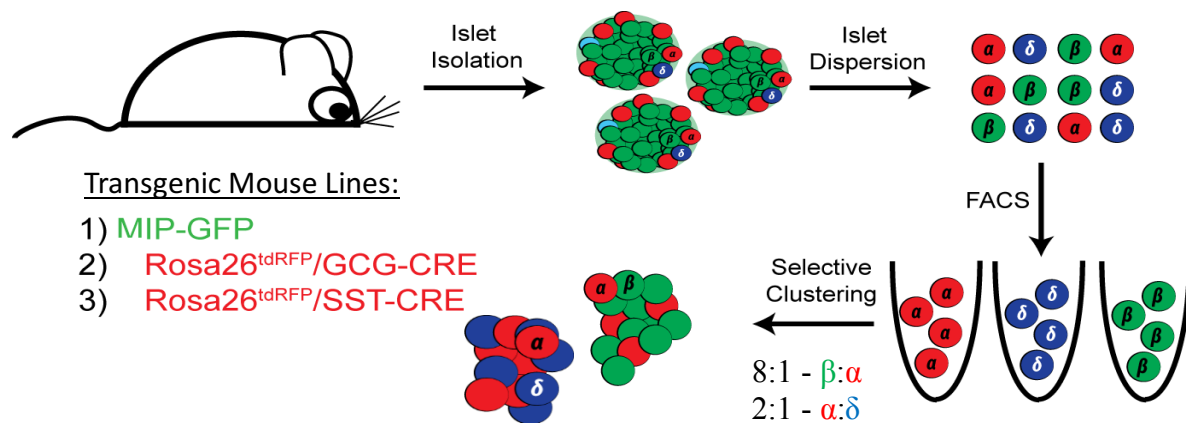
Figure 25: Schematic of Selective Pseudo-Islet Generation. In this protocol, transgenic mouse islets, expressing fluorescent proteins in specific islet cells, are isolated, dispersed, FAC sorted, then recombined in selective combinations to form novel pseudo-islets. Combined ratios of the islet cells are designed to mimic the ratios observed in intact mouse islets.

4.2   Methods

4.2.1   Transgenic Animals, Cell Sorting, and Pseudo-Islet Generation

Islets were isolated as described in Chapter 2.2.1. To generate pseudo-islets of selective combinations, transgenic mice expressing fluorescent labels were utilized. To obtain

fluorescently labeled beta cells, transgenic mice in which the insulin promoter drives expression of green fluorescent protein (MIP-GFP) were used (181). Fluorescently labeled alpha cells were generated as described in Chapter 3.2.1. To obtain fluorescently labeled delta cells, ROSA26-tdRFP expressing mice were crossed with transgenic mice expressing a somatostatin promoter-driven CRE recombinase (64,182). Pseudo-islets were generated as described in Chapter 3.2.2. Selective pseudo-islets were plated at islet-like ratios (8:1 beta:alpha, 2:1 alpha:delta).

4.2.2   Modulating and Measuring Glucagon Secretion

Glucagon secretion was measured from islets, dispersed cells, or pseudo-islets as described in Ch. 3.2.3, with or without the following conditions: 100 µM 3-isobutyl-1-methylxanthine (IBMX), 50 µM forskolin (Fsk), 4 µg/mL ephrinA5-Fc (R&D Systems), 100 nM somatostatin, 12.5 µM 4-(2,5-dimethyl-pyrrol-1-yl)-2-hydroxy-benzoic acid (DPHBA) (Santa Cruz), 1 µM S961 (Novo Nordisk), and 1 µM insulin.

4.2.3   Pseudo-islet cAMP and F-Actin Semi-Quantitative Fluorescence

For immunofluorescence imaging, islets, dispersed alpha cells, and pseudo-islets were subjected to the secretion protocol described above, but prior to lysis, samples were fixed in 2% paraformaldehyde in PBS at 4°C for 30 minutes, permeabilized, and blocked overnight in 0.3% Triton X-100, 5 mM sodium azide, 1% BSA, and 5% goat serum. Samples were incubated with the primary antibodies, mouse anti-cAMP (Cell Signaling Technologies) and guinea pig anti-glucagon (EMD Millipore), for 72 hours at 4°C, washed three times, then incubated with the secondary antibodies labeled with Alexa Fluor 488 and 546 (Life Technologies), and the F-actin binding phalloidin, conjugated to Alexa Fluor 633 (Invitrogen) for 72 hours at 4^0C. Long

incubations with antibodies were utilized to allow antibody penetrance into the core of larger islets. Samples were mounted with DAPI Fluoromount-G (Southern Biotech). Data analysis used background subtracted raw images, while representative images included were linearly adjusted for presentation purposes only. Fluorescence intensities were normalized to cAMP or F-actin fluorescence levels in the islet at 1 mM glucose.

4.3   Results

4.3.1   Recovery of Regulated cAMP and F-Actin Density in Pseudo-Islets

As previously published (34,36), semi-quantitative immunofluorescence was used to measure the static levels of cAMP and F-actin in response to glucose in alpha cells from islet, dispersed cells, and pseudo-islets containing all cell types (Figure 26A). In islet and pseudo-islet alpha cells, the levels of cAMP decreased in response to elevated glucose. As was seen for glucagon secretion, this glucose-dependent decrease was lost upon islet dispersion, but returned in pseudo-islets within 72 hours (Figure 26B). In alpha cells within intact islets, F-actin levels showed minimal changes between 1 mM and 11 mM glucose. F-actin levels are significantly decreased upon dispersion, but returned to islet-like levels by 48 hours during pseudo-islet formation (Figure 26C).
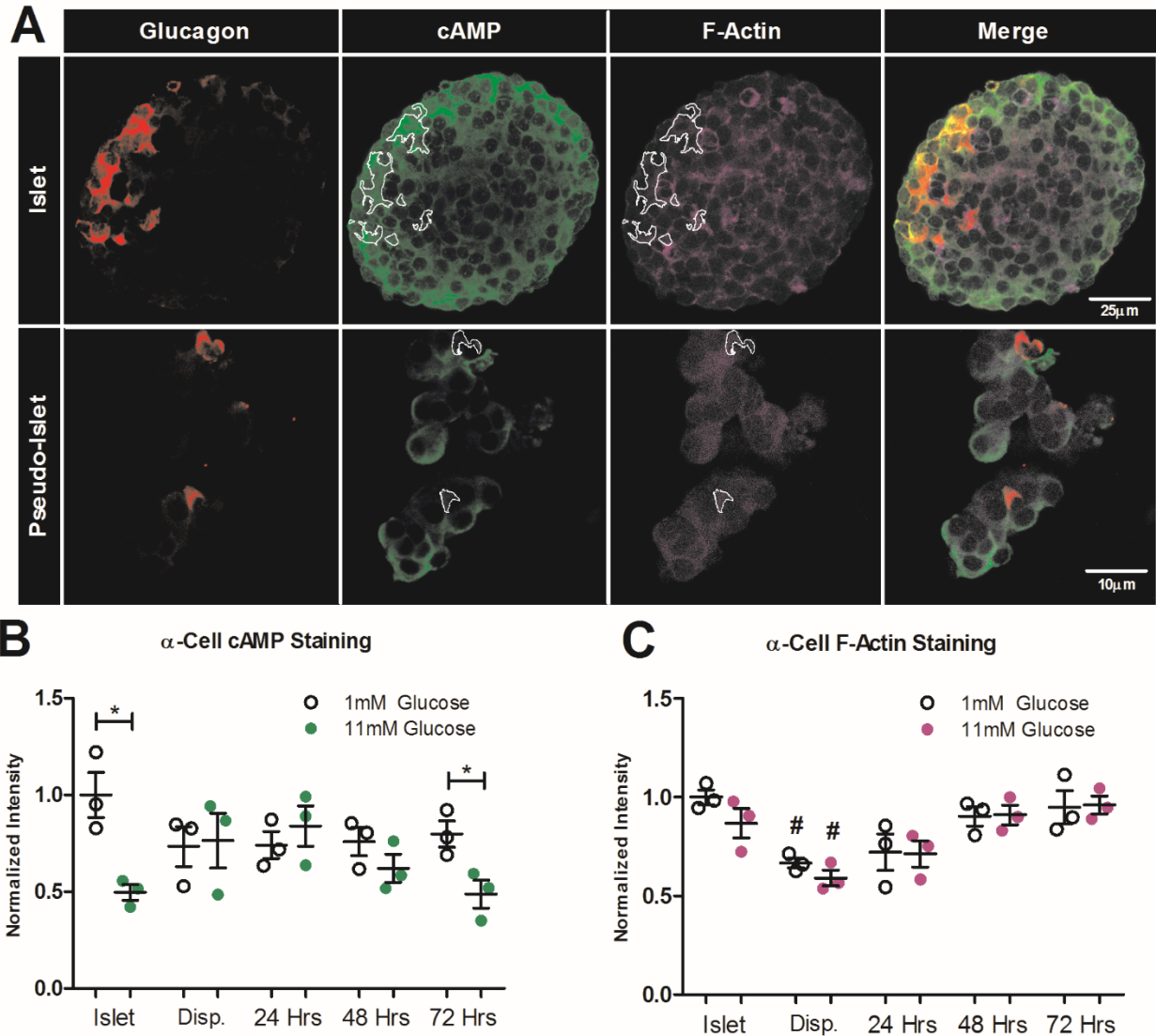
Figure 26: Representative Immunolabeling and Quantitation for Islet and Pseudo-Islet cAMP and F-actin. A) Fixed samples were immunolabed for cAMP (green) and F-actin (magenta) and fluorescence intensities were measured in glucagon positive regions (white overlay). Representative images show immunolabeling for an islet and pseudo-islet. Quantification of alpha cell specific cAMP (B) and F-actin (C) levels in intact islets, dispersed cells, and pseudo-islets at 1 mM and 11 mM glucose (n = 3 mice with >15 α-cells/mouse). Error bars represent standard error of the mean. Asterisks (*) label differences between 1 mM and 11 mM glucose; hash marks (#) label differences between control and conditions at the same glucose concentration ($p < 0.05$ by one-way ANOVA). Adapted with permission from (155).

There was an observed range in the final diameter of formed pseudo-islets. It was unclear if alpha cells were regulated differently across all sizes of formed pseudo-islets. It may be possible that larger pseudo-islets with more beta cells secrete more insulin and have an increased local concentration of insulin. This higher level of insulin may then modulate cAMP levels in neighboring alpha cells. To determine whether the different sized pseudo-islets were under different forms of regulation, we measured the levels of cAMP in alpha cells as a function of the pseudo islet diameter (Figure 27). There was a minimal, but not significant correlation between pseudo-islet size and alpha cell specific cAMP levels (R^2=0.1264).
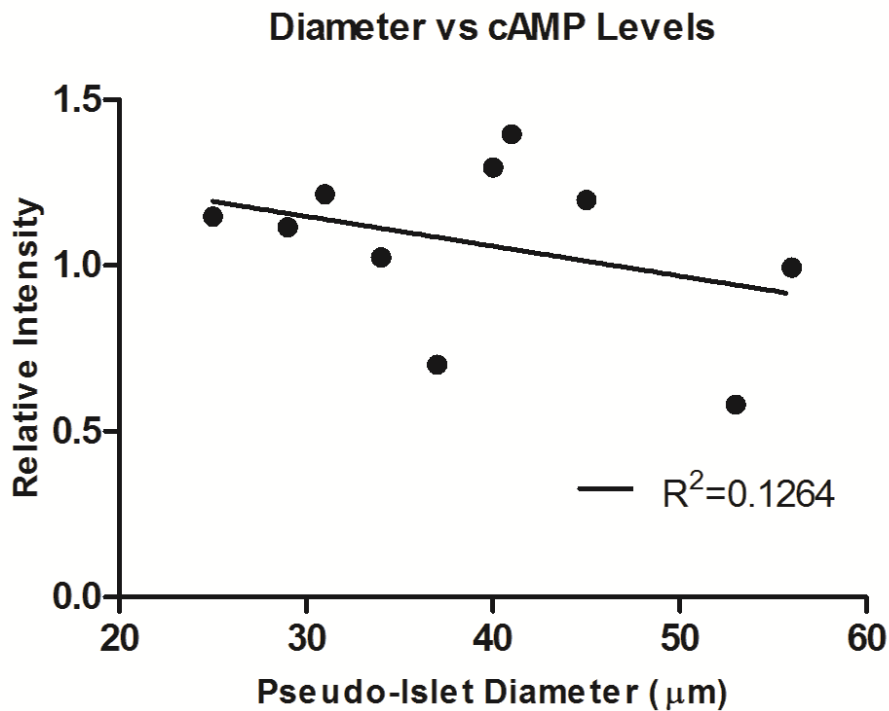
Figure 27: Pseudo-Islet Diameter Versus Relative Alpha Cell cAMP Intensity. The relative fluorescence level of cAMP measured in alpha cells at 1 mM glucose from 10 pseudo-islets plotted against the diameter (μm). A line of best fit was found to have a slope of -0.01 ± 0.01, with $R^2 = 0.1264$ (p = 0.3). Adapted with permission from (155).

4.3.2   Modulating of cAMP, F-Actin Density, and Glucagon Secretion in Pseudo-Islets

Since pseudo-islets recover islet-like levels of both cAMP and F-actin density upon formation, we aimed to determine if these pseudo-islets exhibit cAMP and juxtacrine signaling similar to intact islets. To assess this, pseudo-islets were treated with known stimulators of the cAMP and EphA4 pathways; the phosphodiesterase inhibitor 3-isobutyl-1-methylxantine (IMBX) combined with the adenylyl cyclase stimulator forskolin (Fsk), and the EphA stimulator ephrinA5-fc, or both. In pseudo-islets, IBMX/Fsk stimulates the formation of cAMP in alpha cells at 11 mM glucose, while ephrinA5-Fc does not alter cAMP levels. Dual treatment has no additional effect on cAMP levels (Figure 28A). Neither IBMX/Fsk, ephrinA5-Fc, nor the combination of both alters F-actin density in pseudo-islet alpha cells (Figure 28B).
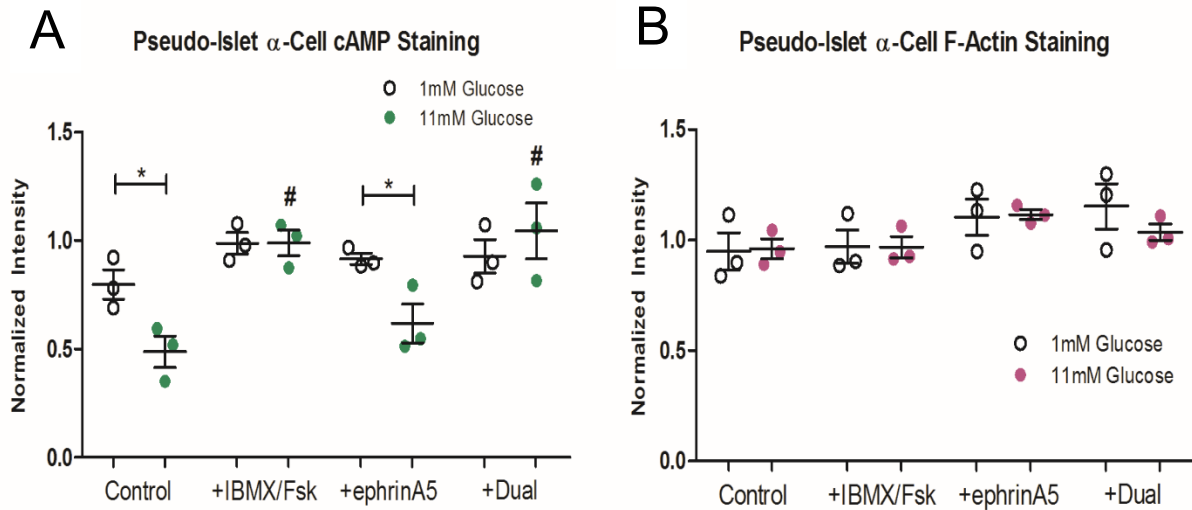
Figure 28: Pseudo-Islet Alpha Cell cAMP and F-actin In Response to IMBX/Fsk and EphrinA5. Quantification of alpha cell specific cAMP (A) and F-actin (B) levels in pseudo-islets at 1 mM and 11 mM glucose in response to 100 μM IMBX and 50 μM Fsk, 4 μg/mL ephrinA5-Fc, or both; normalized to islet levels at 1 mM glucose (n = 3 mice with >15 alpha cells/mouse). Data represent samples collected from 3 mice. Error bars represent standard error of the mean. Asterisks (*) label differences between 1 mM and 11 mM glucose; hash marks (#) label differences between control and conditions at the same glucose concentration (p < 0.05 by one-way ANOVA). Adapted with permission from (155).

The levels of cAMP and F-actin in pseudo-islet alpha cells both appear to be regulated in a similar manner to alpha cells within intact islets. Even though we know cAMP and F-actin are involved in glucagon release, it is unclear how, or whether these pathways have crosstalk in order to regulate glucagon secretion in response to glucose. We hypothesized that while IMBX/Fsk can potently stimulate glucagon secretion, EphA4 forward signaling can block glucagon secretion downstream of cAMP-dependent regulators. To test this hypothesis, glucagon secretion was measured from islets and pseudo-islets treated with IMBX/Fsk, ephrinA5-Fc, or both (Figure 29A-B). Treatment with IMBX/Fsk, which elevates cAMP in alpha cells, stimulated glucagon release at 11 mM glucose. Treatment with ephrinA5-Fc, which does not increase F-actin density in alpha cells, further inhibited glucagon release at 1 mM glucose. In

combination, IMBX/Fsk failed to overcome ephrinA5-Fc inhibition of glucagon secretion in both

islets and pseudo-islets.
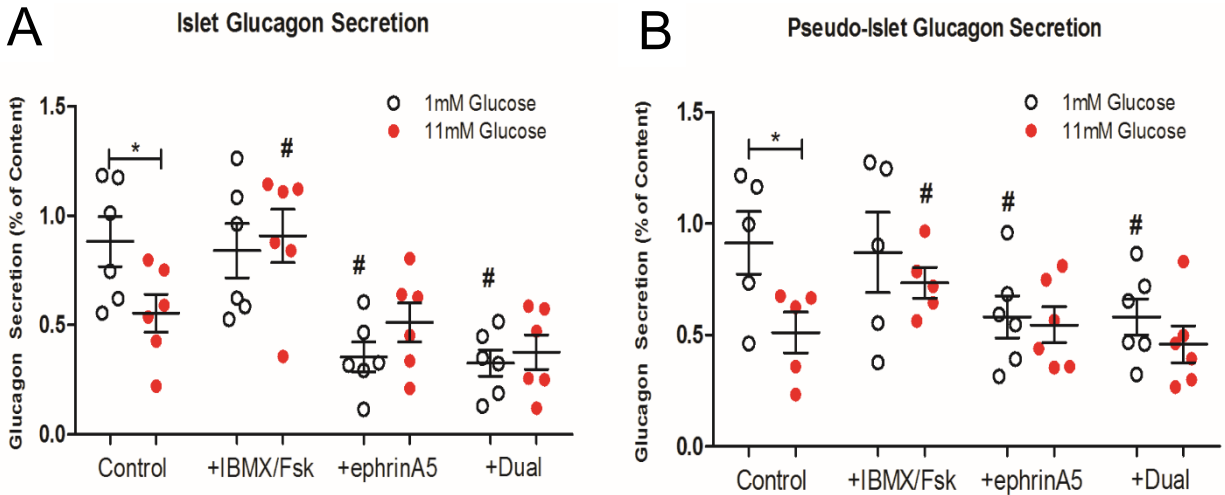
Figure 29: Pseudo-Islet Glucagon Secretion in Response to IMBX/Fsk and EphrinA5. Glucagon secretion at 1 mM and 11 mM glucose was measured from mouse intact islets (A), and pseudo-islets at 72 hours (B) in response to 100 μM IMBX and 50 μM Fsk, 4 μg/mL ephrinA5-Fc, or both (n = 6 mice). Error bars represent standard error of the mean. Asterisks (*) label differences between 1 mM and 11 mM glucose; hash marks (#) label differences between control and conditions at the same glucose concentration ($p < 0.05$ by one-way ANOVA). Adapted with permission from (155).

The observed changes in cAMP and F-actin in the dispersed state suggest these pathways

have become misregulated by dispersion (Figure 26). However, it is unclear if these pathways

remain functional and responsive after dispersion. To test this, we treated dispersed alpha cells

with both IMBX/FSK and ephrinA5-Fc, and measured glucagon secretion and F-actin density.

IBMX/Fsk treatment had no effect on dispersed alpha cell glucagon secretion or F-actin density.

EphrinA5-Fc treatment increased F-actin density in dispersed alpha cells, which correlated with

a decrease in glucagon secretion (Figure 30A-B). The ability of ephrinA5-Fc to stimulate the

formation of F-actin density in dispersed alpha cells, but not pseudo-islets suggests that alpha

cells in pseudo-islets have reestablished islet-like EphA4/7 forward signaling with neighboring

beta cells.
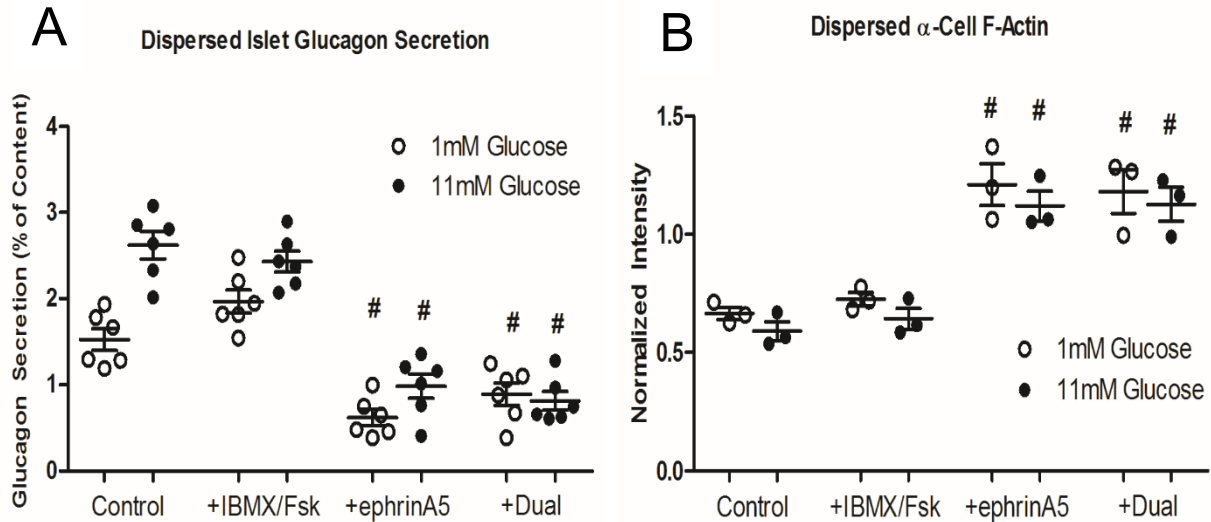
Figure 30: Dispersed Alpha Cell Glucagon Secretion in Response to IMBX/Fsk and EphrinA5. Glucagon secretion (A) and F-actin levels (B) were measured from dispersed α-cells at 1 mM and 11 mM glucose treated with 100 µM IMBX and 50 µM Fsk, 4 µg/mL ephrinA5-Fc, or both. Data represent samples collected from 6 (A) and 3 (B) mice. Error bars represent standard error of the mean. Asterisks (*) label differences between 1 mM and 11 mM glucose; hash marks (#) label differences between control and conditions at the same glucose concentration ($p < 0.05$ by one-way ANOVA). Adapted with permission from (155).

4.3.3   Selective Pseudo-Islets: Alpha/Beta

Using transgenic animals that express fluorescent proteins in specific islet cells, we were

able to isolate pure populations of alpha and beta cells and generate pseudo-islets independent of

other islet cell types. Using this approach, any treatments will affect only the present cell types,

minimizing complex indirect effects that could modulate glucagon secretion. We generated

pseudo-islets composed of only alpha and beta cells (Figure 31A) and measured glucagon

secretion in response to proposed regulators at 72 hours (Figure 31B). Without the presence of

delta cells and somatostatin, glucagon release in response to high glucose was not suppressed in

alpha/beta pseudo-islets. The addition of exogenous somatostatin potently inhibited glucagon

secretion, similar to what was measured from intact islets at 11 mM glucose. We also blocked

proposed glucagon modulators originating from the beta cell. Treatment with the selective

EphA2/4 inhibitor DPHBA disrupted the reformed EphA4 forward signaling, and resulted in

glucagon secretion similar to dispersed alpha cells. Antagonism of the insulin receptor with the

small molecule S961 enhanced glucagon secretion, supporting the paracrine model in which

insulin secreted by neighboring beta cells contributes to the inhibition of glucagon secretion.
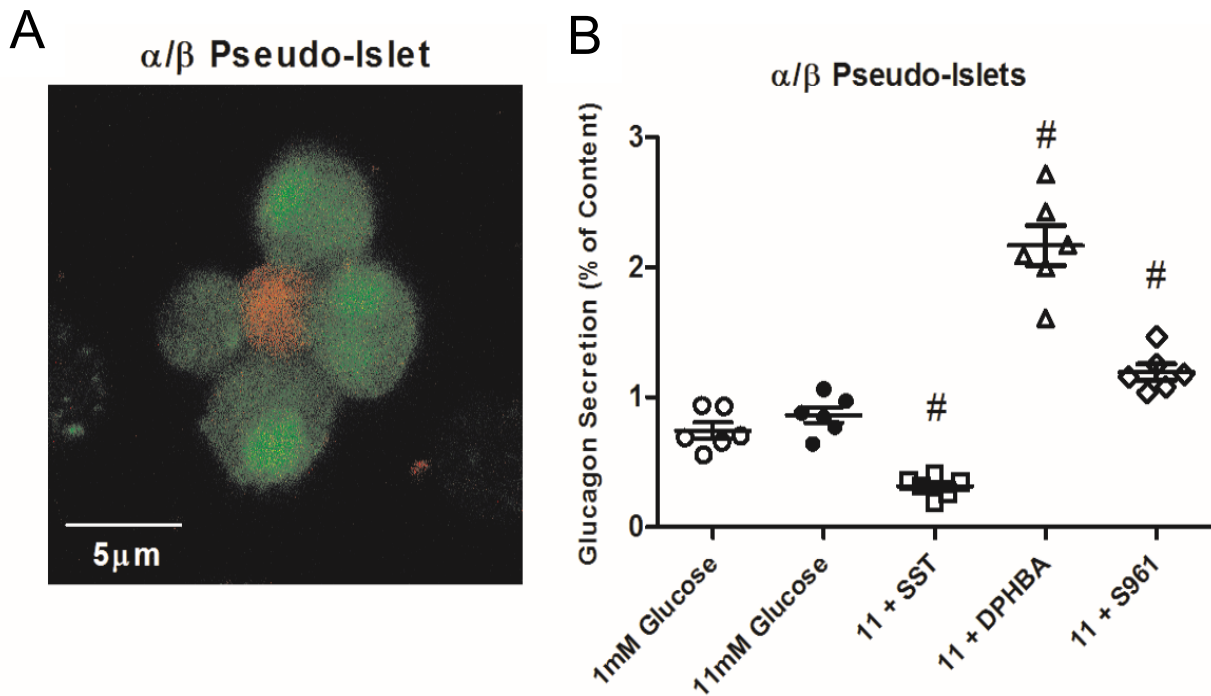
Figure 31: Glucagon Secretion from Alpha/Beta Pseudo-Islets. A) Representative image of an alpha cell surrounded by beta cells in an alpha/beta cell pseudo-islet at 72 hours. Alpha cells (red) were identified by transgenic RFP expression while beta cells (green) were identified by GFP expression. B) Glucagon secretion from alpha/beta cell pseudo-islets treated with 1 mM or 11 mM glucose with or without 100 nM somatostatin (SST), 12.5 μM DPHBA, or 1 μM S961 (n = 6 mice). Asterisks (*) label differences between 1 mM and 11 mM glucose; hash marks (#) label differences between control and conditions at the same glucose concentration ($p < 0.05$ by one-way ANOVA). Adapted with permission from (155).

4.3.4   Selective Pseudo-Islets: Alpha/Delta and Alpha Only

Due to the heterogeneity reported for alpha cell and delta cell arrangement in islets, it is unclear if alpha cells form functional cell-to-cell contacts with delta cells or other alpha cells in an intact islet (23,25,26). To examine this further, we attempted to create pseudo-islets from purified alpha cells with or without delta cells. Unfortunately, alpha cells fail to aggregate with delta cells or by themselves, even after 72 hours in culture. Both purified alpha cells with delta

cells and alpha cells alone secreted glucagon similar to dispersed cells (Figure 32A-B). The

addition of paracrine factors, including insulin and somatostatin, and the EphA4/7 stimulator

ephrinA5-Fc all inhibited glucagon secretion, which is maximally inhibited by combination

treatment. The basal level of glucagon secretion from alpha and delta cell co-cultures also

appeared lower than purse alpha cell cultures (Figure 32A vs B). This difference might be due to

tonic inhibition by the release of somatostatin from delta cells in culture.
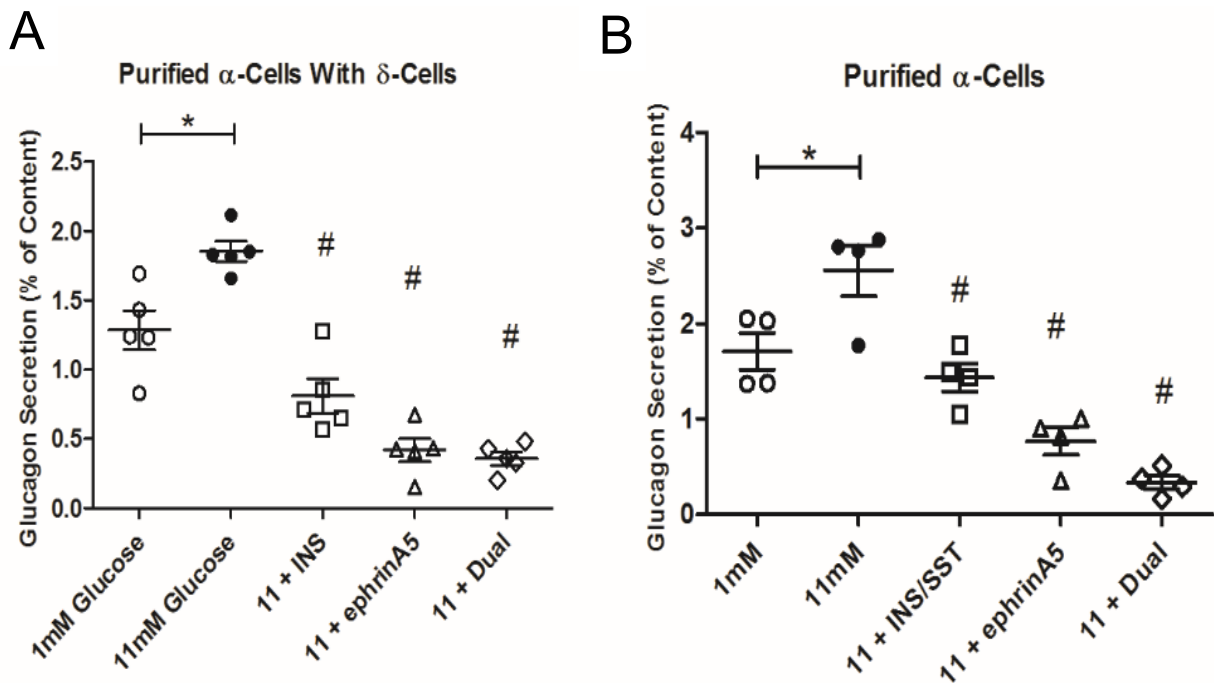
Figure 32: Glucagon Secretion from Alpha/Delta Cells and Alpha Cells Alone. A) Glucagon
secretion from alpha cells in co-culture with delta cells treated with 1 mM or 11 mM glucose
with or without 1 μM insulin, 4 μg/ml ephrinA5-Fc, or both (n = 5 mice). B) Glucagon
secretion from alpha cells alone treated with 1 mM or 11 mM glucose with or without 1 μM
insulin and 100 nM somatostatin, 4 μg/ml ephrinA5-Fc, or both (n = 4 mice). Asterisks (*)
label differences between 1 mM and 11 mM glucose; hash marks (#) label differences
between control and conditions at the same glucose concentration ($P < 0.05$ by one-way
ANOVA). Adapted with permission from (155).

4.3.5   Selective Pseudo-Islets: Beta Only

The inability of purified alpha cells and delta cells to form aggregates may be due to the lack of supporting beta cells. Data suggest that beta cells are the cell type that drives the formation of islets and pseudo-islets (25). To determine if pure beta cell populations form functional pseudo-islets, we generated beta cell only pseudo-islets (Figure 33A) and measured insulin secretion (Figure 33B). Beta cell pseudo-islets exhibited near-normal GSIS and secrete insulin at islet-like levels at 72 hours. The incomplete inhibition of insulin secretion at low glucose in beta cell pseudo-islets might be due to the lack of delta cells and somatostatin signaling.
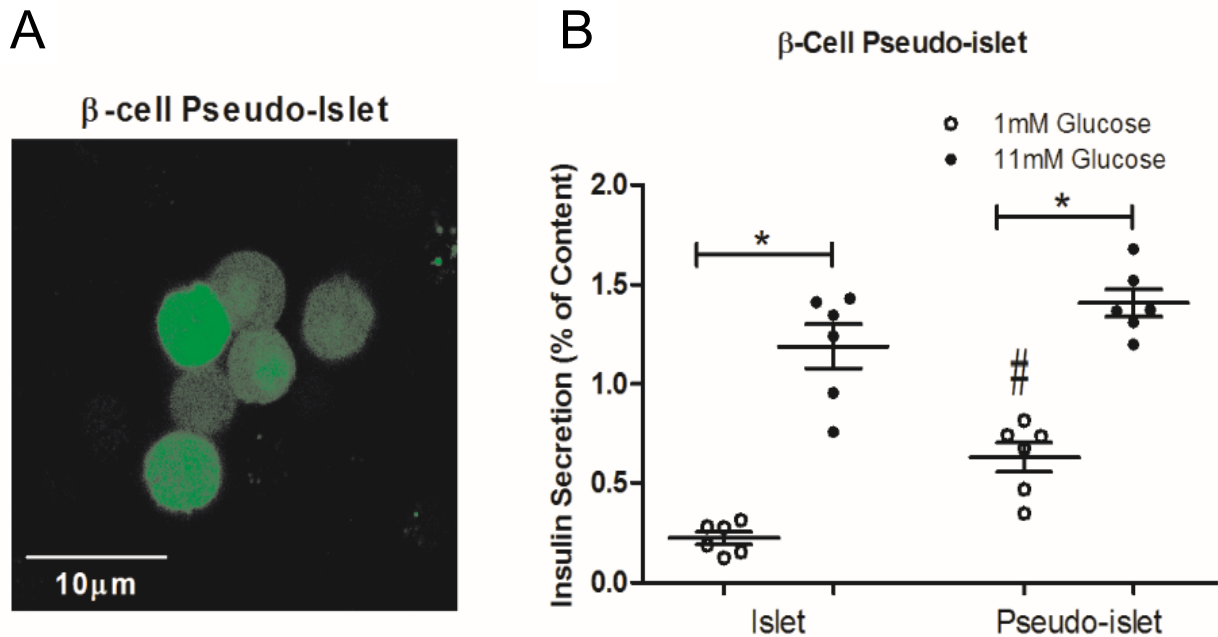
Figure 33: Insulin Secretion from Beta Only Pseudo-Islets. A) Representative image of beta cell pseudo-islets at 72 hours. B) Insulin secretion from beta cell pseudo-islets treated with 1 mM or 11 mM glucose (n = 6 mice). Error bars represent standard error of the mean. Asterisks (*) label differences between 1 mM and 11 mM glucose; hash marks (#) label differences between control and conditions at the same glucose concentration (p < 0.05 by one-way ANOVA). Adapted with permission from (155).

4.3.6   Recovery of Pseudo-Islets after FACS

It was unclear whether the partial or incomplete recovery of hormone secretion from both alpha and beta cells in selective pseudo-islets was due to the specific absence of certain factors or a possible artifact introduced by FACS. To eliminate the possibility that FACS causes cellular damage that alters pseudo-islet recovery or function, we generated all cell-type pseudo-islets using cells that had undergone the same FAC sorting as labeled cells. The FACS pseudo-islets did not show a difference in either the rate of recovery or the amplitude of glucagon secretion compared to other non-sorted pseudo-islet samples (Figure 34).
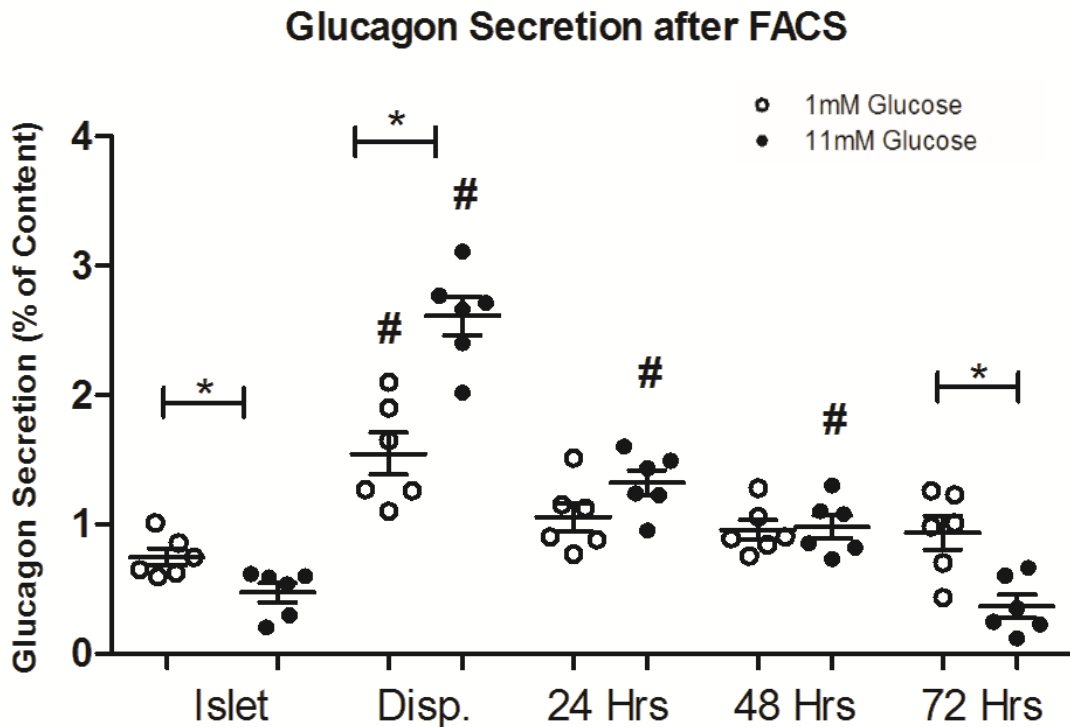
Figure 34: Glucagon Secretion from FAC Sorted Pseudo-Islets. Glucagon secretion was measured from intact, dispersed, and forming pseudo-islets from all cells recovered after FACS. The small percentage (<5%) of undispersed doublet cells or any remaining cell clusters were removed by FACS. Data represent samples collected from 6 mice. Error bars represent standard error of the mean. Asterisks (*) label differences between 1 mM and 11 mM glucose; hash marks (#) label differences between control and conditions at the same glucose concentration ($p < 0.05$ by one-way ANOVA). Adapted with permission from (155).

4.4   Discussion

4.4.1   Recovery and Modulation of cAMP and F-Actin in Pseudo-Islet Alpha Cells

The recovery of regulated glucagon secretion appears to depend on the restoration of GSIS from beta cells, as well as cell-to-cell contacts between alpha and beta cells. Some of these changes might also be attributed to the recovery of proper somatostatin secretion from delta cells in response to glucose. As insulin becomes maximally secreted from beta cells, cAMP levels

recover in pseudo-islet alpha cells (Figure 14B and 26B). The levels of cAMP do not appear to depend on pseudo-islet diameter (Figure 27).

The contact of beta and alpha cells in small pseudo-islets appears to provide the necessary ephrin stimulation to activate EphA4/7 forward signaling and stimulate the formation of F-actin like seen in intact islets (Figure 26C). In beta cells, F-actin density is dynamically regulated in order to prevent aberrant insulin release at low glucose and enhance secretion at high glucose (180). In alpha cells, F-actin density appears more static and in place to tonically inhibit maximal glucagon secretion (34,155). When EphA signaling is lost and this density decreases, as seen in the dispersed state or when EphA4 signaling is inhibited, glucagon secretion increases significantly. Recovery of this basal density by pseudo-islet formation or EphA4/7 activation by ephrinA5-Fc potently inhibits glucagon secretion (Figure 29 and 30).

It is important to indicate that ephrinA5-Fc only minimally increases F-actin density in islets and pseudo-islets, but potently inhibits glucagon secretion. This may indicate a change in F-actin matrix stability or remodeling, rather than an increase in the overall F-actin density. In neurons, changes in intracellular calcium and cAMP have been shown to alter F-actin density through downstream regulators (183,184). This is not the case in alpha cells, where the forced formation of cAMP does not alter the levels of F-actin, even in the dispersed state when EphA4/7 stimulation is absent.

4.4.2   Selective Pseudo-Islets

While flow sorted populations of individual islet cells have been successfully isolated and interrogated previously (64,159,185), the creation of pseudo-islets from specific fluorescent-protein labeled islet cells provides additional insights into islet signaling and glucagon

regulation. Cellular damage from dispersion or FACS appears negligible in the recovery of normal hormone secretion from pseudo-islets (Figure 15 and 34). Selective pseudo-islets composed of only alpha and beta cells do not recapitulate normal glucose inhibition of glucagon secretion (Figure 31). In this case, though, the addition of somatostatin, independent of any other delta cell signal, leads to proper inhibition of glucagon secretion. This inhibition is consistent with the lowering of cAMP levels by insulin and somatostatin as previously described (36).

We observed that alpha cells cultured alone or with delta cells fail to form pseudo-islets, suggesting no additional cell-to-cell contacts exist between themselves or delta cells. This is further supported by the observed enhanced glucagon secretion in response to glucose from those alpha cells. Basal glucagon secretion is lowered when alpha cells are co-cultured delta cells, putatively due to the basal release of somatostatin (Figure 32A vs Figure 32B). Combination treatment with insulin and somatostatin to suppress cAMP levels and ephrinA5-Fc to stimulate F-actin formation appears to maximally inhibit glucagon secretion from pure alpha cells or alpha/delta cell co-cultures. These secretion data are consistent with our hypothesis that the ephrinA ligand signal, coming from the beta cells and not the delta cells, works additively with insulin and somatostatin to inhibit glucagon release. The ability of beta cells to form pseudo-islet in pure cultures argues that the beta cell provides the necessary cell-to-cell contacts required for islet integrity and might act as a sight of nucleation during formation (Figure 33).

4.4.3 Glucose Regulated Glucagon Secretion

Altogether, our data indicate that glucagon secretion is regulated by multiple independent pathways. EphA4/7 signaling in alpha cells appears to require ephrinA ligands from beta cells, and upon stimulation, these receptors regulate F-actin density to provide a secretion barrier for

glucagon release. However, this F-actin density appears permissive and independent of glucose, which is consistent with glucagon secretion falling only to ~50% of its maximal level during glucose stimulation. EphA4/7 signaling does not appear to directly affect alpha cell cAMP levels, which instead are lowered by paracrine signals, including insulin and somatostatin. Thus, regulation of cAMP acts as an independent mechanism of indirect glucose regulation on glucagon secretion. Finally, our data support a mechanism where the independent paracrine and juxtacrine pathways, which fail to fully suppress glucagon release alone, act additively to maximally inhibit glucagon secretion in response to glucose.

Chapter 5

General Discussion, Implications, Future Directions, and Significance

5.1   Summary

In this dissertation, we studied GPCR regulation of hormone secretion from islet cells, recovery of normal hormone secretion in pseudo-islets, and how pseudo-islets can be utilized to interrogate regulatory mechanisms central to islet cell function. The hypotheses and experiments presented were designed to further our understanding of the mechanisms utilized *in vivo* to maintain glucose homeostasis through regulated hormone secretion. The field of beta cell research, encompassing nearly 100 years of publications, has yielded countless discoveries and a well-characterized model for the regulation of insulin secretion in response to glucose and other endogenous ligands. We introduced additional mechanistic details in response to endogenous ligands that target the beta cell. These findings will aid in the development and implementation of future T2D therapies.

While the field of alpha cell research is rapidly expanding, a consensus model for the regulation of glucagon secretion has yet to be established. The characterization of pseudo-islets and the recovery of regulated secretion demonstrated in Chapter 3 enabled subsequent studies on selective pseudo-islets and alpha cell function. In Chapter 4, we described many of the factors that are expected to contribute to a complex, multifactorial model for the regulation of glucagon secretion. Based on our data, we hypothesize that paracrine and juxtacrine signals inhibit glucagon secretion by acting downstream of pathways that regulate calcium. Specifically, our results suggest that EphA4 signals, along with insulin and somatostatin, inhibit glucagon secretion independent of calcium (Figure 35).
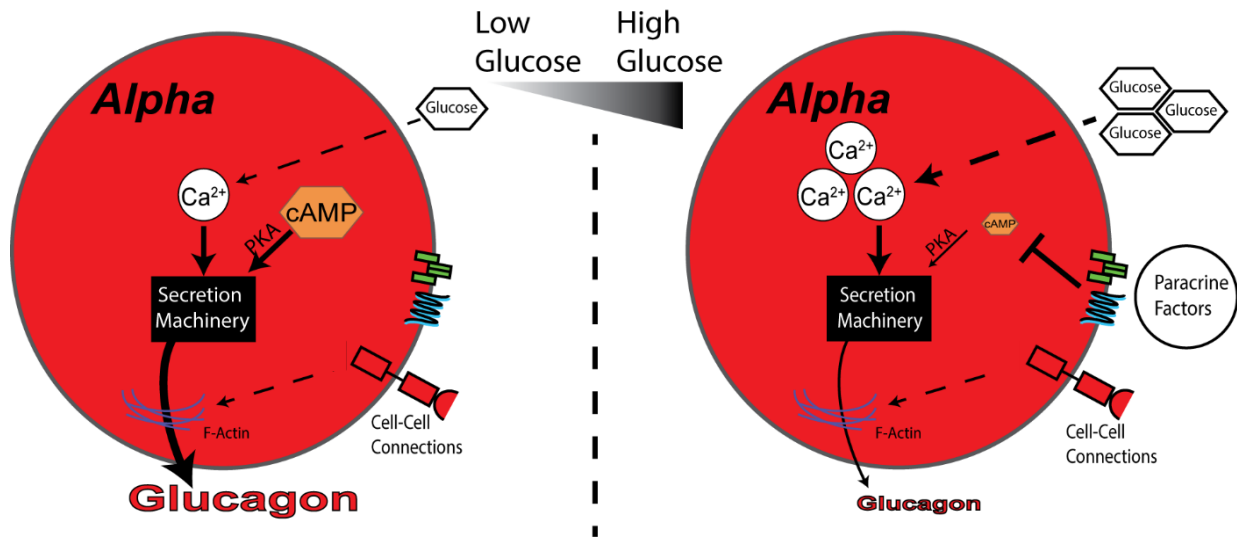
Figure 35: Regulation of Glucagon Secretion from Alpha Cells within an Intact Islet. At low glucose, glucagon secretion is regulated, in part, by the levels of calcium, cAMP and the presence of cell-to-cell connections. As glucose levels rise, so does calcium. However, the increased secretion of paracrine factors from other cell types in the islet inhibit the formation of cAMP and suppress glucagon secretion independent of calcium.

To support this model, more research needs to be conducted in several key areas. While studying alpha cells within the intact islet is possible in select cases, it is difficult due to alpha cells being a minority cell population within the overall islet. The creation and use of pseudo-islets, enables novel, targeted approaches to understanding the mechanisms underlying alpha cell function. Future studies should aim to elucidate specific steps of the signaling cascades that include calcium, cAMP, and F-actin. Identifying specific differences between the alpha cell and the other cell types, like alpha cell-specific EphA4, will be a crucial step toward discovering therapies that only modulate glucagon secretion. To elaborate, if the alpha cell has a unique protein or receptor not found in beta cells, it may be possible to target that factor to decrease glucagon secretion independent of changes to insulin secretion. As a future therapeutic avenue, it may be possible to target EphA4 on alpha cells to suppress glucagon from alpha cells.

5.2   GPCR Regulation of Beta Cells

5.2.1   Implications

GPCR signaling is a prime candidate for pre-clinical research and potential therapeutic intervention since these receptors have an extracellular, targetable domain and their downstream signaling often affects pathways specific to that receptor (105). The abundance of GPCRs present on the surface of islet cells supports the need for studies to identify putative ligands that can modulate hormone secretion (117,186). Identified ligands, like KP and GLP-1, show great promise due to their ability to potentiate insulin secretion, as shown in Chapter 2.

While the current body of work on KP in the islet is limited in scope, it is clear that KP has an insulin-potentiating effect on beta cells (126,129,187). It is unclear how KP exerts its effect on beta cells and whether the effect is physiologically relevant. Since KP affects other organ systems (123,124,188), systemic treatment with KP-based therapies would need thorough analysis prior to utilization as a T2D therapy due to possible off-target effects.

On the other hand, GLP-1 analogs have already been developed and are used clinically (189–191). GLP-1 potentiates insulin secretion through a $G_{\alpha s}$ mechanism that increases the levels of intracellular cAMP (120,132,180). While this might be the primary signaling avenue of the GLP-1 receptor, data suggest that alternative signaling cascades may also be present in the beta cell (129,192). For both KP and GLP-1, detailed mechanisms of action within the beta cell are incomplete. While data presented in Chapter 2 adds to our understanding of those mechanisms, uncertainties still remain.

5.2.2   Future Directions

Based on our KP data and previous publications looking at KP signaling through GPR54, we hypothesize that KP signals through a calcium-independent $G_{\alpha q}$ mechanism in beta cells (123,125,126,129). This hypothesis could be tested using simple, but direct follow up. Since the $G_{\alpha q}$ mechanism has a characteristic signaling cascade (105,108), we would expect to see increases in the levels of DAG and PLC/PKC activity in beta cells upon KP stimulation. To rule out a possible $G_{\alpha s}$ mechanism, it would be beneficial to determine if beta cell cAMP levels change in response to KP treatment. Subsequent studies should also aim to understand the *in vivo* role for KP in insulin secretion and glucose homeostasis.

While we assume the primary signaling pathway goes through $G_{\alpha q}$ for KP and $G_{\alpha s}$ for GLP-1, our data (Figure 9A vs 11) suggest that secondary pathways may also be utilized by the receptors. For example, GLP-1 can alter calcium dynamics in dispersed beta cells but not in intact islets, suggesting GLP-1 signaling directly alters calcium dynamics in some fashion (120,128,129). Since the effect of GLP-1 on cAMP is well characterized, future work should determine if the $G_{\beta \gamma}$ subunits have relevant roles in the beta cell within an intact islet. $G_{\beta \gamma}$ subunit activation has been demonstrated to modulate calcium (193–196) and potassium channels (116,197) in other tissues. To determine if this is the case in beta cells, fluorescence resonance energy transfer (FRET) assays, utilizing tagged $G_{\beta \gamma}$ subunits along with other tagged membrane ion channels, could reveal novel interactions between GLP-1 signaling and ion regulation. Using FRET, changes in association between the fluorescent markers on the $G_{\beta \gamma}$ and the tagged ion channels could be measured when beta cells are treated with GLP-1. If there is an increase in association, as determined by the increase in FRET ratio, this would suggest that the $G_{\beta \gamma}$ subunits of GLP-1 signaling may modulate calcium regulation independent of cAMP. If this

is the case, consequent studies should aim to determine if this signaling has regulatory roles in glucose stimulated insulin secretion.

5.3   Pseudo-Islets and Transplantation

5.3.1   Implications

The interplay and collaboration between the islet cell types through paracrine signals and cell-to-cell contacts to regulate hormone secretion should not be overlooked when experimenting with intact islets and comparing data from dispersed and intact islets. For example, the direct and indirect effects of GLP-1 treatment (Figure 12) highlight the importance of a holistic view of the islet when considering the effects of a hormone or drug on a single hormone's secretion. Based on the initial observation that GLP-1 potentiated insulin secretion and inhibited glucagon secretion *in vivo*, it was assumed that GLP-1 could be used as an anti-glucagon therapy in T1D (88,131,189,190). This turned out to be only partly true. Since GLP-1 inhibits glucagon secretion indirectly through both beta and delta cells (Figure 12), much of the inhibitory effect is lost when beta cells are absent, like in T1D. While GLP-1 does slightly improve glycemia in T1 diabetics, this treatment paradigm in these patients has had marked deleterious effects, including pancreatitis (189,198,199). Complications might have been avoided with a better understanding of the possible effects of GLP-1 on non-beta cells.

The problem of indirect effects is a difficult hurdle to overcome when studying alpha cells since they are a minor population in the islet compared to beta cells. While intact islet studies are the "bread and butter" model for the field, the pseudo-islet model deserves strong consideration in islet studies due to its ability to limit and control indirect effects. Pseudo-islets provide an easily manipulated, reductionist model to study specific paracrine and cell-to-cell

interactions. The resulting insulin and glucagon responses to glucose and other stimuli are remarkably similar to what is measured *in vivo* and *ex vivo* (Figures 14 and 18). While the importance of the size component of pseudo-islets needs further exploration, it is clear that small pseudo-islets can also recapitulate the islet state like larger pseudo-islets (140,155). This is of particular relevance since it has long been postulated that size is a critical component to islet function and pseudo-islet dimensions must be comparable to intact islets for proper hormone secretion and function (139–141,144,200).

A primary use for pseudo-islets has been in transplantation and beta cell mass replacement studies (141,144). This current research direction is based upon the idea that pseudo-islets are superior to intact islets for transplantation and can further optimize beta cell mass transplantation therapies. It has been demonstrated that fully-formed pseudo-islets had improved vascularization compared to intact islets after transplantation (140,144,201). The increased transplant success was attributed to the homogeneous nature of the pseudo-islets. It may also be possible to further enhance engraftment and vascularization by transplanting forming, rather than formed, pseudo-islets.

As we followed the process of pseudo-islet formation, the islet cells appear to enter a plastic state between the time of dispersion and full formation. Here, plastic refers to being easily shaped or molded, which is different from plasticity, or genotypic flexibility. In an intact islet, the signals that regulate islet cell migration are maintained at low levels, but increase upon dispersion (202). This is visibly observed in the enhanced movement and shaping of islet cells post-dispersion when pseudo-islets are forming (Figure 13). The plastic state might be the key to optimizing transplant studies since forming pseudo-islets are primed to migrate and aggregate. If

pseudo-islet formation occurs within the host tissue, this may improve vascularization and engraftment.

5.3.2   Future Experiments

To examine the possibility that transplantation of forming pseudo-islets might improve transplant success, a kidney capsule transplant model can be used. In this experiment, islets, forming pseudo-islets, and formed pseudo-islets would be transplanted under the kidney capsule of healthy and diabetic mice. Changes in glycemia, glucose tolerance, and circulating levels of insulin would be measured. At a fixed end-point, kidneys containing the transplants would be recovered, fixed, sectioned, and analyzed to quantitate vascularization and other aspects of transplant health. Since infiltrating endothelial cells and macrophages will be incorporated in the pseudo-islet as it forms, we might expect higher levels of vascularization and lower levels of pro-inflammatory macrophages or cytokines in these transplants. This would suggest that the forming state was optimal for transplantation. If forming pseudo-islets perform best, this would merit a reconsideration of current methodologies of beta cell mass transplant therapies, wherein human donor islets should be dispersed and allowed to start forming pseudo-islets prior to transplantation in the recipient to promote vascularization and engraftment.

5.4   Selective Pseudo-Islet Studies

5.4.1   Implications

As shown in Figure 31-33, selective pseudo-islets can be used to interrogate specific functions of various islet cell types in novel combinations. Data collected from selective pseudo-islets support an alpha cell model in which glucagon secretion is regulated by multiple signals

originating from other islet cells (34,36,155). Furthermore, these data support the idea that the correction of dysfunctional glucagon secretion in diabetic patients will require combination therapies that modulate different pathways simultaneously. Future studies and experiments should consider perturbations to more than one pathway simultaneously. Undiscovered interplay and crosstalk may exist between intrinsic, paracrine, and juxtacrine regulation within the alpha cell. While multi-variable experiments will be inherently complicated, the current models suggest regulation is more complex than previously assumed. However, some of this complexity can be overcome using the selective pseudo-islet combinations formed from FAC sorted islet cells as described in Chapter 4. With fewer indirect effects, novel experimental paradigms can be established to give more specific alpha cell results. A few of the new possibilities are included and described in the following sections.

The ability to readily identify the islet cells at different time points throughout pseudo-islet formation opens the door to novel RNA-based studies too. For example, RNA from intact islets, dispersed cells, forming, and formed pseudo-islets could be collected and analyzed with a RNA-Seq-based approach to measure gene expression changes throughout recovery. These data could reveal unique transcriptional changes relevant to islet cell dysfunctional and pseudo-islet formation/recovery.

5.5   Alpha Cell Heterogeneity and Calcium Dynamics

5.5.1   Implications

The cell-intrinsic model of alpha cell function fails to fully explain the regulation of glucagon secretion in response to glucose (64,65,167). While it is accepted that calcium is required for secretion (32,70,162), the role calcium plays between low and high glucose remains

unclear. Unlike gap junction coupled beta cells (33,60,61,133), islet alpha cells appear electrically isolated from neighboring cells (64). To this effect, alpha cells within an intact islet and in the dispersed state have different populations of calcium responses and dynamics (64). The differences in calcium dynamics between dispersed alpha cells and those in the intact islet suggest the islet contributes to alpha cell calcium regulation in some fashion. Our data suggest that an inclusion of paracrine or cell-to-cell influences may reconcile the discrepancies observed in the intrinsic model.

In Figure 14D, we showed that the percentage of active cells, as defined by the presence of significant elevations in Fluo-4 intensity during the time-course, does not change between the intact, dispersed, and pseudo-islet states. While these data appear contradictory to the idea proposed above, it may be possible to parse out novel results if additional parameters are included in Fluo-4 calcium data analysis, like oscillation frequency, duration, or alpha cell proximity to the other cell types. This begs the question: do alpha cells behave in specific ways when in close proximity with beta or delta cells? For instance, an alpha cell next to a delta cell might have a different response compared to a lone alpha cell.

5.5.2   Future Directions

The question as to whether alpha cells behave differently depending on their proximity to beta or delta cells leads to a variety of experiments, which would benefit greatly from the selective pseudo-islet technique described in Chapter 4. Calcium activity measurements could be gathered from alpha/beta pseudo-islets or co-cultures of alpha and delta cells. In addition, selective pseudo-islets would be treated with modulators that stimulate or disrupt insulin, somatostatin, or EphA4/7 signaling. One possible outcome could be that alpha cells in close

proximity to delta cells have decreased calcium activity compared to independent alpha cells. Altogether, these data could aid in reconciling the heterogeneity of calcium responses observed in both intact islets and dispersed alpha cells.

5.6   Alpha Cell Paracrine and Juxtacrine Regulation

5.6.1   Implications

Based on observations that calcium levels increase in alpha cells as glucose levels increase (50,64,167), it appears calcium has only a minor role in the regulation of glucagon secretion. While this point remains highly debated in the field (14,64,151,153,173), other recent publications have demonstrated that paracrine and juxtacrine signaling pathways have roles in the proper regulation of glucagon secretion (34,36,64,155). Our data supports a model where the paracrine and juxtacrine signaling pathways, acting independently and additively, regulate glucagon secretion of alpha cells independent of intrinsic calcium regulation (Figure 29, 31, and 32). However, several uncertainties arose from these studies.

First, it is unclear to what extent each pathway contributes to glucagon regulation *in vivo*. In our studies, pharmaceutical inhibitors and stimulators, like DPHBA or ephrinA5-Fc, appear to have potent on/off effects. These modulators might be a poor representation of the *in vivo* properties of these receptor/ligand interactions. For example, EphA4-ephrin interactions between alpha and beta cells *in vivo* may be more transient, while ephrinA5-Fc stimulation is maximal and long-lived. Furthermore, potent perturbations to one pathway, like ephrinA5-Fc stimulation of EphA4 signaling, might mask other regulatory changes occurring in other pathways, like calcium or cAMP signaling.

Second, the addition of ephrinA5-Fc can potently inhibit glucagon secretion without a measureable increase in F-actin density (Figure 28B and 29B). These data suggest averaged static fluorescence measurements collected might overlook dynamic changes in F-actin density that contribute to the regulation of glucagon secretion during our static secretion assay.

Third, the downstream regulators and intracellular cascades involved in both juxtacrine and paracrine signaling are unclear. We know EphA4 receptor stimulation results in a change in F-actin density, but no intracellular mediators have been identified in alpha cells. In beta cells, the inactivation of RhoA, a component of the cytoskeletal arrangement machinery, enhances insulin secretion (179). A similar mechanism might be present in alpha cells.

Additionally, we know that PKA plays an important role in cAMP signaling; however, it is unknown what PKA targets within alpha cells or how these targets might modulate secretion. In beta cells, complexin-1, a protein associated with calcium-dependent exocytosis regulated by PKA, plays a significant role in GSIS (203,204). Recent human alpha cell transcriptome data support the possibility that complexin-2 might be specific and critical to the regulation of glucagon secretion (55). This differential expression, which might also occur in other proteins, may allow a targeted approach to understand islet cell-specific secretion in future experiments (55,159–161).

5.6.2   Future Directions

As described in Chapter 4, the beta cell appears to provide both paracrine and juxtacrine signals that regulate glucagon secretion. Without beta cells, alpha cells fail to suppress glucagon secretion, even in the presence of delta cells and somatostatin (Figure 31). It would be of particular interest to knockdown or overexpress beta cell ephrinA5 in alpha/beta pseudo-islets

using adeno or lenti virus. One major caveat to viral expression systems in intact islet studies is the inability to infect all islet cells. Since the time course of infection and knockdown/overexpression is similar to pseudo-islet recovery (i.e. days), we could infect any or all islet cell types in the dispersed state to greatly increase the infection rate and allow pseudo-islet formation to occur simultaneously with viral changes. Specifically, knockdown of ephrinA5 could have two possible outcomes: enhanced glucagon secretion, confirming previous results, or a partial effect on glucagon secretion, suggesting other ephrins on the beta cells are also stimulating EphA4/7 signaling. With the overexpression of ephrinA5, we may observe more inhibition of glucagon secretion from EphA4/7 signaling. If overexpression does not further suppress glucagon secretion, these data would suggest EphA4/7 is fully bound by the endogenous levels of ephrinA5 in beta cells. These are important considerations when developing future therapies. If the EphA4/7 is partially bound in the normal state, then future drugs or molecules would have to have similar on/off kinetics to recapitulate the in vivo properties. If EphA4/7 is maximally bound in the normal state, then future drugs would need to potently bind and have slow off kinetics to continually stimulate forward signaling.

In T1D, patients lose beta cell mass from autoimmune destruction. The loss of beta cells and ephrinA stimulation results in a decrease in F-actin density in alpha cells and hypersecretion of glucagon. It is possible to imagine a therapy where the cells in close proximity of alpha cells are forced to express the ephrins necessary for glucagon inhibition. The preliminary experiments for this therapy would involve overexpressing ephrinA5 on alpha or delta cells in culture. We would infect sorted alpha or delta cells and determine if these cells, when cultured in close proximity with other alpha cells, recapitulate the cell-to-cell connections normally found

between beta and alpha cells. Even in the absence of insulin, we would hope to observe an inhibition of glucagon secretion through a recovery of F-actin density in alpha cells.

Our data suggest that the EphA4/7 signaling pathway modulates F-actin density and glucagon secretion (Figure 28-30). However, it is unclear if this density is static or dynamic during the steady-state. To determine real-time changes in F-actin, we could utilize Lifeact mice, which express a GFP-tagged F-actin (205). If crossed with our RFP alpha cell reporter mouse, we could determine alpha cell specific F-actin changes in response to glucose and additional stimuli over time using confocal microscopy in living islet cells. A decrease in GFP fluorescence should be observed upon the addition of DPHBA and in dispersed alpha cells that lack of EphA4 signaling. Conversely, an increase in GFP fluorescence should be observed upon the addition of ephrinA5 and during the formation of pseudo-islets. The fluorescence data may reveal rates of change upon modulation or possible fluctuations over time. If the average level of fluorescence remains constant, but the levels fluctuate greatly in response to stimuli, this may suggest a dynamic role for F-actin in secretion. If GFP fluorescence does not fluctuate, this confirms our current immunofluorescence data and model that F-actin is more permissive, not regulatory. In either case, intracellular mediators central to F-actin density need to be elucidated.

In sorted alpha cells, we could specifically target RhoA, a protein central to cytoskeletal regulation, for knockdown or overexpression to interrogate intracellular components of the juxtacrine signaling cascades. If RhoA does indeed regulate alpha cell F-actin density, then we would expect inhibited secretion when RhoA is overexpressed and enhanced secretion when RhoA is knocked-down. Also, if treatment with EphrinA5 fails to suppress glucagon secretion when RhoA is knocked-down, these data together would support the hypothesis that RhoA is involved in EphA4 signaling.

Similarly, we could specifically knockdown or overexpress Complexin-2 in sorted alpha cells to interrogate how paracrine signaling cascades regulate exocytosis elements. If complexin-2 knock-down decreases secretion while its overexpression inhibits secretion, this may suggest complexin-2 is required for glucagon secretion. Additionally, we can stimulate cAMP formation with IMBX/Fsk and determine if PKA specifically targets complexin-2 in alpha cells. Together, these data would support the possibility that complexin-2 is a novel piece of the exocytosis machinery in alpha cells.

5.7   Significance

The studies described, experiments completed, and future directions proposed in this dissertation are ideas that are central to modulating hormone secretion. We expect that better understanding of the pathways that govern regulated hormone secretion from the islet and dispersed beta and alpha cells will lead to improved maintenance of glucose homeostasis in patients with all forms of diabetes. Insulin-based therapies, including insulin injections and GLP-1 analogs, are routinely used in the clinical setting to alleviate hyperglycemia (132,189,191). Additional investigation of these treatments and the pathways they utilize may reveal novel methodologies to further enhance insulin-based treatment.

Insulin replacement therapy, while essential for managing the disease, has not provided a cure since the abnormalities in glucagon secretion still remain. Accordingly, glucagon-based therapies have been investigated, but they have yet to be implemented successfully in the clinic. Researchers have considered blocking glucagon receptor signaling through a variety of methods, all of which have great potential (18,206–209). These studies have demonstrated that blocking glucagon signaling can improve glycemia, but this treatment also results in hyperglucagonemia and alpha cell hyperplasia (18,210,211). Future work needs to determine if these deleterious

side-effects can be minimized or managed in order for glucagon-based therapies to be a viable clinical option (210,212,213).

As highlighted throughout this dissertation, we are only starting to understand and develop a complete model for the regulation of glucagon secretion *in vivo* and *in vitro*. As shown and described here, the complexity of alpha cell function has impeded the progress of developing glucagon-based therapies. Though there is great complexity, we can see that the alpha cell may contain important untapped therapeutic avenues for the treatment of both T1D and T2D.

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