The role of c-Kit and insulin receptor tyrosine kinases in beta cell function and insulin secretion

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Graduate Program in Pathology and Laboratory Medicine
A thesis submitted in partial fulfillment of the requirements for the degree in Doctor of Philosophy
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Abstract

The receptor tyrosine kinases (RTKs) c-Kit and insulin receptor (IR) initiate similar intracellular signalling pathways in pancreatic beta cells to regulate beta cell proliferation, survival, and insulin secretion. Mice with a c-Kit overexpression specifically in beta cells (c-KitβTg) demonstrated improved beta cell proliferation and insulin secretion compared to control mice, and islets from c-KitβTg mice also demonstrated increased IR expression. The potential interplay between c-Kit and IR and their roles during ageing and metabolic stress are not currently known. This work reports the examination of c-Kit and IR signalling using in vitro and in vivo models to determine their effects on beta cell proliferation and insulin secretion.

To examine the effects of prolonged c-Kit signalling, c-KitβTg mice were analyzed at 60 weeks of age. The results from this section demonstrated that c-KitβTg mice developed impaired insulin release due to reduced soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) expression. Increased IR and phosphorylated IRS-1<sup>S612</sup> and reduced insulin-induced Akt signalling were observed in islets from 60-week c-KitβTg mice. The effect of IR deficiency was examined by knocking down beta cell IR at 40 weeks in c-KitβTg mice and resulted in partial restoration of glucose tolerance. To determine the role of postnatal IR function on insulin secretion, a mouse model with beta cell-specific IR knockout (MIP-βIRKO) was generated and exposed to high-fat diet (HFD). MIP-βIRKO mice on HFD developed glucose intolerance due to reduced insulin secretion and SNARE expression. Islets from MIP-βIRKO HFD mice also demonstrated reduced Akt phosphorylation and GLUT2 levels. In vitro examination of INS-1 832/13 cells showed that co-stimulation of c-Kit and IR with ligands stem cell factor (SCF) and insulin, respectively, did not lead to synergistic intracellular signalling or proliferation when compared to single ligand treatments. This may be due to negative feedback from IRS-1<sup>S612</sup> phosphorylation, which can be inhibited with rapamycin.
In summary, this work presents a regulatory role for altered c-Kit and IR activity in maintaining beta cell intracellular signalling and insulin release. Results from these studies may be useful when considering RTK-based treatments to optimize islet function.

Keywords

Receptor tyrosine kinase, diabetes mellitus, beta cell, insulin release, c-Kit, insulin receptor, SNARE proteins, receptor interplay, tamoxifen-inducible Cre recombinase
Co-Authorship Statement

Amanda Oakie is the main author and contributor to the experimental work featured within this thesis. The following authors contributed to the chapters highlighted below:

**Chapter 1** is adapted from the following manuscript: Oakie A and Wang R. β-cell receptor tyrosine kinases in controlling insulin secretion and exocytotic machinery: c-Kit and insulin receptor. *Endocrinology*. 2018;159(11):3813-3821. RW contributed to the generation of the manuscript outline and the editing of the review.

**Chapter 3** is adapted from the following manuscript: Oakie A, Feng Z-C, Li J, Silverstein J, Yee S-P, and Wang R. Long-term c-Kit overexpression in beta cells compromises their function in ageing mice. Accepted to *Diabetologia* (in press, # Diab-18-1549). AO and ZCF share co-authorship on this manuscript. ZCF performed the partial collection and analysis of data from Figures 3.1, 3.2, and 3.3 and contributed to the drafting and revision of the manuscript. JL contributed to data collection and design, manuscript drafting, and the revision of the final approved manuscript. JS contributed to immunofluorescence staining and data collection and to the preparation of the submitted and revised manuscript. SPY contributed to the generation of the mouse lines used in this study and critical comments for both manuscript and revision preparation. RW contributed to the experimental design, interpretation of data, and manuscript preparation and revisions.

**Chapter 4** is adapted from the following manuscript: Oakie A, Zhou L, Rivers S, Cheung C, Li J, and Wang R. Postnatal deletion of beta cell insulin receptor affects insulin secretion in mice exposed to high-fat diet stress. Submitted to *American Journal of Physiology- Endocrinology and Metabolism*, April 2019 (#E-00165-2019). LZ contributed to the normal diet experiments in Figures 4.1, 4.2, 4.3, and 4.4 and contributed to the experimental design and drafting of the submitted manuscript. SR contributed to the acquisition and interpretation of immunofluorescence data and to manuscript drafting. CC contributed to the acquisition and interpretation of islet morphology and manuscript drafting. JL contributed to data collection design and
acquisition and to manuscript preparation. RW contributed to the design of the experiments in this draft, data interpretation, and drafting and preparation of the submitted manuscript.

**Chapter 5** is adapted from the following manuscript: Oakie A, Li J, and Wang R. The co-stimulation of receptors tyrosine kinase c-Kit and insulin receptor in the INS-1 832/13 cell line. Manuscript in preparation (2019). JL contributed to the acquisition of data and manuscript preparation. RW contributed to the design of the experiments conducted, interpretation of data, and manuscript preparation.
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<th>Full Form</th>
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<tbody>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>AUC</td>
<td>Area under curve</td>
</tr>
<tr>
<td>c-KitβTg</td>
<td>Beta cell-specific c-Kit-overexpressing transgenic</td>
</tr>
<tr>
<td>c-KitβTg;βIRKO</td>
<td>Beta cell-specific inducible insulin receptor knockout in c-KitβTg</td>
</tr>
<tr>
<td>DDR1</td>
<td>Discoidin domain receptor tyrosine kinase 1</td>
</tr>
<tr>
<td>e</td>
<td>Embryonic day</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epidermal growth factor receptor</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular signal-regulated kinase</td>
</tr>
<tr>
<td>FAK</td>
<td>Focal adhesion kinase</td>
</tr>
<tr>
<td>FGFR1</td>
<td>Fibroblast growth factor receptor 1</td>
</tr>
<tr>
<td>FOXO1</td>
<td>Forkhead box transcription factor O1</td>
</tr>
<tr>
<td>Grb</td>
<td>Growth factor receptor bound protein</td>
</tr>
<tr>
<td>GLUT</td>
<td>Glucose transporter</td>
</tr>
<tr>
<td>GPCR</td>
<td>G protein-coupled receptor</td>
</tr>
<tr>
<td>GSIS</td>
<td>Glucose-stimulated insulin secretion</td>
</tr>
<tr>
<td>GSK3β</td>
<td>Glycogen synthase kinase-3β</td>
</tr>
<tr>
<td>HFD</td>
<td>High-fat diet</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Name</td>
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<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>hGH</td>
<td>Human growth hormone</td>
</tr>
<tr>
<td>HLA</td>
<td>Human leukocyte antigen</td>
</tr>
<tr>
<td>IAPP</td>
<td>Islet amyloid polypeptide</td>
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<tr>
<td>IGF</td>
<td>Insulin-like growth factor</td>
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<tr>
<td>IGF-1R</td>
<td>Insulin-like growth factor-1 receptor</td>
</tr>
<tr>
<td>Ins</td>
<td>Insulin</td>
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<td>IPGTT</td>
<td>Intraperitoneal glucose tolerance test</td>
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<td>IPITT</td>
<td>Intraperitoneal insulin tolerance test</td>
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<tr>
<td>IR</td>
<td>Insulin receptor</td>
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<td>IRS-1</td>
<td>Insulin receptor substrate-1</td>
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<tr>
<td>K&lt;sub&gt;ATP&lt;/sub&gt;</td>
<td>ATP-sensitive potassium channels</td>
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<tr>
<td>Kir6.2</td>
<td>Inward rectifying potassium 6.2</td>
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<td>MAFA</td>
<td>v-maf musculoaponeurotic fibrosarcoma oncogene homolog A</td>
</tr>
<tr>
<td>mTORC</td>
<td>Mammalian target of rapamycin complex</td>
</tr>
<tr>
<td>MUNC18-1</td>
<td>Mammalian homologue of uncoordinated18-1</td>
</tr>
<tr>
<td>NEUROD</td>
<td>Neuronal differentiation 1</td>
</tr>
<tr>
<td>NGF</td>
<td>Nerve growth factor</td>
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<td>NGN3</td>
<td>Neurogenin 3</td>
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<td>Neurogenin 3</td>
</tr>
<tr>
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<td>NK-homeodomain factor 2.2</td>
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<tr>
<td>NKX6.1</td>
<td>NK-homeodomain factor 6.1</td>
</tr>
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<td>NOD</td>
<td>Non-obese diabetic</td>
</tr>
<tr>
<td>p</td>
<td>Postnatal day</td>
</tr>
<tr>
<td>PARP</td>
<td>Poly(ADP-ribose) polymerase</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Name</td>
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<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>PAX4</td>
<td>Paired box gene 4</td>
</tr>
<tr>
<td>PDGFR</td>
<td>Platelet-derived growth factor receptor</td>
</tr>
<tr>
<td>PDX-1</td>
<td>Pancreatic and duodenal homeobox-1</td>
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<tr>
<td>PECAM</td>
<td>Platelet endothelial cell adhesion molecule</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphatidylinositol 3-kinase</td>
</tr>
<tr>
<td>PPARγ</td>
<td>Peroxisome proliferator-activated receptor γ</td>
</tr>
<tr>
<td>PTB</td>
<td>Phosphotyrosine-binding domain</td>
</tr>
<tr>
<td>PTF1A</td>
<td>Pancreas associated transcription factor 1a</td>
</tr>
<tr>
<td>RTK</td>
<td>Receptor tyrosine kinase</td>
</tr>
<tr>
<td>RyR</td>
<td>Ryanodine receptor</td>
</tr>
<tr>
<td>SCF</td>
<td>Stem cell factor</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
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<tr>
<td>SERCA</td>
<td>Sarco-endoplasmic reticulum Ca$^{2+}$ ATPase</td>
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<tr>
<td>SH2</td>
<td>Src homology 2</td>
</tr>
<tr>
<td>SHB</td>
<td>Src homology-2 domain containing protein B</td>
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<td>SNAP25</td>
<td>Synaptosome-associated protein of 25 kDa</td>
</tr>
<tr>
<td>SNARE</td>
<td>Soluble N-ethylmaleimide-sensitive factor attachment protein receptor</td>
</tr>
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<td>SOX9</td>
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<td>STZ</td>
<td>Streptozotocin</td>
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<td>Sulfonylurea receptor</td>
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<td>T2DM</td>
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<tr>
<td>TF</td>
<td>Transcription factor</td>
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<tr>
<td>Trk-A</td>
<td>Tropomyosin receptor kinase-A</td>
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<tr>
<td>VAMP2</td>
<td>Vesicle-associated membrane protein 2</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>VEGFR</td>
<td>Vascular endothelial growth factor receptor</td>
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</table>
Chapter 1

1 Introduction

1 Text and figures from this chapter have been adapted from the following manuscript:
1.1 Significance of study

Insulin production and exocytotic events from beta cells are necessary to avoid the development of hyperglycemia and diabetic complications. Various subfamilies of receptor tyrosine kinases (RTKs) are found on beta cells and on cells within the islet microenvironment. RTKs are required for normal pancreatic development and play a role in mature islet function. The receptors c-Kit and insulin receptor (IR) are present on beta cells and exploit similar intracellular signalling pathways to promote proliferation, survival, and insulin release. This study examines the role of c-Kit and IR activation on the regulation of beta cell function and insulin secretion using in vitro and in vivo models. The findings in this thesis provide new insights into the effects of RTK interplay on beta cell function and how RTK therapies can influence signalling pathways in islets and affect the long-term alleviation of hyperglycemia in diabetes.

1.2 Islet cell biology

1.2.1 General islet development and morphology

The pancreas is an endoderm-derived organ that contributes to both digestive and endocrine functions through two distinct secretory cell types: [1] the exocrine acinar cells, which release digestive enzymes through the pancreatic ducts to the duodenum in order to aid in the breakdown of macronutrients; and [2] the endocrine cells comprising the islets of Langerhans, responsible for the release of hormones that regulate glucose availability and energy expenditure. The mature pancreas first emerges in the foregut at rodent embryonic day (e) 8.5 as dorsal and ventral buds that express early pancreatic transcription factors (TFs) pancreatic and duodenal homeobox-1 (PDX-1), SRY-box 9 (SOX9), and pancreas associated transcription factor 1a (PTF1A) (1). By the time the two buds fuse at e12.5, the pancreas has formed an outer ‘tip’ layer, committed to acinar cell differentiation, and an inner ‘trunk’ region, where endocrine cells emerge from the early duct compartment (2,3). Neurogenin 3 (NGN3) TF expression in the duct promoted the migration of endocrine-committed cells through the mesenchyme to form initial islet clusters, which then undergo vascularization and TF-induced maturation to form the islet microenvironment (1,4,5).
The islets are composed of five distinct endocrine cell types that secrete specific hormones: alpha cells (glucagon), beta cells (insulin), delta cells (somatostatin), PP cells (pancreatic polypeptide), and epsilon cells (ghrelin). The ratio of each endocrine cell type within the islet varies drastically throughout species. However, beta cells compose the highest percentage of endocrine cells found in both rodent (~75-80%) and human (~55-60%) islets (6,7). Insulin release can be regulated by hormone secretion from the other four cell types found in the islet. In turn, insulin exocytosis from the beta cell is able to prevent the release of glucagon (8,9). The islet microenvironment is therefore able to control insulin secretion through paracrine signalling.

1.2.2 Beta cell transcription factors

The temporal expression of beta cell TFs is required for beta cell commitment during development (1). NGN3 is the major TF that has been shown to drive the commitment of all endocrine cells within the islet population (10). Subsequent expression of neuronal differentiation 1 (NEUROD), paired box gene 4 (PAX4), NK-homeodomain factors 2.2 and 6.1 (NKX2.2 and NKX6.1), and V-maf musculoaponeurotic fibrosarcoma oncogene homolog A (MAFA) during the second transition of pancreatic development (e12.5-18.5) are required for developing beta cells to reach a functionally mature state (11-15). Downregulation of beta cell-associated TFs has been shown to reduce postnatal beta cell mass and induce glucose intolerance in mice (16,17). The beta cell TFs examined in the following chapters are important for adult beta cell function and their roles in the postnatal beta cell are discussed in further detail below.

Pancreatic and duodenal homeobox-1 (PDX-1) is a key TF in pancreatic development and is essential for maintaining Insulin (Ins) 1/2 transcription in the postnatal beta cell. Recent studies have identified that activation of specific enhancer regulatory areas in Pdx-1 were required during different stages of development and maturation, with area IV being key for postnatal function (18). Pdx-1 transcription was also deemed essential for maintaining insulin secretion in mature islets and for the expression of additional beta cell-associated TFs (18).
V-maf musculoaponeurotic fibrosarcoma oncogene homolog A (MAFA) is a beta cell-specific TF that has been identified as an essential factor for the maintenance of beta cell mass and for insulin vesicle docking and release in mice following weaning (3 weeks of age) (15,19). Studies conducted using a beta cell-specific knockout of MAFA resulted in decreased beta cell mass and Cyclin D2 expression in adult islets, loss of beta cell identity due to dedifferentiation, and reduced glucose transporter 2 (GLUT2) expression in islets (17,20). Along with PDX-1 and NEUROD, MAFA has been identified as one of the essential TFs that interact with the insulin promoter to promote Ins1/2 transcription (21).

NK-homeodomain factor 6.1 (NKX6.1) has been identified as an essential TF for the maintenance of MAFA and GLUT2 expression and beta cell proliferation (16). The postnatal loss of NKX6.1 at approximately 4 weeks of age induced in the mature beta cell demonstrated a similar phenotype to mouse models with NKX6.1 lost during development (reduced insulin secretion and beta cell proliferation), indicating the importance of NKX6.1 in the maintenance of normal beta cell function (22).

Forkhead box O1 (FOXO1) has been shown to down-regulate Pdx-1 gene expression. This has been reported to initiate the following: [1] inhibition of beta cell emergence during development; [2] decreased proliferation of mature beta cells; [3] reduced expression of the Insulin2 gene; and [4] induction of endoplasmic reticulum (ER) stress and subsequent beta cell apoptosis through binding to the Chop promoter (23-27). FOXO1 also inhibited peroxisome proliferator-activated receptor γ (PPARγ), which further contributed to impaired proliferation and compromised glucose tolerance (28). However, increased nuclear FOXO1 expression in beta cells during oxidative stress has been found to up-regulate beta cell MAFA expression, which led to improved glucose tolerance (29-31). These findings suggest that FOXO1 also maintains a protective role in diabetic islets until its eventual down-regulation in failing beta cells (29,32).

1.2.3 Regulation of beta cell mass

The endocrine cells that comprise adult islets first emerge during the second transition (e12.5) of murine pancreatic development and form islet clusters through beta cell
neogenesis from the trunk compartment (33). At postnatal day (p) 0, the neonatal rodent pancreas undergoes a period termed ‘beta cell remodeling’, where both high beta cell neogenesis and apoptosis are present and result in a maintained beta cell mass until weaning (p20) (33,34). A similar period of remodeling was identified in the developing human pancreas, where beta cell turnover occurred from 15 weeks of fetal age to 2 months postnatal age after expansion of the beta cell mass from 8-21 weeks of fetal age (35,36). The remodeling phase can also exhibit high beta cell regeneration capacity as seen in studies utilizing streptozotocin (STZ)-induced destruction of beta cells (37,38). After the remodeling phase is complete, beta cell expansion is maintained through proliferation and is regulated by the expression of Cyclin D cell cycle proteins (39-41).

The replication of existing beta cells is the most frequently reported mechanism of beta cell generation in the adult rodent islet (42). Rodent beta cells maintain their replicative expansion throughout their lifespan and undergo proliferation multiple times, although their observed frequency of replication has been shown to decrease with age (43). Reported beta cell proliferation rates are low (<1%) in adult (6-week) mice and have been observed to be lower than 0.1% in mice over 7 months of age (44,45). However, dietary stress and obesity in rodents have been shown to induce beta cell mass expansion due to the increased demand for insulin to overcome elevated glucose levels. Beta cell hyperplasia in high-fat diet (HFD) mouse models occurred within the first week of HFD initiation but was lost as cells underwent apoptosis with prolonged feeding (46-48). 7- to 8-month old mice also did not demonstrate HFD-induced beta cell expansion, which demonstrates an age-dependent loss of beta cell compensatory proliferation (45).

Although beta cell neogenesis has not been observed in unchallenged islets, it has been reported to occur with injury-induced pancreatic damage. Ductal ligation, a technique where 50-60% of the pancreas (gastric and splenic segments) is subjected to acinar destruction due to blocked ductal drainage, has been shown to contribute to islet neogenesis through ductal cell reprogramming (49,50). Regulation of the postnatal and ageing beta cell compartment can therefore be altered by metabolic challenges and injuries.
1.3 Mechanisms of insulin section in beta cells

1.3.1 Glucose-stimulated insulin secretion

The major physiological mechanism that induces insulin secretion is increased intracellular glucose levels. Glucose-stimulated insulin release is a biphasic response that has two distinct phases: first-phase insulin secretion, which peaks approximately 5 minutes after glucose challenge and releases 0.15% of the total insulin content per minute per beta cell, and second-phase insulin secretion, which begins 10 minutes after glucose challenge and releases 0.05% of the total insulin content per minute per beta cell (51). Glucose stimulation induces a multiple step process, which is outlined in the following sections:

1) Glucose entry and glycolysis

Unstimulated beta cells have a resting membrane potential between -56 to -75 mV (52). Glucose initially enters the beta cell through the glucose transporter GLUT1 (human) or the lower affinity transporter GLUT2 (rodent) (53), located on the lateral membrane of beta cells (54). The expression of glucose transporters is essential for the rapid release of insulin associated with the first phase of exocytosis (55). Glucokinase primarily serves as a sensor for intracellular glucose levels in beta cells (56). The influx of glucose into beta cells initiates glycolysis, producing ATP and initiating membrane depolarization (57).

2) Potassium channel–induced depolarization and calcium influx

Beta cells express ATP-sensitive potassium (K$_{ATP}$) channels on their membrane composed of four sulfonylurea receptors (SUR1) and four inward rectifying potassium 6.2 (Kir6.2) isoforms, with each SUR1 unit linked to one pore-forming Kir6.2 unit in order to create a functional channel (58,59). ATP binds to the Kir6.2 subunits and inhibits the potassium channel, preventing K$^+$ efflux that promotes depolarization and Ca$^{2+}$ influx (60). Maintaining the expression of both SUR1 and Kir6.2 in K$_{ATP}$ channels is required for physiological control of insulin secretion under low and high glucose conditions as previously observed in knockout mouse models (61-63). Extracellular Ca$^{2+}$ influx through channels located on the cell membrane increases cytosolic Ca$^{2+}$ levels, priming
the beta cell for insulin exocytosis from docked vesicles close to Ca$^{2+}$ channels (64-67). The ryanodine receptors (RyR) found on the endoplasmic reticulum (ER) can regulate Ca$^{2+}$ levels in both the ER and cytosol, which affect insulin secretion and mitochondrial function (68).

3) Cytoskeletal remodeling and exocytosis of insulin vesicles through SNARE machinery assembly

Increased intracellular Ca$^{2+}$ in beta cells induces phosphorylation of focal adhesion kinase (FAK) and paxillins, components of the focal adhesion complex, which leads to actin depolymerization and remodeling (69). Gelsolin is activated by calcium and is involved in the remodeling process for glucose-stimulated insulin secretion (70). Soluble N-ethylmaleimide-sensitive factor attachment receptor (SNARE) protein assembly allows for vesicle tethering and is a necessary step involved in insulin release. The assembly of SNARE complexes at the beta cell membrane consists of two synaptosome-associated protein 25 (SNAP25), one syntaxin protein, and one vesicle-associated membrane protein (VAMP), with mammalian homologue of uncoordinated 18 (MUNC18) proteins serving to stabilize the formed complex (71). The first and second phases of insulin secretion involve specific SNARE isoforms (Table 1.1). Although SNARE proteins are found throughout the beta cell membrane, insulin vesicles are released on the vascular-facing side of the beta cell (72). Syntaxin 1A has also been shown to bind to and inhibit K$_{ATP}$ channels, which demonstrates that SNARE proteins can play multiple roles during insulin secretion (73,74). Synaptotagmins found on the insulin vesicle are Ca$^{2+}$ sensor proteins that facilitate SNARE complex-beta cell membrane contact and results in insulin exocytosis (71,75).
Table 1.1: SNARE protein isoform assembly during insulin secretion.

<table>
<thead>
<tr>
<th>Isoform</th>
<th>Phase</th>
<th>Key roles (associated SNAREs)</th>
<th>Refs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Munc18-1</td>
<td>First</td>
<td>Located on cell membrane and cytoplasm. Required for docking of granules during first-phase secretion (<em>Syntaxin-1a, Syntaxin-4</em>)</td>
<td>(76)</td>
</tr>
<tr>
<td>Munc18-2</td>
<td>Both</td>
<td>Involved in vesicle fusion to beta cell membrane and to other insulin secretory granules (<em>Syntaxin-2, Syntaxin-3</em>)</td>
<td>(77)</td>
</tr>
<tr>
<td>Munc18-3</td>
<td>Second</td>
<td>Required for docking of newcomer granules (<em>Syntaxin-4</em>)</td>
<td>(78)</td>
</tr>
<tr>
<td>Syntaxin-1a</td>
<td>Both</td>
<td>Responsible for granule docking of readily releasable pool and newcomer granule pool. Highly associated with first-phase insulin release (<em>VAMP2, VAMP8, MUNC18-1, SNAP25</em>)</td>
<td>(79-81)</td>
</tr>
<tr>
<td>Syntaxin-2</td>
<td>Both</td>
<td>Located on secretory insulin granules. Inhibitory: reduces fusion of readily releasable pool and newcomer granule docking (<em>MUNC18-2, SNAP23, SNAP25, VAMP2, VAMP8</em>)</td>
<td>(82)</td>
</tr>
<tr>
<td>Syntaxin-3</td>
<td>Both</td>
<td>Located on secretory granules. Fusion of newcomer granules to beta cell membrane and to other insulin granules (<em>VAMP8</em>)</td>
<td>(83)</td>
</tr>
<tr>
<td>Syntaxin-4</td>
<td>Both</td>
<td>Released from gelsolin binding upon glucose stimulation. Granule docking of readily releasable pool and newcomer granule pool (<em>VAMP2, VAMP8, MUNC18-3</em>)</td>
<td>(84,85)</td>
</tr>
<tr>
<td>SNAP23</td>
<td>First</td>
<td>Inhibitory: reduces docking and fusion of the readily releasable pool granules by replacing SNAP25 in formation of SNARE complex (<em>Syntaxin 1a, VAMP2</em>)</td>
<td>(86)</td>
</tr>
<tr>
<td>SNAP25</td>
<td>Both</td>
<td>Localized to the cell membrane. Involved in the docking of readily releasable insulin granules (<em>Syntaxin-1a to 4, VAMP2 and 8, MUNC18-1-3</em>)</td>
<td>(87)</td>
</tr>
<tr>
<td>VAMP2</td>
<td>Both</td>
<td>Present on insulin secretory granules. Tethers to syntaxin-SNAP complexes for vesicle docking (<em>Syntaxin 1a, Syntaxin 2, Syntaxin 4</em>).</td>
<td>(88,89)</td>
</tr>
<tr>
<td>VAMP8</td>
<td>Both</td>
<td>Interacts with SNARE proteins to initiate GLP-1 and glucose-induced release of short-lived docked newcomer granules (<em>Syntaxin 1a</em>)</td>
<td>(79,90)</td>
</tr>
</tbody>
</table>
1.3.2 Intracellular signalling pathways in beta cell insulin secretion

The activation of multiple signalling pathways can induce insulin secretion from beta cells. The phosphatidylinositol 3-kinase (PI3K)–Akt signalling axis has been shown to promote beta cell survival and increase proliferation through increased Cyclins D1 and D2 levels (40, 91). Loss of class IA PI3Ks in beta cells reduced intracellular Ca²⁺ levels and SNARE proteins, which ablated insulin exocytosis (92). The α isoform of class II PI3Ks has also been linked to downstream Akt1 activation and glucose-stimulated insulin release (93). FAK-induced cytoskeletal remodeling and insulin vesicle trafficking has been shown to stimulate the phosphorylation of the Akt–AS160 complex, responsible for stimulating glucose-induced insulin release (94–96). Interestingly, the selective PI3K inhibitor LY294002 has been found to increase glucose-stimulated insulin release through Kv channel inhibition in rat insulinoma line INS-1 832/12 cells (97, 98), and inhibition of this pathway has been linked to increased insulin exocytosis (99). Further support for an inhibitory role of the PI3K–Akt pathway was found in experiments that suppressed the p85 regulatory subunit of PI3K, which resulted in enhanced insulin release (100). Taken together, the activation of the PI3K–Akt pathway appears to have differing effects on insulin secretion, yet it likely remains an important regulator of exocytosis in beta cells.

The mitogen-activated protein kinase (MAPK) pathway is another signalling cascade linked to insulin secretion. Extracellular signal-regulated kinase (ERK), part of the MAPK pathway, has been shown to be required for glucose- and secretagogue-induced insulin release (101, 102). Cytoskeletal remodeling, which is critical for exocytosis during the second-phase of insulin secretion, required signalling through the MAPK–ERK pathway (103). Phosphorylated FAK has been shown to stimulate ERK phosphorylation and induce the release of insulin (69). Interestingly, phosphorylated ERK proteins, triggered by chronic high glucose levels, led to reduced insulin secretion and was not restored by ERK inhibition despite normalized actin cytoskeletal dynamics (104). These studies are informative in understanding ERK signalling in relation to cytoskeletal remodeling and insulin release but further research is required to fully elucidate its role.
1.4 Etiology of diabetes mellitus

Diabetes mellitus is a metabolic disorder that is characterized by high circulating glucose levels. Diabetes may be caused by either insufficient insulin production or a defect with insulin signalling, classified as Type 1 (T1DM) or Type 2 (T2DM), respectively. Both types of diabetes result in beta cell failure and apoptosis, resulting in a lack of endogenous insulin production (105). The predicted rate of global diabetes diagnoses in patients over 18 years was approximately 451 million people in 2017 and is currently predicted to reach 693 million people by the year 2045 (106). The estimated economic burden from this disease has also been predicted to reach approximately $2.5 trillion dollars by 2030 (107). In addition to the loss of normoglycemia and the development of metabolic dysregulation, diabetes can promote vascular disorders that result in cardiovascular complications and peripheral tissue damage (neuropathy, nephropathy, and retinopathy) (108). The two major types of diabetes mellitus, Type 1 and Type 2, are discussed in detail below.

1.4.1 Type 1 diabetes mellitus

T1DM develops from the autoimmune recognition and destruction of a patient’s beta cells. Up to 90% of the functional beta cell mass is lost and patients require an exogenous source of insulin to maintain normoglycemia. A genetic association between first-degree family members and the inheritance of the disease has been found to exist (109). Environmental factors have also been suggested to induce beta cell destruction (110). Islets in T1DM patients were found to be infiltrated by multiple immune cells including T-cells (CD4+ and CD8+), B-cells (CD20+), and macrophages (CD68+) (111). In the initial stages of T1DM, proteins that are produced by beta cells are recognized as autoantigens through antigen presentation (112). Examples of candidate autoantigens include a peptide sequence in the B chain of insulin, zinc transporter ZnT8, and chromogranin A (113-115). The inheritance of certain antigen presenting haplotypes, such as HLA-DQ8, have been shown to induce autoimmunity and present select C-peptide epitopes to CD4+ cells (110,116). As autoantigens induce immune cell migration, the progressive islet infiltration with CD45+ (leukocyte) cells has been shown to destroy the surrounding islet basement membrane, which contributes to the progressive loss of
islets (117). Beta cells are then lost through the induction of apoptotic pathways, including CD8\(^+\)-induced perforin and granzyme B release, increased Fas receptor-induced Caspase 8 activation, and cleaved Caspase 3 activation (105).

One study was able to identify *INSULIN* mRNA in the pancreatic tissue of T1DM patients even when there was an insufficient number of mature beta cells (118). Insulin secretion from the islets of T1DM patients followed a similar response when compared to control islets and normalized to total insulin content, indicating that the function of remaining beta cells in T1DM may not be damaged (119). Non-obese diabetic (NOD) mice also displayed an immature population of beta cells that are preserved from immune cell-mediated apoptosis (120).

### 1.4.2 Type 2 diabetes mellitus

Similar to T1DM, T2DM is associated with elevated circulating glucose levels. However, the pathogenesis of the disease is different from the autoimmune attack observed in T1DM. Genetic variants as well as nutritional and lifestyle conditions, resulting in obesity, are established links to the development of T2DM (51). T2DM patients develop insulin resistance in peripheral tissue sites, including the skeletal muscle, adipose tissue, and liver, that are important for the control of glucose reuptake (insulin-induced GLUT4 up-regulation in adipocytes and skeletal muscle) and circulating glucose regulation (121). This results in increased beta cell mass and proliferation through the replication of pre-existing beta cells (122,123). Unfortunately, it has been noted that many beta cell complications arise with prolonged exposure to hyperglycemia in insulin-resistant patients. Excess glucose and free fatty acids have been shown to induce oxidative and ER stresses in the beta cell, which resulted in misfolded proteins and the induction of apoptosis (124). Increased islet vasculature was also shown in obese and T2DM mouse models, which may facilitate infiltrating macrophages and the development of subsequent islet inflammation (125,126). One of the common histological markers exclusively associated with T2DM in humans is the accumulation of islet amyloid polypeptide (IAPP) plaques in the islet microenvironment, which has been reported to promote beta cell apoptosis through enhanced ER stress (127). Islets from T2DM patients also demonstrated a loss of essential SNARE proteins involved in insulin vesicle tethering and
exocytosis (128,129). Recent evidence confirmed that T2DM islets have reduced vesicle docking when stimulated with glucose (130). Although weight loss induced through nutritional and lifestyle interventions has been shown to improve the liver and pancreas fat content and return glucose-stimulated insulin secretion to some obese T2DM patients, a significant proportion of patients were unable to regain the same insulin secretory function, indicating that the alleviation of obesity-induced metabolic stresses may not be sufficient to rescue beta cell dysfunction in all patients (131).

Diet-induced obesity mouse models have been used to examine the progression of beta cell compensation and failure in a T2DM-like model. It has been shown that feeding C57BL/6 mice a high-fat diet (HFD), in which 60% of total available kcal is derived from fat, for a 12 to 16-week duration induced multiple metabolic changes that manifested as glucose intolerance and peripheral insulin resistance (132). The accumulation of metabolic stresses in glucose-regulating peripheral tissues and reduced insulin sensitivity in HFD-fed mice resulted in hyperinsulinemia in order to maintain normal glycemic levels (133). The increased insulin demand induced in long-term HFD mice also led to the progressive loss of beta cell TFs and increased ER stress (134).

1.4.3 Potential therapeutics for insulin restoration

Diabetic patients that require an exogenous source of insulin to maintain normal glucose levels are required to monitor their blood glucose levels and regulate their insulin administration through injection or abdominal pump delivery. However, the optimal treatment would be the design of a user-independent system where patients would not be required to regulate their insulin input. The most promising form of endogenous insulin restoration is islet transplantation. This technique, known as the Edmonton Protocol, was first published in 2000 and involved the isolation of islets from two or more cadaveric donor pancreata and the infusion of these islets into the hepatic portal vein (135). Recent experiments have also examined the omentum, a peritoneal organ rich in vascularization, as an ideal site for islet transplantation since it is able to maintain insulin secretion while avoiding the toxic environment of the hepatic portal vein (136). Although there were promising results achieved with the Edmonton Protocol in the initial time following its publication, many limitations had developed at later time-points following
transplantation. A large pool of donor islets (minimum of 9000 islets per kilogram of body weight) was identified as a requirement for successful insulin independence in diabetic patients (137). Furthermore, the ability of transplanted islets to relieve patients of exogenous insulin has been shown to decrease over a 5-year period, with 90% of transplanted islet recipients requiring exogenous insulin delivery at 5 years (138). Other therapies are currently being researched to alleviate these limitations. The management of insulin deficiency in patients through the use of an ‘artificial pancreas’ has gained more traction in the past few years. Both single hormone (insulin only) and dual hormone (insulin and glucagon) artificial pancreas devices managed to achieve optimal glucose levels when compared to conventional insulin pump therapy (139). T1DM patients have also been treated with immunoglobulin therapy, which is aimed at targeting the T-cell autoimmune attack against beta cells. Although normoglycemia can be achieved by preventing the elimination of the patient’s beta cell mass, the frequent recurrence of T-cell-mediated beta cell destruction has been noted with this treatment (140). When considering all available therapies, it is evident that maintaining beta cell mass and function is required to produce sufficient insulin in patients. The maintenance of islet function can be achieved under normal conditions through receptor activation and intracellular signalling, and examining the interplay of receptor activation and signalling in beta cells could provide information for the development of future therapies.

1.5 Receptor tyrosine kinases

1.5.1 General structure of RTKs

RTKs are a class of cell membrane receptors that are subdivided into 20 distinct families. The structural composition of the extracellular region varies between each receptor class and can include repeats of a single type of molecule (e.g. class III/IV receptors with immunoglobulin-rich domains) or different molecular components (e.g. class II receptors with cysteine-rich domains, L domains, and fibronectin type III domains) (141,142). RTKs contain a single transmembrane region and an intracellular region consisting of tyrosine residues. RTKs exist as monomers and dimerize upon ligand binding, with the exception of class II receptors insulin receptor (IR) and insulin-like growth factor 1 receptor (IGF-1R) that remain heterodimeric in the absence of ligands (141). In their
activated state, the intracellular region of RTKs undergo conformational changes and autophosphorylation of tyrosine kinase residues, which leads to the initiation of intracellular signalling through proteins containing regulatory phosphotyrosine-binding domain (PTB) or Src homology 2 (SH2) regions (e.g. the p85 regulatory unit of PI3K) (141,143-145). Activation of these signalling proteins can also be initiated through the binding of corresponding adaptor and scaffold proteins, which dock to the tyrosine residues and regulate multiple downstream signalling pathways (144). Adaptor and scaffold proteins that control intracellular signalling through RTK phosphorylation include Shc, the Growth factor receptor bound protein (Grb) family, Src homology-2 domain containing protein B (SHB), adaptor protein with a PH and SH2 domain (APS), and insulin receptor substrates (IRS) (144,146). Many of these proteins regulate insulin secretion and insulin sensitivity (147-151), highlighting the importance of RTK-initiated signalling in the islet microenvironment.

1.5.2 Expression of islet RTKs

Cells in the islets express various RTKs that regulate beta cell proliferation, function, and insulin synthesis and secretion (Figure 1.1). Pancreatic development is one process that relies on the expression of multiple RTKs to achieve islet maturation. Epidermal growth factor receptor (EGFR) expression is required for embryonic beta cell maturation, islet migration, and maintenance of beta cell mass and proliferation (152,153). Other members of the ErbB receptor family are also present during murine pancreatic development (154). The receptor for nerve growth factor (NGF), tropomyosin receptor kinase-A (Trk-A), follows a specific pattern of expression within endocrine cells that is dependent on the stage of embryonic development (155). Discoidin domain receptor tyrosine kinase 1 (DDR1) has recently been identified during early pancreatic development in emerging endocrine cells and in injury-induced ductal ligated pancreata (156). Platelet-derived growth factor receptor (PDGFR), which shares structural homology to c-Kit, demonstrated islet proliferation with activation (157). The contributions of c-Kit and IR to beta cell development and postnatal function are discussed in subsequent sections.

The current literature regarding RTK expression on the non-beta islet endocrine cells is limited. Unlike the receptor for insulin, the receptors for other islet hormones belong to
the G protein-coupled receptor (GPCR) family (158,159). The presence of IR has been confirmed on alpha cells and is required for cell proliferation and glucagon secretion regulation through the Akt/P70S6K1 axis (160,161). IR expression is also found in isolated single delta cells from human islets and in the somatostatin-secreting TGP52 cell line (162,163). Recent findings have identified that glucagon release from alpha cells can be manipulated through the erythropoietin-producing hepatoma A (EphA) receptor, a RTK class that is unique due to ‘forward’ and ‘reverse’ signalling through beta cell-beta cell and beta cell-alpha cell receptor-ligand interactions (164,165). However, the presence of RTKs and their activity in non-beta cell endocrine cells requires further investigation.

RTKs are also implicated in integrin-extracellular matrix (ECM) communication and islet vascularization. One study identified that beta cell fibroblast growth factor receptor 1 (FGFR1) increased ERK phosphorylation in beta cells, but its activation depended on the binding of α6β1 integrin to specific ECM ligands produced by endothelial cells (166). This coordinated activity enhanced both islet-microvasculature remodeling and beta cell insulin secretion and survival (167). Vascular endothelial growth factor (VEGF) has also been shown to be released by beta cells and to bind to its receptor (VEGFR) on islet endothelial cells to promote angiogenesis. Reduced islet vascularization, decreased beta cell mass, and impaired insulin and glucagon secretion were noted in mice with beta cell VEGF-A loss during embryonic development (168-170). While VEGF-A inactivation in the postnatal adult beta cell also lowered islet vascularization after 3 months, these mice developed mild glucose intolerance and had unchanged beta cell mass (169). These results indicate that islet endothelial cell regulation of beta cell mass occurred during select developmental time-points. Hypervascularization of the islets through enhanced VEGFR expression can also detrimentally affect beta cell survival and lead to the development of hyperglycemia. Overexpression of VEGF-A during islet development or induced in adult beta cells led to enhanced endothelial cell density, but this expansion impaired the formation of islet clusters and decreased beta cell proliferation and mass in both mouse models (171,172). NOD mice also demonstrated increased VEGF-A production from beta cells, which increased the expression of VEGFR on endothelial
cells and subsequently led to islet inflammation and T-cell-mediated beta cell destruction (173).

In addition to controlling overall islet function, the activation of certain RTKs can directly regulate insulin release. NGF signalling has been shown to promote Trk-A internalization, F-actin reorganization, and insulin exocytosis, which maintained glucose-stimulated insulin secretion in mouse islets (174-176). Signalling through the c-Met receptor has also been observed to regulate secretion. Its disruption in murine beta cells resulted in impaired insulin release with minimal effect on islet morphology (177). IGF-1R, which shares structural homology with IR, is required for glucose-stimulated insulin release and glucose sensing through the maintenance of GLUT2 expression (178,179). FGF-21, through binding to its receptor, stimulated insulin release from isolated islets of diabetic rodent models under high glucose conditions (180). RTKs have further been found to play a role in the negative feedback of insulin release, as observed with bidirectional signalling through EphA (164,181). Examining the role of RTKs signalling in insulin secretion is therefore important when determining their contribution towards maintaining glucose homeostasis.
Figure 1.1: Summary of reported RTKs in the islet microenvironment.

The mature murine islet is composed of insulin-secreting beta cells in the core of the islet (blue) and other endocrine hormone–secreting cells along the islet’s periphery (red). Beta cells express multiple RTKs that influence their growth and development. The direct effect of specific RTK activation on insulin secretion has been previously demonstrated. Beta cells regulate and are also influenced by RTKs expressed on islet vasculature.
1.6 c-Kit receptor

1.6.1 Receptor structure and activation

The c-Kit (CD117 or Kit) receptor is a 21-exon class III RTK located on the Kit/W locus of chromosome 5 in mice (chromosome 4 in humans) (182). The extracellular portion of the receptor is composed of five immunoglobulin (Ig)-like domains (183). c-Kit interacts with the ligand stem cell factor (SCF), a 9-exon growth factor expressed from the Steel locus (chromosome 10 in mice and 12 in humans) (184,185). SCF is produced as a cell membrane-bound ligand with two different isoforms: one that contains exon 6 and one that is lacking exon 6 (185). SCF isoforms containing exon 6 have a proteolytic cleavage site that is susceptible to cleavage by proteases, producing a soluble form of the ligand, while SCF lacking exon 6 would be a solely membrane-bound ligand (185,186). These forms of SCF have been found to have differing functions in vitro, where the membrane-bound form of SCF promoted sustained c-Kit phosphorylation while the soluble form promoted receptor internalization (187-189). In order to stimulate c-Kit dimerization, SCF itself must form a homodimer (190). SCF dimers interact with the first 3 Ig-like domains (N-terminal) of the extracellular portion of c-Kit to allow for receptor dimerization of the remaining two Ig-like domain regions (183). Once SCF-induced c-Kit dimerization has been initiated, the intracellular region tyrosine residues Y568 and Y570 are phosphorylated and initiate intracellular signalling through multiple subsequent tyrosine residues (141,185). c-Kit phosphorylation promotes downstream intracellular signalling through different pathways, including PI3K–Akt, MAPK–ERK, and phospholipase C-γ1 (PLC-γ) pathways, in various cell types (191-194).

Early studies tracing the expression of c-Kit during murine organogenesis using LacZ labeling identified multiple cell types, including hematopoietic cells, germ cells, melanocytes, and intestinal cells (195,196). Various mutations in the intracellular region of c-Kit have been well-characterized, with missense loss-of-function mutations at select sites leading to death shortly after birth in mice (182). In contrast, mutations in the intracellular region that are associated with constitutive activity are associated with the development of neoplasia (197).
1.6.2 c-Kit activity in the islet

c-Kit is expressed in human and murine beta cells and is also present in pancreatic carcinoma-derived cell lines (195,198-200). Similar to the islet RTKs discussed above, c-Kit expression is high in embryonic and developing endocrine cells, yet expression of the receptor is reduced to a small subpopulation (10-20%) of beta cells in postnatal and adult rodents (195,200,201). The role of c-Kit signalling in maintaining beta cell survival and function has been studied in both *in vitro* and *in vivo* models (Figure 1.2). SCF treatment increased insulin expression in the human pancreatic ductal carcinoma PANC-1 cell line, implicating its role in beta cell development (195,198). Silencing of the c-Kit receptor using *in vitro* siRNA administration has also demonstrated that it is required to maintain both PDX-1+ and insulin+ cell expression, cell proliferation, and cell survival in human fetal islet-epithelial cell clusters (200). c-Kit activity in islet function and insulin secretion have been further examined *in vivo* using mouse models with either a point mutation or an over-expression of c-Kit. The *c-Kit<sup>Wv/Wv</sup>* mouse line has been previously used as a global c-Kit-deficient mouse model since a point mutation located in the intracellular site of the receptor produced mice with almost no functional receptor activity (182). Interestingly, *c-Kit<sup>Wv/+</sup>* mice displayed reduced islet insulin content and glucose-stimulated insulin secretion (202). This impaired insulin release was associated with reduced signalling through the c-Kit–Akt–glycogen synthase kinase-3β (GSK3β) pathway, reduced mature beta cell transcription factors, and increased apoptosis through Fas receptor–p53–caspase-3 activation (203,204). In contrast to the *c-Kit<sup>Wv/+</sup>* model, mice with a beta cell-specific overexpression of the human c-*KIT* receptor (c-KitβTg) had markedly improved islet mass, proliferation, and insulin release as well as increased phosphorylation of Akt at serine 473 when compared to wild-type controls (205). Increased vascularization was also noted in the islets of c-KitβTg mice with increased beta cell VEGF-A production, which promoted insulin secretion in control diet mice. However, inflammation-induced beta cell apoptosis and islet dysfunction were observed when these mice were subjected to 22 weeks of HFD feeding (199,205). In summary, information gathered through *in vivo* studies has demonstrated that c-Kit signalling is required for beta cell development, survival, and function, but that prolonged activation of the receptor may promote detrimental effects under metabolic stresses.
c-Kit has been shown to induce multiple intracellular signalling pathways, including MAPK–ERK, Akt, and PLC-γ. The Akt signalling pathway is of interest to this thesis due to its previously determined role in beta cell function. A schematic illustrating the main findings from our laboratory is provided above (206). Numbers within brackets indicate the position of the residue that is phosphorylated. [Y, tyrosine; S, serine].

Figure 1.2: Summary of established c-Kit signalling pathways in the beta cell.
1.6.3 The role of c-Kit on insulin release

c-Kit activation in beta cells has been established to increase insulin production and release, but the exact role of c-Kit signalling on the exocytosis of insulin is unknown (205). The secretory control of granules from mast cells through c-Kit activation has been extensively assessed, where histamine and interleukin 6 (IL-6) release were shown to be stimulated with SCF administration (207,208). In mast cells, c-Kit stimulated PI3K pathway signalling and cytoskeletal remodeling to promote granule secretion (209,210). Mice with a mutation that rendered c-Kit inactive exhibited reduced downstream PI3K–Akt signalling and decreased granule release response from mast cells (211). Although the direct role of c-Kit signalling on the regulation of beta cell insulin granule exocytosis has not yet been documented, our group has previously found increased insulin release in c-KitβTg mice (203,205). In light of these results, additional research is warranted to determine whether c-Kit directly regulates vesicle release in beta cells or requires the assistance of additional beta cell RTKs.

1.7 Insulin receptor

1.7.1 Receptor structure and activation

Insulin receptor (IR, CD220) is a heterodimeric receptor that is 22 exons long and is located on chromosome 8 in mice and chromosome 19 in humans (212,213). IR is a dimer made up of two alpha chains (extracellular domain) and two beta chains (extracellular, transmembrane, and intracellular domains) linked by disulfide bonds (214). The extracellular domain of the receptor is composed of two leucine-rich repeat domains surrounding one cysteine-rich region towards the N-terminal, type III fibronectin domains, and insert domains where the alpha and beta chains are bound (214). IR in its non-stimulated state is inhibited by the activation loop of the intracellular region of the receptor (141). The binding of insulin to IR leads to phosphorylation of Y1146, Y1150, and Y1151 residues in the kinase regulatory region and activates the intracellular portion of the receptor (215). The two functional isoforms of IR found in the islet are IR-A and IR-B. The difference in the isoforms is the inclusion (IR-B) or exclusion (IR-A) of
exon 11 (216). IR-B has been reported to be the dominant IR isoform in beta cells, while IR-A is expressed in alpha cells and a smaller percentage of beta cells (163).

The insulin gene is located on chromosome 11 in humans. However, mice have two nonallelic insulin genes lying on chromosomes 19 and 7 (217,218). These two genes encode for Insulin 1 and Insulin 2, with Insulin 2 displaying higher expression in the beta cell and detectable expression in the hypothalamus (219,220). Insulin mRNA is translated to preproinsulin before proinsulin formation in the rough ER (disulfide bonds directly joining A and B chains with a C chain located between the two), and is processed to its mature monomer form in the Golgi apparatus through C chain removal (C-peptide) via prohormone convertase 1/3 (221). The affinity of insulin for its receptor is low (approximately 18.4 pmol/L for human IR-A and 22.7 pmol/L for human IR-B) (222). Insulin-like growth factors (IGF-1 and -2) are also able to bind to IR-A due to the lack of exon 11 in this isoform (approximately 863 pmol/L for IGF-1 and 95.3 pmol/L for IGF-2) (222,223).

1.7.2 IR signalling on glucose regulation

IR knockout in murine models has demonstrated drastically different effects based on the severity of its loss. Mice homozygous for a mutation in the IR intracellular kinase region died shortly after birth due to hyperglycemia and ketoacidosis but heterozygous mice displayed a lifespan and glucose tolerance similar to wild-type litter-mates, although this may be due in part to high insulin levels in heterozygous mice (224,225). The IR–insulin pathway is required for normal and controlled islet cell development, and mouse models with either a loss of insulin (Ins1<sup>-/-</sup>, Ins2<sup>-/-</sup>) or a loss of beta cell IR (MIP-βIRKO) demonstrated islet hyperplasia and vascularization by the end of embryonic development (e18.5-e20.5) (226,227). The earliest study of mice with a beta cell-specific IR knockout (βIRKO) demonstrated that mice suffered from the loss of beta cell mass, beta cell GLUT2 expression, and first-phase insulin secretion, leading to the development of glucose intolerance and overt diabetes in 7-month old βIRKO mice (228,229). The expression of IR on peripheral tissue types can also influence the maintenance of normoglycemia due to the lack of IR-induced glucose reuptake. Hepatocyte IR knockout mice displayed an insulin resistance-like phenotype with increased glucose intolerance.
In contrast, mice with a skeletal muscle cell-specific IR knockout were able to maintain normoglycemia (231). The knockout of IR specifically on adipocytes of 8-week old mice promoted a compensatory increase in islet mass and hyperinsulinemia following a brief period of glucose intolerance (232).

The capacity to affect glucose tolerance, beta cell survival, and insulin secretion is also shared with IGF-1R–IGF-1 signalling. However, manipulations to the IGF-1R–IGF-1 pathway did not affect beta cell mass, unlike that observed with IR–insulin signalling (178,233). Signalling through the IGF-1R axis may partially compensate for the loss of IR as mice with a loss of both receptors on the beta cells showed severe glucose intolerance compared to mice with a loss of either IR or IGF-1R alone (234).

1.7.3 Insulin receptor substrates

Insulin receptor substrates (IRS) are a class of docking proteins that bind to the phosphorylated intracellular region of IR, inducing interactions with SH2-containing proteins (235). To date, a total of six IRS proteins have been identified in rodents and humans, yet IRS-1 and IRS-2 are the only major substrates currently shown to play a major role in beta cell function (236). Beta cell IRS-1 loss (Irs-1−/−) in mice was linked to a mild change in pancreatic beta cell mass and stunted insulin secretion due to reduced sarco-endoplasmic reticulum Ca^{2+} ATPase (SERCA) expression (237,238). IRS-2 was found to be important for maintaining beta cell mass and proliferation and for promoting compensatory beta cell hyperplasia in HFD-treated mice (239,240). In addition, inducing IRS-2 expression exclusively in the beta cells of Irs-2−/− mice maintained normoglycemia even with global IRS-2 loss, highlighting the importance of these insulin substrates in maintaining the insulin–IR–IRS axis in islets (241).

Docking of IRS-1 to the phosphorylated region of IR resulted in the tyrosine (Y) phosphorylation of the substrate, which then interacted with the p85 subunit of PI3K and promoted downstream phosphorylation of Akt, mTOR, GSK3β, and P70S6K1 (235,242). Interestingly, downstream signalling from phosphorylated IRS tyrosine sites resulted in negative feedback phosphorylation of serine (S) and threonine (T) sites on IRS-1, which has been shown to cause signalling disruption of phosphorylated tyrosine IRS residues
and down-regulate their association with PI3K (243,244). This negative feedback on IRS is hypothesized to be initially initiated through maintained PI3K–Akt–mTOR pathway signalling and can be prevented with the mTOR inhibitor rapamycin (151,245). The over-activation of the IR–IRS pathway and signalling through the PI3K–Akt pathway demonstrated a reduction of both IRS-1 and IRS-2 and compromised insulin–IR–IRS signalling (246,247).

1.7.4 The role of IR signalling on insulin secretion

In contrast to c-Kit, numerous studies have examined the capacity of IR activation to stimulate insulin release. There is little consensus on the role of insulin-stimulated insulin secretion since multiple studies have presented both stimulatory and inhibitory roles (summarized in Figure 1.3). Findings from both in vitro cell models and in vivo rodent studies indicate that maintenance of the insulin–IR–IRS signalling axis is important for biphasic insulin signalling and insulin granule exocytotic machinery. Transfection of mouse insulinoma cell line βTC6-F7 cells with a kinase-inactivating mutation in IR was shown to impair glucose-stimulated insulin secretion (248). βIRKO mice demonstrated that reducing the presence of IR interferes with first-phase insulin secretion (229). More recently, the adaptor protein APPL1, a regulator of Akt and an anchor between IR and IRS-1 (249), was identified as a potential upstream factor for insulin granule exocytotic SNARE protein (Syntaxin 1A, SNAP25, and VAMP2) up-regulation in murine beta cells (250). Isolated single murine beta cells also demonstrated increased exocytotic events when stimulated with high doses of insulin (100 nmol/L) and impaired secretion when pretreated with IR neutralization antibody (251).

A review of the literature suggests that IR–IRS activity differentially affects intracellular Ca\(^{2+}\) influx from the cell membrane and ER, which may explain the contrasting effects of autocrine insulin action during insulin release. It has been shown that IR signalling does not affect membrane depolarization-induced secretion but is required for mobilizing Ca\(^{2+}\) from intracellular stores (251). Insulin stimulation of isolated murine beta cells demonstrated hyperpolarization of the cell and mitochondrial membranes through activated K\(_{ATP}\) channels, which subsequently reduced the levels of intracellular Ca\(^{2+}\)
necessary for insulin exocytosis (252). However, another report showed that treating murine beta cells with 100 nmol/L of insulin heightened intracellular Ca\textsuperscript{2+} release from the ER, resulting in enhanced insulin secretion (253). This pathway relied on activation through the IR–IRS-1–Akt axis and was independent of $K_{\text{ATP}}$-induced depolarization. IRS-1 co-localized with SERCA3b in βTC6-F7 cells, which inhibited Ca\textsuperscript{2+} re-uptake into the ER and increased insulin secretion (254). Irs\textsuperscript{-1/-} mice also displayed reduced insulin granule exocytosis due to a shortened transient period of high intracellular Ca\textsuperscript{2+} levels following glucose stimulation (238), indicating that the IR–IRS-1 signalling axis is important for ER-regulated Ca\textsuperscript{2+} release. It has also been reported that isolated rat islets increased insulin release when IRS-1 was inhibited in a high glucose level environment (255), which suggests that IRS-1-induced insulin secretion can also be affected by glycemic levels. This indicates that additional research is required to determine the contrasting effects of insulin stimulation on intracellular Ca\textsuperscript{2+} levels and the downstream signalling pathways that connect receptor activation to granule exocytosis.
Figure 1.3: Summary of the role of IR activation on insulin secretion.

Autocrine insulin stimulation of beta cells can induce positive or negative feedback on insulin release. Positive feedback (right, green arrowheads): activation of the IR–IRS-1 pathway induced Akt phosphorylation, which increased intracellular Ca$^{2+}$ release from the ER and SNARE protein levels, which led to enhanced insulin release from beta cells. Negative feedback (left, red arrowheads): insulin stimulation activated the IR–IRS–PI3K pathway, which resulted in hyperpolarization of membrane $K_{ATP}$ channel and a reduction in Ca$^{2+}$-stimulated insulin release.
1.7.5 Interplay between c-Kit and IR signalling on insulin exocytosis

Mice with beta cell-specific c-Kit overexpression (c-KitβTg) exhibited increased expression of *Ir* and *Irs-1* in isolated islets at 8-weeks of age (205). Enhanced insulin release was also initially observed in 8-week c-KitβTg mice and continued to 28 weeks of age (199). This up-regulation of the IR signalling axis in islets of mice with c-Kit overexpression established a link between the c-Kit and IR–IRS pathways. Therefore, the improved insulin secretion and increased beta cell mass and proliferation observed in 8-week c-KitβTg mice may be due to intact and enhanced signalling through the IR pathway. The previous findings from this mouse model resulted in two suggested mechanisms where c-Kit signalling in beta cells led to IR–IRS signalling up-regulation: [1] a direct interaction that affects the activity of the IR–IRS pathway (previously proposed in reference (206)); and/or [2] indirect IR activation through increased insulin secretion from c-Kit signalling (Figure 1.4). The focus of the work included in this thesis was to examine if signalling through both c-Kit and IR were required for maintaining beta cell function and insulin release.
c-Kit and IR both promote PI3K–Akt signalling, which is required for beta cell proliferation and the maintenance of factors that control insulin secretion. c-Kit receptor activation is proposed to increase IR–IRS signalling (via phosphorylation of tyrosine sites, green) through the following: [1] direct activation, and [2] indirect activation through increased insulin secretion. Y, tyrosine; S, serine.
1.8 Rationale and hypothesis

1.8.1 General hypothesis of project

The main objective of the work in the following chapters was to determine the role of c-Kit and IR interplay using *in vitro* and *in vivo* models. I hypothesized that the regulation of c-Kit and IR signalling in beta cells under normal and diabetic stresses affects beta cell function, proliferation, and insulin release.

1.8.2 Beta cell function in 60-week c-KitβTg mice

RTKs in beta cells are essential to maintain beta cell mass and insulin secretion. 8-week old c-KitβTg mice have been shown to increase the expression of IR–IRS-1 in islets (205). However, the prolonged expression of RTKs in islets has not been well characterized. Mice with beta cell overexpression of platelet-derived growth factor receptor (PDGFR), a RTK found in the same family as c-Kit, developed improved glucose tolerance and increased beta cell mass at 60-weeks of age (157). However, it was not known if c-Kit signalling would display a similar phenotype and maintain the enhanced beta cell proliferation and insulin secretion seen in younger mice. In addition, while the increase in IR expression and insulin secretion may be beneficial under transient conditions, chronic insulin signalling may result in complications including insulin resistance and reduced lifespan in mice (256,257). The examination of prolonged c-Kit overexpression in mouse beta cells is therefore important. In Chapter 3, 60-week c-KitβTg and c-KitβTg;βIRKO mice were examined to determine the effect of chronic c-Kit overexpression on insulin release, beta cell mass and transcription factor expression, intracellular signalling, and regulation of the IR axis. I hypothesized that prolonged c-Kit signalling would produce glucose intolerance and beta cell defects due to c-Kit-induced over-activation of the IR–IRS-1 pathway, and that reducing the expression of IR on beta cells of c-KitβTg mice would restore glucose tolerance.

1.8.3 Postnatal IR loss in beta cells under normal and high-fat diet conditions

IR expression on beta cells can influence beta cell mass, proliferation, and insulin transcription. The presence of IR on beta cells throughout development ensures that
normal beta cell mass and function is maintained. The loss of beta cell IR during development (βIRKO) produced adult mice that displayed reduced beta cell mass and first-phase insulin secretion, leading to the development of glucose intolerance (228,229). These mice are also unable to compensate for high-fat diet (HFD)-induced beta cell mass expansion (258). Previous research from our laboratory has shown that mice with a tamoxifen-inducible loss of beta cell IR (MIP-βIRKO; MIP, mouse insulin promoter) induced at e13.5 had compensatory beta cell expansion induced through IGF-1R–IGF-2 signalling, which indicates that the stage at which IR loss is induced may produce differing effects on islet mass and function (227). Preliminary studies from our group also found that postnatal beta cell IR loss (4-week) did not alter glucose tolerance or beta cell morphology and proliferation in mice fed a short-term HFD for 6 weeks. However, long-term (12-16 weeks) administration of HFD feeding in C57BL/6 mice has been shown to promote multiple metabolic changes not present on short-term HFD, including peripheral insulin resistance and the progressive loss of beta cell transcription factors and increased ER stresses (132-134). In Chapter 4, MIP-βIRKO mice with postnatal beta cell IR loss induced at 4 weeks of age were exposed to long-term (18-week) HFD feeding to examine the effects on glucose tolerance, insulin secretion, and beta cell mass and intracellular signalling pathways. I hypothesized that the loss of postnatal beta cell IR signalling in mice exposed to a prolonged HFD would produce beta cell dysfunction and loss of beta cell mass that resulted in the development of hyperglycemia.

1.8.4 In vitro c-Kit and IR interplay

Co-stimulation of RTKs has been shown to lead to synergistic effects in cells (259,260). Both c-Kit and IR have been shown to individually activate the PI3K–Akt signalling pathway in beta cells (205,261). However, it is not currently known if co-activation enhances signalling along this pathway. Since c-Kit overexpression initiated increased islet IR expression, there is the potential that the enhanced proliferation and signalling observed in c-KitβTg mice is due to activity from both receptors (205). Chapter 5 of this thesis used the INS-1 832/13 cell line to examine the effects of c-Kit and IR co-stimulation on the activation of intracellular signalling pathways and cell proliferation at various time-points (15 minutes, 1 hour and 24 hours). I hypothesized that c-Kit and IR
co-stimulation would produce a synergistic effect on intracellular signalling and proliferation that is greater than single receptor stimulation alone.
1.9 References

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Chapter 2

2 General materials and methods for Chapters 3, 4, and 5
2.1 Experimental mouse models

2.1.1 General maintenance of mouse models

Experimental male mice utilized in Chapters 3 and 4 were housed with their control male littermates, with a minimum of 2 mice per cage and a maximum of 5 mice per cage. Mice were maintained on a 12-hour light/12-hour dark cycle. All mice were fed their assigned diet ad libitum. Weekly health checks were performed on all mice, and mice that developed health complications were excluded from experimental studies. Tamoxifen (>99%, Sigma-Aldrich, Saint Louis, MO; product #T5648) was prepared at a concentration of 30 mg/mL in corn oil vehicle (Sigma-Aldrich) and delivered using intraperitoneal injection or oral gavage (1,2). All animal work was conducted after protocols were approved by the University of Western Ontario Animal User Subcommittee in accordance with the Canadian Council of Animal Care guidelines (Appendix A). Biosafety permits were also approved prior to mouse experiments (Appendix B).

2.1.2 Mouse models in Chapter 3

Generation of the c-KitβTg mouse model: The c-KitβTg mouse line was generated with the injection of a pKS/RIP plasmid containing human c-KIT (2.9 kbp), with IRES2-linked enhanced green fluorescence protein (eGFP), into the pronuclei of one-cell C57BL/6J embryos as described previously (3). Mice that were positive for c-Kit expression and did not demonstrate any phenotypic abnormalities were used as founder mice and crossed with wild-type C57BL/6J mice (The Jackson Laboratory, Bar Harbor, ME). Genotyping primers c-Kit/Globin5 and eGFP/Globin3 were used to confirm c-Kit expression in mice (Figure 2.1a). Sequences can be found in Table 2.1. Male wild-type and c-KitβTg mice were aged to 60 weeks for analysis. All mice were maintained on a normal chow diet (Harlan Teklad, product #2919, Indianapolis, IN, USA) ad libitum throughout the study.
Figure 2.1: Representative PCR product from genotyping of c-KitβTg, c-KitβTg;MIP-CreER;IR, and MIP-CreER;IRfl/+ mouse lines.

Representative images for (a) eGFP, (b) IRfl/fl, and (c) MIP-CreERT bands from PCR gel electrophoresis. Base pair (bp) length is listed for each band of interest.
Table 2.1: Primers for PCR genotyping of c-KitβTg, c-KitβTg;βIRKO, and MIP-βIRKO mice featured in Chapters 3 and 4.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Primer sequence 5’-3’</th>
<th>Fragment</th>
<th>Annealing Temp</th>
</tr>
</thead>
<tbody>
<tr>
<td>eGFP</td>
<td>5’-CAG TCC GCC CTG AGC AAA GAC C-3’</td>
<td>303bp</td>
<td>57°C</td>
</tr>
<tr>
<td>Globin3</td>
<td>5’-GGT ATT TGT GAG CCA GGG CAT TG-3’</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Globin5</td>
<td>5’-GCT CCT GGG CAA CGT GCT GG-3’</td>
<td>481bp</td>
<td>57°C</td>
</tr>
<tr>
<td>c-Kit</td>
<td>5’-GTG AGA GGA CAG CGG ACC AGC-3’</td>
<td></td>
<td></td>
</tr>
<tr>
<td>oIMR6765</td>
<td>5’-GAT GTG CAC CCC ATG TCT G-3’</td>
<td>KO 313bp</td>
<td>60°C</td>
</tr>
<tr>
<td>oIMR6766</td>
<td>5’-CTG AAT AGC TGA GAC CAC AG-3’</td>
<td>WT 279bp</td>
<td></td>
</tr>
<tr>
<td>MIP</td>
<td>5’-CCT GGC GAT CCC TGA ACA TGT CCT-3’</td>
<td>268bp</td>
<td>57°C</td>
</tr>
<tr>
<td>CreER</td>
<td>5’-TGG ACT ATA AAG CTG GTG GGC AT-3’</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

WT, wild-type; KO, knockout
Generation of c-KitβTg;βIRKO mouse model: Tg(Ins1-Cre/ERT)1Lphi mice (MIP-CreERT) were obtained from Dr. Louis Philipson at the University of Chicago (Chicago, IL). The verification of Insulin 1 (MIP) promoter-driven Cre recombinase expression in pancreatic beta cells was performed by crossing MIP-CreERT mice with the B6.Cg-Gt(Rosa)26Sor tm9(CAG-tdTomato)Hze/J reporter strain (stock number: 007909; The Jackson Laboratories) as previously reported (4). MIP-CreERT mice were crossed to B6.129S4(FVB)-Insr tm1Khn/J mice (IR fl/fl) from Jackson Laboratories (stock number: 006955) to produce a beta cell-specific Cre recombinase IR knockout model that was induced with tamoxifen administration (MIP-CreER+/−;IR fl/+ or MIP-CreER−/−;IR fl/fl) (Figure 2.2) (4). PCR genotyping was performed using primers oIMR6765 and oIMR6766 for floxed IR detection and primers MIP and Cre-ERT for MIP-CreERT driven Cre recombinase detection (Table 2.1 and Figure 2.1bc). The MIP-CreER;IR fl/+ line was crossed to c-KitβTg mice to generate the following experimental groups: control (WT;MIP-CreER+;IR fl/+ or WT;MIP-CreER −;IR fl/fl to increase the number of control mice available), c-KitβTg (c-KitβTg;MIP-CreER+;IR fl/+), and c-KitβTg;βIRKO mice (c-KitβTg;MIP-CreER+;IR fl/fl) (Figure 2.3). In order to induce beta cell IR knockout at 40 weeks of age, 4 mg/20 g bodyweight of tamoxifen was administered daily to male mice from all three experimental groups via oral gavage for three consecutive days (Figure 2.4a) (5). Mice were maintained on ad libitum normal chow diet and examined at 60 weeks of age.

2.1.3 Mouse model in Chapter 4

The MIP-CreER;IR fl/+ mouse line described above was used to generate male control (MIP-CreER+;IR fl/+ or MIP-CreER−;IR fl/fl) and MIP-βIRKO (MIP-CreER+;IR fl/fl) mice (Figure 2.2). Genotyping using the IR and MIP Cre-ERT primers was used to confirm floxed IR or MIP-CreERT expression (Table 2.1 and Figure 2.1bc). Control and MIP-βIRKO mice at 4 weeks of age received daily intraperitoneal tamoxifen injections (4 mg/20 g bodyweight) for three days (Figure 2.4b) (5). At six weeks of age, control and MIP-βIRKO mice were placed either on ad libitum normal chow (23% protein, 22% fat, 55% carbohydrate, 3.3 kcal/g; product #2919; Harlan Teklad) or high-fat diet (20% protein, 60% fat, 20% carbohydrates, 5.21 kcal/g; product # D12492; Research Diets Inc, New Brunswick, NJ, USA) for 18 weeks. Mice were analyzed at 24 weeks of age.
Figure 2.2: Breeding schematic for the MIP-CreER;IR\textsuperscript{fl/+} mouse line.

Tg(Ins1-Cre/ERT)\textsuperscript{1Lphi} and B6.129S4(FVB)-Insr\textsuperscript{lm1khn/J} lines were crossed to generate heterozygous MIP-CreER\textsuperscript{+/-};IR\textsuperscript{fl/+} breeding mouse pairs. The offspring from MIP-CreER\textsuperscript{+/-};IR\textsuperscript{fl/+} mouse breeding pairs are listed with the percentage for potential generation of each genotype. Mice used for the control group (MIP-CreER\textsuperscript{+} or MIP-CreER;IR\textsuperscript{fl/fl}) are outlined in green, while MIP-βIRKO mice (MIP-CreER\textsuperscript{+};IR\textsuperscript{fl/fl}) are outlined in red.
Figure 2.3: Breeding schematic for the c-KitβTg;βIRKO mouse line.

Established mice from c-KitβTg and MIP-CreER\textsuperscript{+/−};IR\textsuperscript{fl/fl} mouse lines were crossed to produce c-KitβTg;MIP-CreER\textsuperscript{+/−};IR\textsuperscript{fl/+} breeding pairs. Control (green, WT;MIP-CreER\textsuperscript{+};IR\textsuperscript{+/-} or WT;MIP-CreER\textsuperscript{+};IR\textsuperscript{fl/fl}), c-KitβTg (blue, c-KitβTg;MIP-CreER\textsuperscript{+};IR\textsuperscript{+/-}), and c-KitβTg;βIRKO mice (red, c-KitβTg;MIP-CreER\textsuperscript{+};IR\textsuperscript{fl/fl}) were generated from the breeding pairs. The percentage of offspring that are predicted to result from each crossing are listed beside each genotype.
Figure 2.4: Timeline design for studies involving the c-KitβTg;MIP-CreER;IR and MIP-CreER;IR mouse lines.

Timelines showing the week of tamoxifen administration, HFD feeding initiation, and end-point analysis of (a) c-KitβTg;βIRKO and (b) MIP-βIRKO mouse cohorts discussed in Chapters 3 and 4 of this work, respectively.
2.2 Genotyping of mouse models

In order to identify mouse genotypes for experimental groups in Chapters 3 and 4, tail snips (1-2 mm) were obtained at postnatal day 21 and dissolved in base solution (25 mmol/L NaOH and 0.2 mmol/L EDTA) at 95°C for 30 minutes. Tail samples were incubated at room temperature for 1 hour and then immersed in 40 mmol/L Tris HCl (pH 5.0). Primers (Table 2.1) and tail samples were added to 2x Taq FroggaMix (FroggaBio Inc., Toronto, ON) and amplified using a thermocycler. Annealing temperatures were dependent on the primers utilized. PCR samples were run on a 2% ethidium bromide (Invitrogen, Burlington, ON) agarose gel and imaged on the GeneGenius BioImaging System (Syngene, Frederick, MD) (Figure 2.1).

2.3 Metabolic studies of mouse models

Experimental mouse models used in Chapters 3 and 4 had their body weight measured at experimental end-points. Food intake was monitored in individual 60-week wild-type and c-KitβTg mice every 24 hours for a 72-hour period. Overnight fasting (16-hour) blood glucose measurements were taken in all experimental mouse models. All blood glucose readings were taken from tail vein samples and were read on a FreeStyle Lite glucometer (Abbott, Mississauga, ON, Canada).

Intraperitoneal glucose tolerance tests (IPGTT), which bypass incretin-induced insulin release, were performed in all mouse models after 16-hour fasting using a 2 mg/g bodyweight injection of D- (+)-glucose (Dextrose; Sigma, Saint Louis, MO) (6). Glucose levels were measured at baseline (0 minutes) and at 15, 30, 60, 90, and 120 minutes following administration. Intraperitoneal insulin tolerance tests (IPITT) were performed in 4-hour fasted mice using 1 U/kg bodyweight of insulin (Humulin, Eli Lilly, Toronto, ON), and glucose levels were measured at baseline and 15, 30, 60, and 120 minutes following administration. Differences between control and experimental groups were determined using the area under the curve (AUC), which represented glucose levels over a period of 120 minutes (mmol/L x min) or percentage of baseline glucose over a period of 120 minutes (% x min) (7,8). In vivo glucose-stimulated insulin secretion (GSIS) was conducted after 16 hours of fasting by injecting 2 mg/g bodyweight glucose. Plasma
insulin was collected at baseline (0 mins), 5, and 35 minutes following glucose injection and blood glucose measurements were taken in parallel. Samples were centrifuged and serum was stored at -20°C for insulin measurement using ELISA.

2.4 Islet isolation

The islet isolation protocol described below was used for islet purification for ex vivo GSIS, ex vivo insulin stimulation experiments, and protein analyses. Mouse pancreata were saturated with collagenase V (1 mg/mL, Sigma-Aldrich) through ductal injection and digested at 37°C for 30 minutes in dissociation buffer (Hank’s Balanced Salt Solution and HEPES; 0.6% g/mL). Islets were separated from exocrine tissue by Ficoll density gradient followed by hand picking, as previously described (9,10).

2.5 Ex vivo glucose-stimulated insulin secretion and insulin-treated stimulation of isolated islets

For ex vivo GSIS experiments, 10 islets from wild-type and c-KitβTg mice (Chapter 3) and from control and MIP-βIRKO mice (Chapter 4) were picked and recovered overnight in RPMI-1640 media (FBS; Gibco) supplemented with 10% fetal bovine serum (Gibco), 2 g/L sodium bicarbonate, 10 mmol/L HEPES, and 1 mmol/L sodium pyruvate. Low and high glucose stimulation was initiated by incubating islets for 1 hour in RPMI-1640 supplemented with 0.5% bovine serum albumin (BSA; Sigma-Aldrich) and containing either 2.2 mmol/L or 22 mmol/L glucose, respectively (3,11). Media from the low and high glucose conditions were collected for insulin measurement. Ex vivo GSIS measurements were completed in technical duplicates.

For insulin stimulation of islets from wild-type and c-KitβTg mice (Chapter 3), islets from each mouse were divided into two separate wells in a 12-well plate and incubated in RPMI-1640 supplemented with 1% BSA media for 3 hours. Islets were either stimulated with 2 nmol/L insulin (Sigma-Aldrich) or were left unstimulated (12). Cells were harvested 24 hours after insulin stimulation for protein lysis analysis.
2.6 ELISA analysis of *in vivo* and *ex vivo* insulin secretion and islet insulin content

Fed plasma insulin levels were measured from cardiac serum withdrawal following mouse euthanasia. Islet insulin content was measured by extracting insulin from fresh islets using Acid-Ethanol extraction (165 mmol/L HCl in 70% ethanol). Insulin release and content was detected using the ALPCO (Salem, NH) ultrasensitive insulin mouse ELISA kit (0.15 ng/mL sensitivity) (3). Insulin levels in plasma and media were reported as ng/mL. Islet insulin content was normalised to total islet protein content, measured using a Bradford Assay (Bio-Rad Dye; Bio-Rad Laboratories Inc, Mississauga, ON), and expressed as µg/mg.

2.7 *In vitro* cell culture and treatment

**INS-1 cell line culture:** The INS-1 832/13 cell line provided by Dr. C. Newgard (Duke University, Durham, North Carolina) was maintained in RPMI-1640 media (Gibco) supplemented with 10% FBS (Gibco), 2 g/L sodium bicarbonate, 10 mmol/L HEPES, 1 mmol/L sodium pyruvate, and 0.05 mmol/L 2-mercaptoethanol (Sigma-Aldrich). Cells were incubated at 37°C in 5% CO₂. Experiments involving the INS-1 cell line (Chapters 4 and 5) were performed when cell growth reached 80-90% confluency. Treated INS-1 cells were harvested for western blot and immunofluorescence staining at the designated time-points. The use of the INS-1 832/13 cell line was approved and can be found on the Biosafety protocol (Appendix B).

*Insulin-stimulated SNARE protein analysis:* For Chapter 4, INS-1 cells were serum-starved in RPMI-1640 supplemented with 1% BSA media overnight (16-24 hours) before treatment. Cell wells were either left untreated (control) or received 100 nmol/L insulin (Sigma-Aldrich) for 1 hour. Cells were harvested, fixed, and embedded in 2% agarose gel for paraffin-embedded immunofluorescence staining.

*SCF and insulin time-dependent co-stimulation studies:* For Chapter 5, INS-1 cells were serum-starved in RPMI-1640 with 1% BSA media overnight (for 15-minute and 1-hour stimulation) or for 3 hours (for 24-hour stimulation) before treatment. Experimental wells received no treatment (control), SCF only (50 ng/mL; ID Labs, London, ON), insulin
only (0.2 nmol/L; Sigma), or a combination of SCF (50 ng/mL) and insulin (0.2 nmol/L) for a 15-minute, 1-hour, or a 24-hour period (3,13). Cells were either harvested and lysed in a Nonidet-P40 (NP40)-containing lysis buffer for western blot analysis or were fixed and embedded in 2% agarose gel for paraffin-embedded immunofluorescence analysis.

**Rapamycin treatment of INS-1 cells:** For Chapter 5, cells were incubated in RPMI-1640 with 1% BSA media overnight once they had reached 80-90% confluency. 100 nmol/L of rapamycin (LC Laboratories, Woburn, MA) was used as a 1-hour pre-treatment before SCF (50 ng/mL) and insulin (0.2 nmol/L) co-stimulation was initiated for 1 hour (7). Experimental groups were as follows: [1] no treatment (control), [2] 1-hour SCF and insulin co-stimulation, [3] 1-hour rapamycin pre-treatment, [4] 1-hour rapamycin pre-treatment with subsequent 1-hour SCF and insulin co-stimulation. Cells were harvested and lysed in NP40 lysis buffer for western blot analysis.

### 2.8 Histological staining and morphometric analyses

#### 2.8.1 Immunofluorescence and immunohistochemical staining

Pancreata from mice and treated INS-1 cells were fixed overnight (4°C) in 4% paraformaldehyde. INS-1 cell pellets were embedded in 2% agarose gel. Fixed cells and pancreata were dehydrated and embedded in paraffin wax then sectioned at 4 μm and placed on slides. Cell and pancreatic sections were probed with the primary antibodies listed in Table 2.2 for single immunohistochemical or double immunofluorescence staining. Sections were treated with heat-induced antigen retrieval solution (citrate pH 6.0 or Tris-EDTA pH 9.0) to improve antibody detection (indicated in Table 2.2).

Double immunofluorescence staining was performed using fluorescein isothiocyanate (FITC)- and tetramethyl rhodamine isothiocyanate (TRITC)-conjugated secondary antibodies (Jackson Immunoresearch, West Grove, PA) and nuclear counterstaining was conducted with 4’-6’-diamidino-2-phenylindole (DAPI) (1:1000 dilution; Sigma-Aldrich). Immunohistochemical detection of a single antigen was achieved using the Zymed Histostain Plus IHC kit and aminoethyl carbazole (AEC) substrate kit (Invitrogen) with hematoxylin counterstaining (Invitrogen). Negative control slides were prepared by adding secondary antibody alone to rule out non-specific binding.
Antibodies, which were used for both histological and western blot analyses, were validated in prior publications (4,7,14). Islet sections and cultured cells were captured using the Leica DMIRE2 fluorescent microscope and analyzed using Image Pro Plus software version 7.0.1 (MediaCybernetics, Rockville, MD). Representative double immunofluorescence images in this thesis were imaged using the Nikon Eclipse Ti2 confocal microscope (Nikon, Melville, NY).

2.8.2 Histological analysis and islet quantification

Islet morphology was measured using area measurement software in Image Pro Plus. Alpha and beta cell mass were calculated using the following previously described formula (15,16):

\[
\text{alpha, beta cell mass [mg]} = \frac{\text{alpha, beta cell area} \times \text{pancreas mass [mg]}}{\text{pancreas section area}}
\]

Islet density was calculated by dividing the total number of islets per section by the total pancreatic area. Quantification of beta cell proliferation (Ki67), beta cell transcription factors (PDX-1, NKX6.1, MAFA, FOXO1), and beta cell SNARE proteins (Syntaxin 1A, VAMP2, SNAP25, MUNC18-1) was conducted using manual cell counting of marker positive beta cells (marker+ / insulin+ cells) divided by the total number of beta cells in each islet (11,16). Immunohistochemistry of phosphorylated (p-) AktS473 staining in islets was performed by dividing the number of p-AktS473+ cells over the total number of islet cells. Islet capillary morphology was measured using the capillary marker PECAM-1 and calculated using the following formulas: Islet capillary area was calculated by dividing the total islet capillary area over the total islet area; islet capillary density was quantified by dividing the number of islet capillaries over total islet area; islet capillary diameter was measured by dividing capillary diameter by the number of islet capillaries (7). A minimum of ten islets per section were used for marker-positive quantifications.
2.9 Protein isolation and western blot analyses

Isolated mouse islets and INS-1 cells were lysed in Nonidet-P40 (NP40; Sigma-Aldrich) lysis buffer (100 mmol/L sodium othovanadate; 100 mmol/L phenylmethylsulfonyl fluoride; complete Protease Inhibitor Cocktail Tablet, Roche) for 20 minutes on ice and sonicated. Protein samples were separated by centrifugation (15,800 rcf) for 20 minutes at 4°C. To determine the protein concentration of each sample, a Bradford assay with Bio-Rad Dye (Bio-Rad Laboratories Inc.) was run in parallel with a BSA standard curve (0-0.5 mg/mL BSA). All protein samples were normalised to 15-20 μg before loading on to a western blot gel.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) ranging from 5-12% was used to separate bands of a targeted kDa size. After the samples had run through and separated, gel bands were transferred to a nitrocellulose membrane and run on ice for 120 minutes at 250 mA. Ponceau S dye (Sigma-Aldrich) was used to confirm successful protein transfer. After washing in Tris buffered saline containing 0.1% Tween-20 (TBST), membranes were blocked in 5% non-fat dry milk (2.5 mL TBS, 50 μL NP-40 in 50 mL double distilled water with pH adjusted to 7.4) at room temperature for 1 hour before overnight primary antibody incubation at 4°C (Table 2.2). Anti-rabbit and anti-mouse IgG secondary antibodies were conjugated with horseradish peroxidase and used to detect primary antibodies (Cell Signalling Technology, Whitby, ON). Chemiluminescence detection (PerkinElmer, Waltham, MA) was used to visualize proteins of interest and membranes were imaged on the Versadoc imaging system, version 4.6.9 (Bio-Rad Laboratories Inc.). Reference proteins (α-tubulin, β-actin) and total proteins were used to normalise protein bands of interest. The probing for total protein bands of signalling proteins was completed after immersing the membrane in membrane stripping buffer (3.5 mL 2-mercaptoethanol, 3.79 g Tris base, 10 g SDS in 500 mL double distilled water with pH adjusted to 6.7) at 55°C and blocking in milk blocking solution for 1 hour at room temperature. Densitometry analysis of bands was completed with the Bio-Rad Image Lab program, version 5.2 (Bio-Rad Laboratories Inc.).
2.10 Statistical analyses

All data presented are represented as mean ± SEM and were generated using GraphPad Prism, version 5.0 (GraphPad Software, Inc.). An unpaired Student’s t-test was used when examining two groups, while a one-way ANOVA with a Tukey’s post hoc test was used when comparing groups of three or more. Statistical significance was obtained when \( p < 0.05 \).
Table 2.2: Antibodies for immunohistochemistry/immunofluorescence and western blot analysis.

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\(^W\): Western Blot, \(^B\): Blot, \(^A\): Antibody
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<td>1:100/1:1000</td>
<td>Abcam Inc. Cambridge, MA, USA</td>
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<td>Cell Signaling Technology, Boston, MA, USA</td>
</tr>
<tr>
<td>Mouse anti-SNAP25</td>
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<td>Mouse anti-Syntaxin 1A</td>
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<td>Rabbit anti-VAMP2</td>
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\( ^w \) Dilution selected for optimal western blot probing

\( ^A \) Citrate (pH 6.0) antigen retrieval used

\( ^B \) Tris-EDTA (pH 9.0) antigen retrieval used
2.11 References


3 Long-term c-Kit overexpression in beta cells compromised their function\textsuperscript{2}

\textsuperscript{2} Text and figures from this chapter have been adapted from the following manuscript:


** Partial data from Figures 3.1, 3.2, and 3.3 were collected and analyzed by Z-C Feng (co-author in the manuscript above)
3.1 Introduction

The receptor tyrosine kinase (RTK) c-Kit, expressed during pancreatic development and in a subset of adult beta cells, has been shown to activate the PI3K/Akt signalling pathway to regulate beta cell mass, increase beta cell proliferation, and enhance insulin secretion (1-6). c-Kit activation also improved islet cell viability and vascularization through suppression of Fas receptor-induced apoptosis and up-regulated VEGF-A production, respectively (7,8). Importantly, mice with beta cell-specific c-Kit overexpression (c-KitβTg) had increased expression of insulin receptor (IR) and insulin receptor substrate-1 (IRS-1) at 8 weeks (4). Although c-KitβTg mice demonstrated improved beta cell function up to 28 weeks on control chow diet, feeding c-KitβTg mice a high-fat diet for 22 weeks resulted in islet vessel dilation and subsequent infiltration of inflammatory cells, which led to beta cell apoptosis and reduced insulin release (7). These results highlighted the importance of controlled c-Kit activation in beta cells to maintain beta cell function and insulin secretion.

Ageing has been shown to deleteriously impact the islet microenvironment through the development of insulin secretion defects and decreased Ins1/Ins2 and Glut-2 mRNA expression in beta cells (9,10). Islets from ageing mice were found to be more susceptible to Fas-induced apoptosis and showed reduced PDX-1 expression, which indicated beta cell dysfunction in older mice (11). Beta cell proliferation was also lower in ageing mice. However, some studies have found that beta cells in older mice were able to replicate in response to beta cell damage (12-17). The induction of beta cell senescence in the islets of ageing mice has also been reported to enhance insulin secretion, emphasizing that the effects of ageing on islets are not well established (18).

We have recently shown that enhanced c-Kit signalling can improve pancreatic transcription factor expression and beta cell proliferation, viability, and insulin secretion during the age-dependent decline of beta cell function (4,8). However, the effects of long-term c-Kit overexpression on beta cells are currently not known. This chapter examined c-KitβTg mice at 60 weeks of age to determine the effects of chronic c-Kit overexpression towards the maintenance of beta cell mass and insulin release. In this study, we also used a mouse model generated through crossing tamoxifen-inducible, beta
cell-specific insulin receptor (IR) knockout mice with c-KitβTg mice (c-KitβTg;βIRKO) in order to determine the role of insulin receptor signalling in 60-week c-KitβTg mice.

3.2 Materials and methods

*Generation of c-KitβTg and c-KitβTg;βIRKO mice:* Male wild-type (WT) and c-KitβTg mice were generated from established in-house breeding colonies. c-KitβTg mice were aged to 60 weeks and subjected to metabolic studies and islet morphology and protein expression analyses. c-KitβTg;βIRKO mice (with littermate control and c-KitβTg mice) were generated by crossing c-KitβTg mice with MIP- CreER;IR°/+ mice from the MIP-βIRKO colony. c-KitβTg;βIRKO mice and littermate controls were treated with tamoxifen (4 mg/ 20 g bodyweight, i.p.) at 40 weeks to induce beta cell IR knockout and analyzed at 60 weeks of age. More information on these mouse models can be found in Section 2.1.2.

*Metabolic experiments:* c-KitβTg mouse groups had body weight and overnight (16-hour) fasting blood glucose measured at 40 and 60 weeks of age. c-KitβTg;βIRKO groups had body weight and overnight blood glucose levels measured at 60 weeks. IPGTT, IPITT, and in vivo and ex vivo GSIS assays were performed and analyzed as detailed in Sections 2.3 and 2.5.

*Western blot analyses:* Islets from 60-week WT and c-KitβTg mice were probed for Syntaxin 1a, MUNC18-1, IR, and phosphorylated (p-) IRS-1S612 and normalised to the loading protein control β-actin (*Table 2.2*). Signalling and cleaved proteins (p-AktS473, p-ERK mutant T202/Y204, cleaved PARP) were normalised to respective total proteins. Additional information can be found in Section 2.9. For insulin stimulation, islets from 60-week WT and c-KitβTg mice were either untreated or treated for 24 hours with 2 nmol/L insulin and harvested for western blot analysis of p-AktS473 (Section 2.5).

*Immunostaining:* Pancreata from the 60-week c-KitβTg group (WT and c-KitβTg) and c-KitβTg;βIRKO group (control, c-KitβTg, and c-KitβTg;βIRKO) were stained with select primary antibodies listed in *Table 2.2*. Immunofluorescence analysis was completed with fluorophore-labeled secondary antibodies (Jackson Immunoresearch) and DAPI (Sigma-
For immunohistochemical studies, the Zymed Histostain Plus kit and AEC substrate kit (Invitrogen) with hematoxylin counterstain (Invitrogen) were used. Islet morphology (islet density, beta cell mass, alpha cell mass), co-staining (marker+/insulin+ cells), or single label staining (marker+ islet cells) quantification was completed as described in Section 2.8. Sample sizes reflected pancreata sections analyzed from different biological replicates, with a minimum of 10 islets analyzed from each sample.

**Statistical analysis:** All data presented are represented as mean ± SEM and were analyzed using either an unpaired Student’s t-test (2 groups) or a one-way ANOVA test with a Tukey’s post hoc test (3 or more groups). Statistical significance was obtained when $p < 0.05$.

### 3.3 Results

#### 3.3.1 c-Kit overexpression impaired glucose tolerance in c-KitβTg mice after prolonged expression

Previous studies demonstrated no change in body weight between WT and c-KitβTg mice at 8 and 28 weeks of age (4,7). During the current study, there was no change in body weight observed in mice at 40 weeks of age, but c-KitβTg mice exhibited increased body weight compared to WT littermates at 60 weeks of age (Figure 3.1a). Also in contrast to prior studies demonstrating reduced fasting blood glucose in young c-KitβTg mice (4,7), the current study found similar blood glucose levels in WT and c-KitβTg mice at 40 weeks of age and significantly elevated levels in c-KitβTg mice at 60 weeks (Figure 3.1b). Examination of pancreas and fat pad mass in 60-week old mice revealed no significant changes between WT and c-KitβTg mice (Figure 3.1cd). The average 24-hour food intake over a 3-day period at 60 weeks did not change between the two groups (Figure 3.1e).

Although improved glucose tolerance was previously observed in young c-KitβTg mice (4,7), 60-week c-KitβTg mice demonstrated impaired glucose tolerance compared to their WT counterparts and did not return to basal levels at 120 minutes (Figure 3.2a). IPITT tests also revealed similar insulin sensitivity between WT and c-KitβTg mice (Figure 3.2b), suggesting that the poor glucose tolerance observed in older c-KitβTg mice may be
due to impaired insulin release by beta cells. Therefore, insulin release was assayed to measure beta cell insulin secretion in response to a glucose challenge. *Ex vivo* GSIS demonstrated similar insulin release from islets between 60-week WT and c-KitβTg mice at 2.2 mmol/L of glucose stimulation but low insulin secretion from islets of 60-week c-KitβTg mice when incubated in 22 mmol/L glucose (Figure 3.2c). Insulin content from isolated islets of 60-week c-KitβTg mice did not differ from WT mice (Figure 3.2d).
Figure 3.1: c-KitβTg mice had increased body weight and fasted glucose levels at 60 weeks of age.

(a) Body weight measurements at 40 (n=13 WT and n=30 c-KitβTg) and 60 (n=20 WT and n=22 c-KitβTg) weeks of age, and (b) overnight (16 hours) fasting blood glucose at 40 (n=13 WT and n=30 c-KitβTg) and 60 (n=15 WT and n=20 c-KitβTg) weeks of age for WT and c-KitβTg mice demonstrated the progressive increase in weight gain and blood glucose levels. Body weight and fasting blood glucose levels were compared to mice previously analyzed at 8 and 28 weeks of age (4,7). (c) Pancreatic (n= 9 for WT, n= 14 for c-KitβTg) and (d) fat pad weights (n= 11 for WT, n= 15 for c-KitβTg) measured at 60 weeks of age in WT and c-KitβTg mice revealed similar weights between the groups. (e) Average 24-hour food intake measurements (in grams) over a 72-hour duration in WT (n=3) and c-KitβTg (n=6) mice. Closed circles: WT group; open circles: c-KitβTg group. Data are expressed as mean ± SEM. *p < 0.05, **p < 0.01; analyzed using unpaired Student’s t-test.
Figure 3.2: Decreased insulin release was linked to impaired glucose tolerance in 60-week old c-KitβTg mice.

(a) IPGTT with area under curve (AUC), and (b) IPITT with AUC for 60-week WT and c-KitβTg mice demonstrated impaired glucose tolerance in 60-week c-KitβTg mice and unchanged insulin resistance (n=13 WT and n=19 c-KitβTg for IPGTT; n=10 WT and n=13 c-KitβTg for IPITT). (c) Ex vivo GSIS (ng/mL) from 10 isolated islets per biological replicate exposed to low (2.2 mmol/L) and high glucose (22 mmol/L) demonstrated that islets from c-KitβTg mice had reduced insulin release under high glucose conditions (n=5). (d) Total insulin content (µg/mg) from isolated islets of 60-week WT and c-KitβTg mice (n=4). Closed circles: WT group; open circles: c-KitβTg group. Data are expressed as mean ± SEM. *p < 0.05; analyzed using unpaired Student’s t-test.
3.3.2 c-KitβTg mice demonstrated increased beta cell mass and proliferation and nuclear FOXO1 expression at 60 weeks of age

Histological analyses of pancreata from 60-week c-KitβTg mice showed an increase in beta cell mass with no discernable changes in islet number or alpha cell mass (Figure 3.3a-d). The increased beta cell mass in 60-week c-KitβTg mice was not due to changes in beta cell size (Figure 3.3e) but was accompanied by higher beta cell proliferation as shown using Ki67 co-staining with insulin (Figure 3.3f). When analyzing the subcellular localisation of transcription factors in beta cells, it was found that nuclear PDX-1 immunoreactivity was similar between 60-week WT and c-KitβTg mice (Figure 3.4a). In contrast, islets from 60-week c-KitβTg mice showed increased FOXO1 localisation in the nucleus (Figure 3.4b). No changes were observed between mouse groups for the nuclear expression of beta cell maturation factors NKX6.1 and MAFA. GLUT2 staining in beta cells was also similar between the groups (Figure 3.5). Vascularization was analyzed in the islets of 60-week WT and c-KitβTg mice and showed similar capillary density, area, and vessel diameter (Figure 3.6).
Figure 3.3: Beta cell mass and proliferation were increased in 60-week c-KitβTg mice.

(a) Representative immunofluorescence images of islet morphology in 60-week WT and c-KitβTg pancreata, showing insulin staining (red) with DAPI counterstained nuclei (blue). Scale bar: 200 μm. Quantification of (b) islet density (no./mm²), (c) beta cell mass (mg), and (d) alpha cell mass (mg), and (e) individual beta cell size (μm²) in 60-week WT and c-KitβTg mice showed significantly increased beta cell mass in c-KitβTg mice (n=5). (f) Proliferation of beta cells, quantified using cell cycle marker Ki67 co-localisation with insulin⁺ cells, was increased in 60-week c-KitβTg mice (n=4). Closed circles: WT group; open circles: c-KitβTg group. Data are expressed as mean ± SEM. *p < 0.05; analyzed using unpaired Student’s t-test.
Figure 3.4: FOXO1 nuclear localisation was increased in islets of c-KitβTg mice at 60 weeks of age.

Representative double immunofluorescence images and quantification of nuclear (a) PDX-1 and (b) FOXO1 (green) co-localised with insulin+ (red) and DAPI (blue) stained pancreas sections. Quantification of nuclear PDX-1 (n=5 WT and n=6 c-KitβTg) and FOXO1 (n=4 WT and n=5 c-KitβTg) in insulin+ cells of 60-week mice revealed increased FOXO1+ beta cells in 60-week c-KitβTg mice. Nuclei are stained with DAPI (blue). White asterisk marks red blood cell autofluorescence in vessel lumens. Scale bar: 50 μm. Closed circles: WT group; open circles: c-KitβTg group. Data are expressed as mean ± SEM. **p < 0.01; analyzed using unpaired Student’s t-test.
Figure 3.5: NKX6.1, MAFA, and GLUT2 expression was similar between islets from 60-week WT and c-KitβTg mice.

(a) Representative immunofluorescence photomicrographs for the beta cell transcription factors NKX6.1 (green), co-stained with insulin (red), and MAFA (red) IHC staining from pancreatic sections of 60-week WT and c-KitβTg mice demonstrated unaltered immunoreactivity. Nuclei were labeled with DAPI (blue) for IF staining, hematoxylin (blue) for IHC staining. (b) Representative immunofluorescence images of GLUT2 (green) co-localisation in insulin⁺ (red) cells showed similar membrane reactivity between 60-week mouse groups. DAPI, blue. White asterisk marks red blood cell autofluorescence in vessel lumens. Scale bar: 50 μm.
Figure 3.6: Islet vascularization was similar in islets from 60-week WT and c-KitβTg mice.

(a) Representative immunofluorescence photomicrographs of islet vascularization detected using PECAM-1 (green) in 60-week WT and c-KitβTg pancreas section co-stained for insulin (red) and DAPI (blue). Scale bar: 50 μm. (b) Blood vessel density, (c) area, and (d) diameter of 60-week WT (n=6) and c-KitβTg (n=5) pancreas sections revealed similar vessel characteristics. Closed circles: WT group; open circles: c-KitβTg group. Data are expressed as mean ± SEM.
3.3.3 Levels of exocytosis-regulating SNARE proteins were reduced in 60-week c-KitβTg mice

The soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) complex, required for insulin vesicle tethering and fusion to the membrane, was examined by western blot and immunofluorescence microscopy to determine their changes in expression. Significantly reduced levels of Syntaxin 1A (Figure 3.7ab) and the associated chaperone protein MUNC18-1 (Figure 3.7cd) were observed in islets from 60-week c-KitβTg mice. Immunofluorescence imaging found unchanged immunoreactivity for SNAP25 between WT and c-KitβTg mice (Figure 3.7e) and relatively low detection of VAMP2 in islets of 60-week c-KitβTg mice (Figure 3.7e). These data implicated that chronic c-Kit overexpression may impair insulin release kinetics in 60-week c-KitβTg beta cells due to significantly reduced levels of SNARE proteins.
Figure 3.7: 60-week c-KitβTg mice demonstrated reduced levels of islet SNARE proteins.

Representative western blot (above) and densitometry (below) for (a) Syntaxin 1A and (c) MUNC18-1 in islets showed decreased levels in 60-week c-KitβTg mice (n=4). Representative immunofluorescence images of (b) Syntaxin 1A and (d) MUNC18-1; (e) SNAP25 (left) and VAMP2 (right) staining in islets of 60-week WT and c-KitβTg mice. SNARE proteins (green) were shown in insulin+ cells (red) labeled with DAPI (blue). Scale bar: 50 μm. White asterisk, red blood cell autofluorescence in vessel lumens. Closed circles: WT group; open circles: c-KitβTg group. Data are expressed as mean ± SEM. *p < 0.05, **p < 0.01; analyzed using unpaired Student’s t-test.
3.3.4 Increased IR and phosphorylated IRS-1\textsuperscript{S612} levels resulted in an impaired response to insulin in 60-week c-KitβTg mice

Although c-KitβTg mice at 8-28 weeks of age showed increased insulin release and enhanced IR–IRS1/2\textsuperscript{Y608}–Akt\textsuperscript{S473} signalling (4,7), islets from 60-week c-KitβTg mice demonstrated increased levels of IR and phosphorylated IRS-1\textsuperscript{S612} (Figure 3.8a). Immunofluorescence analysis for total IRS-1 in pancreata also demonstrated significantly reduced levels in beta cells of 60-week c-KitβTg mice (Figure 3.8b). Increased Akt\textsuperscript{S473} phosphorylation observed in prior studies of 8-week c-KitβTg mice (4) was abolished in 60-week c-KitβTg mice (Figure 3.9a). Similar levels of phosphorylated ERK1/2 was found in islets of 60-week WT and c-KitβTg mice (Figure 3.9a). The apoptosis marker cleaved poly (ADP-ribose) polymerase (PARP) was increased in the islets of c-KitβTg mice at 60 weeks (Figure 3.9a).

To determine whether impaired IR–IRS-1–Akt signalling in islets of 60-week c-KitβTg mice impacted insulin responses, islets were isolated and treated with exogenous insulin at a concentration of 2 nmol/L for 24 hours. Western blot analyses of the lysates revealed that islets from WT mice had increased Akt\textsuperscript{S473} phosphorylation in response to insulin stimulation compared to untreated control islets (Figure 3.9b). However, islets from 60-week c-KitβTg mice treated with 2 nmol/L insulin showed no increase in Akt\textsuperscript{S473} phosphorylation when compared to untreated controls (Figure 3.9b).
Figure 3.8: Islets from 60-week c-KitβTg mice demonstrated increased IR and phosphorylated IRS-1^{S612} levels but reduced total IRS-1 levels.

(a) Western blot and densitometry for total IR and phosphorylated IRS-1^{S612} expression in islets from WT (n=3) and c-KitβTg (n=4) mice showed increased levels of both proteins. (b) Representative images (left) and quantification (right) of IRS-1 (green) in insulin^+ (red) cells in 60-week mice (n=4 WT and n=7 c-KitβTg) co-stained with DAPI (blue). Scale bar: 50 µm. Closed circles: WT group; open circles: c-KitβTg group. Data are expressed as mean ± SEM. *p < 0.05; analyzed using unpaired Student’s t-test.
Figure 3.9: Islets from 60-week c-KitβTg mice exhibited reduced insulin-induced signalling.

(a) Western blot and densitometry for Akt$^{S473}$ and ERK1/2 phosphorylation (n=4), and cleaved PARP levels (n=4 WT and n=3 c-KitβTg) in islets from 60-week c-KitβTg mice demonstrated a loss of Akt signalling with increased islet apoptosis. Data were normalised to total signalling protein (Akt, ERK, PARP) from islets. Closed circles, WT group; open circles, c-KitβTg group. (b) Western blot and densitometry for Akt$^{S473}$ phosphorylation in isolated islets from WT (left) and c-KitβTg (right) mice in response to insulin stimulation for 24 hours showed increased insulin-induced Akt phosphorylation in WT, but not in c-KitβTg, mice (n=4). Closed circles: control with 0 nmol/L insulin; open circles: 24-hour treatment with 2 nmol/L insulin. Data are expressed as mean ± SEM. *$p$ < 0.05; analyzed using unpaired Student’s t-test.
3.3.5 IR knockout protected against glucose intolerance in 60-week c-KitβTg mice

As islets from c-KitβTg mice showed impaired IR–IRS-1 signalling and decreased response to insulin stimulation, we crossed c-KitβTg mice with the MIP-βIRKO mouse line to generate a model with tamoxifen-inducible loss of beta cell IR in c-KitβTg mice (c-KitβTg;βIRKO) (19). At 40 weeks of age, western blot and immunofluorescence analyses of IR levels in islets from MIP-βIRKO mice showed an approximately 40% reduction (Figure 3.10). The body weight of 60-week c-KitβTg and c-KitβTg;βIRKO mice was significantly increased compared to control littermates (Figure 3.11a). Increased fasting blood glucose levels in 60-week c-KitβTg mice were not found in c-KitβTg;βIRKO mice (Figure 3.11b). IPGTT in 60-week c-KitβTg;βIRKO mice showed similar glucose tolerance compared to control littermates (Figure 3.11c). Fed plasma samples revealed that 60-week c-KitβTg mice had significantly elevated insulin levels compared to control and c-KitβTg;βIRKO mice, indicating high circulating insulin levels (Figure 3.11d). No differences were found in \textit{in vivo} GSIS assays between 60-week c-KitβTg;βIRKO mice and c-KitβTg mice (Figure 3.11e).

Islet morphology from c-KitβTg;βIRKO mice revealed that beta cell mass was decreased compared to 60-week c-KitβTg mice (Figure 3.12a). Alpha cell mass and islet density was unchanged between all three groups (Figure 3.12bc). Beta cell proliferation was modestly increased in islets from 60-week c-KitβTg mice compared to control mice but was not different when compared to c-KitβTg;βIRKO mice (Figure 3.12d). Furthermore, no differences were found in the levels of phosphorylated Akt\textsuperscript{S473} (Figure 3.12e) or syntaxin 1A\textsuperscript{+} beta cells from 60-week mice (Figure 3.12f). Nuclear PDX-1 expression was also unchanged between the three groups (Figure 3.13a). Nuclear FOXO1 in 60-week c-KitβTg mouse islets was increased compared to control mice (Figure 3.13b). FOXO1 nuclear localisation in c-KitβTg;βIRKO mice was not statistically different compared to control mice (Figure 3.13b). Interestingly, nuclear MAFA levels were increased in islets from 60-week c-KitβTg;βIRKO mice compared to both control and c-KitβTg mice (Figure 3.13c). Overall, these results demonstrated that reduced IR signalling in c-Kit-overexpressing beta cells decreased glucose levels and beta cell mass.
Figure 3.10: Confirmation of IR knockdown was observed after tamoxifen administration in c-KitβTg;βIRKO mice.

(a) Western blot and densitometry of IR expression in islets from control and MIP-βIRKO mice after oral gavage tamoxifen administration (n=3). (b) Representative immunofluorescence images of control and MIP-βIRKO pancreas sections for IR (green) and insulin* cells (red), co-stained with DAPI (blue). Scale bar: 25 μm. Closed circles: control; open circles: MIP-βIRKO. Data are expressed as mean ± SEM. *p < 0.05; analyzed using unpaired Student’s t-test.
Figure 3.11: 60-week c-KitβTg;βIRKO mice demonstrated improved glucose tolerance and insulin release compared to c-KitβTg mice.

(a) Fasting body weight and (b) blood glucose levels were increased in c-KitβTg mice compared to control littermates at 60 weeks of age (20 weeks post-tamoxifen), but only fasting body weight was increased in c-KitβTg;βIRKO mice (n=9 control, n=8 c-KitβTg, and n=7 c-KitβTg;βIRKO). (c) c-KitβTg mice showed impaired glucose tolerance compared to littermate controls (n=9 control, n=8 c-KitβTg, and n=7 c-KitβTg;βIRKO). (d) Fed plasma insulin was increased in c-KitβTg mice compared to control littermates. c-KitβTg;βIRKO mice showed no change compared to controls (n=5 control, n=4 c-KitβTg, and n=4 c-KitβTg;βIRKO). (e) In vivo GSIS (ng/mL) at baseline (0 min), 5 min, and 35 min after glucose challenge (n=5 control, n=3 c-KitβTg, and n=4 c-KitβTg;βIRKO). Closed circles: control group; open circles: c-KitβTg group; closed squares: c-KitβTg;βIRKO group. Data are expressed as mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001; analyzed using one-way ANOVA with Tukey’s post hoc test.
Figure 3.12: Beta cell mass was increased in 60-week c-KitβTg mice and reduced in c-KitβTg;βIRKO mice.

Morphometric analysis for (a) beta cell mass, (b) alpha cell mass, and (c) islet density (n=4 control, n=5 c-KitβTg, and n=4 c-KitβTg;βIRKO) in control, c-KitβTg, and c-KitβTg;βIRKO mice demonstrated increased beta cell mass in c-KitβTg mice compared to control and c-KitβTg;βIRKO. (d) Beta cell proliferation measured using Ki67 staining in beta cells of 60-week control, c-KitβTg, and c-KitβTg;βIRKO mice (n=6 control, n=6 c-KitβTg, and n=5 c-KitβTg;βIRKO). (e) Representative immunohistochemical images (left) and quantification (right) of p-AktS473 (red) staining in control, c-KitβTg, and c-KitβTg;βIRKO mice (n=4 control, n=3 c-KitβTg, and n=3 c-KitβTg;βIRKO). Hematoxylin, blue; scale bar: 50 μm. (f) Immunofluorescence images (right) and quantification (left) for SNARE protein Syntaxin 1A (green) in insulin+ (red) cells of 60-week control, c-KitβTg, and c-KitβTg;βIRKO mice were similar between the three groups (n= 4). DAPI, blue; scale bar: 50 μm. White asterisk, red blood cell autofluorescence. Closed circles: control; open circles: c-KitβTg; closed squares: c-KitβTg;βIRKO. Data are expressed as mean ± SEM. *p < 0.05; analyzed using one-way ANOVA with Tukey’s post hoc test.
Figure 3.13: Nuclear transcription factor expression in 60-week c-KitβTg;βIRKO mice demonstrated increased MAFA levels when compared to littermates.

Representative double immunofluorescence images (left) and quantification (right) for nuclear (a) PDX-1 (n=3 control, n=5 c-KitβTg, and n=3 c-KitβTg;βIRKO), (b) FOXO1 (n=5 control, n=5 c-KitβTg, and n=4 c-KitβTg;βIRKO), and (c) MAFA (n=4 control, n=4 c-KitβTg, and n=3 c-KitβTg;βIRKO) (green) in insulin+ (red) cells of 60-week mice. The findings indicate that 60-week c-KitβTg;βIRKO mice have lowered nuclear FOXO1 and increased nuclear MAFA in beta cells compared to c-KitβTg mice. DAPI, blue, scale bar: 50 μm. White asterisk, red blood cell autofluorescence. Closed circles: control; open circles: c-KitβTg; closed squares: c-KitβTg;βIRKO. Data are expressed as mean ± SEM. *p < 0.05 and analyzed using one-way ANOVA with Tukey’s post hoc test.
3.4 Discussion

The studies presented characterized the long-term effects of chronic c-Kit receptor expression in murine beta cells. Results from this section demonstrated that 60-week c-KitβTg mice lost their capacity to regulate glucose levels due to reduced insulin secretion. Although beta cell mass and proliferation were maintained in c-KitβTg mice, high levels of apoptosis and decreased insulin exocytosis were observed in islets from 60-week c-KitβTg mice. 60-week islets from c-KitβTg mice also showed increased IR signalling and phosphorylated IRS-1S612, potentially resulting in negative feedback on the PI3K–Akt pathway and increased shuttling of FOXO1 to the nucleus. Decreased response to insulin stimulation in islets of c-KitβTg mice manifested the development of impaired IR–insulin signalling due to c-Kit overexpression (Figure 3.14) (20). Reducing beta cell IR expression in c-KitβTg mice led to partially restored glucose tolerance and improved insulin secretion through up-regulation of MAFA. This study demonstrates that prolonged overexpression of the c-Kit receptor, although able to improve glucose tolerance and insulin release with short-term activity, can induce beta cell dysfunction and insulin secretory defects through chronic activation of the IR-insulin signalling pathway.
AGING
Glucose Levels
Beta Cell Mass and Proliferation
Nuclear FOXO1
Insulin Signalling
Insulin Release

Young c-KitβTg beta cells
Aging c-KitβTg beta cells
Figure 3.14: Schematic for working model summarizing the effects of c-Kit overexpression on insulin secretion in beta cells.

Initial c-Kit activation, as seen in 8-28 week old c-KitβTg mice, led to improved beta cell function and insulin secretion through enhanced Akt signalling, nuclear localisation of PDX-1, and increased expression of IR and IRS (4). When c-Kit overexpression was maintained for prolonged periods (60 weeks in our current study), c-KitβTg mice developed impaired glucose tolerance linked to phosphorylation of IRS-1<sup>S612</sup> from long-term c-Kit and IR signalling (dashed arrows) (20). Islets from c-KitβTg mice demonstrated reduced Akt signalling that was associated with nuclear FOXO1 (to signal apoptosis) and defects in insulin release due to reduced SNARE proteins. Impaired IR–IRS-1–Akt pathway in 60-week c-KitβTg mice was partially rescued by reducing the expression of beta cell IR in tandem with c-Kit overexpression, which restored insulin secretion and glucose tolerance. This figure was assembled using the Inkscape software (version 0.92). Y: phosphorylated tyrosine site; S: phosphorylated serine site.
Previous studies established that c-Kit overexpression in 8-week-old c-KitβTg mice resulted in improved insulin release under normal diet and short-term HFD challenge (4). The current study demonstrated that long-term beta cell c-Kit overexpression in mice resulted in glucose intolerance due to reduced insulin release, which is similar to the findings of long-term HFD challenge in c-KitβTg mice (7). Although beta cell proliferation has been reported to decrease in older mice (12), 60-week c-KitβTg mice maintained their high beta cell proliferation rate and beta cell mass seen in the 8-week model. c-KitβTg mice also demonstrated increased body weight at 60 weeks of age. This increased body weight may have contributed to the development of glucose intolerance and defects in insulin release despite enhanced beta cell mass and proliferation, which is a common phenotype observed C57BL/6 mice fed a high-fat diet (21). These studies have also demonstrated that 60-week c-KitβTg mice lacked the increased Akt phosphorylation seen in young c-KitβTg mice (4). Islet cell survival, which was decreased in 60-week c-KitβTg mice as indicated by increased levels of cleaved PARP, has also been shown to be regulated through Akt signalling (24). Previous studies have also shown that the percentage of apoptotic beta cells cultured in high glucose was increased when PI3K–Akt signalling was inhibited but was unchanged after ERK inhibition (25). Collectively these findings indicate that chronic c-Kit activation can cause intracellular signalling changes that can impact islet survival rate and proliferation.

The altered expression of SNARE proteins in islets of 60-week c-KitβTg mice may have contributed to decreased insulin release and glucose intolerance. The PI3K–Akt signalling pathway has been linked to regulation of SNARE levels in beta cells and has been shown to affect insulin secretion. Mice with a total PI3K deficiency (βDKO mice) had decreased mRNA and protein levels of exocytotic molecules, including Syntaxin 1A, SNAP25 and VAMP2, and were restored upon introduction of constitutive Akt signalling (26). Similar to 60-week c-KitβTg mice, βDKO mice also showed increased nuclear FOXO1 and ERK signalling (26). Akt phosphorylation regulated the cytoplasmic sequestering of FOXO1 and transcription of Pdx-1 in beta cells, which is important in order to avoid FOXO1-induced apoptosis (27,28). Reductions in SNARE protein levels have been noted in db/db mouse and fa/fa rat models and in individuals with type 2 diabetes (29-31). The high fed plasma insulin secretion and glucose intolerance in c-
KitβTg mice at 60 weeks may reflect this defect, where loss of SNARE proteins due to reduced levels of phosphorylated Akt led to FOXO1 nuclear localisation and dysfunctional insulin release.

Our previous study determined that c-KitβTg mice at 8–28 weeks of age had increased insulin release and enhanced IR–IRS1/2–AktS473 signalling (4). However, while 60-week c-KitβTg mice in the current study displayed increased IR protein levels in islets, these islets also had a significant increase in IRS-1S612 phosphorylation. Serine phosphorylation of IRS-1 has been shown to negatively feed back to the PI3K–Akt pathway, resulting in downregulation of its activity, promotion of IRS-1 protein degradation, and insulin resistance (32,33). Maintenance of the IR–IRS signalling axis has also been implicated in glucose-induced FOXO1 nuclear exclusion and insulin resistant ob/ob mouse islets demonstrated both increased nuclear FOXO1 expression along with reduced levels of SNARE proteins SNAP25 and VAMP2 (28,34). Inhibiting the Akt–mechanistic target of rapamycin kinase (mTOR) signalling axis prevents serine phosphorylation and prolongs tyrosine phosphorylation of IRS-1, which led to maintained IRS-1 signalling (33). Insulin resistance has recently been implicated in the enhanced presence of beta cell senescence markers, as seen in mice with induced hyperinsulinaemia through the IR antagonist S961 (35). The high fed plasma insulin levels found in 60-week c-KitβTg mice in this study may indicate the development of beta cell insulin resistance and may contribute to the dysfunction seen in c-KitβTg beta cells. Inhibiting the development of insulin resistance in islets by reducing IR signalling, which was the aim of the IR knockdown in ageing c-KitβTg;βIRKO mice, may serve as a potential mechanism to alleviate beta cell dysfunction.

The knockdown of IR in c-KitβTg mice at 40 weeks partially restored glucose tolerance. This reduction in beta cell insulin signalling can be beneficial since prolonged circulating insulin has been shown to affect glucose tolerance and insulin sensitivity in mice (36,37). The enhanced proliferation and reduced Syntaxin 1A protein expression found in 60-week c-KitβTg mice was not seen in the MIP-CreER-crossed mouse model, where the cohort of 60-week c-KitβTg mice demonstrated only mild glucose intolerance and similar levels of Ki67-positive and Syntaxin 1A-positive beta cell when compared with control
and c-KitβTg;βIRKO mice. This may be due to confounding effects attributed to both the
MIP-CreER mouse model and the tamoxifen administration used in the second part of
this study. The growth hormone minigene found within the MIP-CreER mouse model is
transcribed and has been shown to produce increased beta cell mass and proliferation
(38,39), while tamoxifen can blunt beta cell proliferation when administered to mice (40).
Another limitation to consider is the incomplete IR knockdown achieved in 60-week c-
KitβTg;βIRKO mice administered tamoxifen at 40 weeks (40% reduction). Previous
knockout mouse models from our laboratory that were crossed with the MIP-CreERT line
achieved a 40–70% protein reduction (19,41). One change that was consistently observed
was increased nuclear MAFA levels in c-KitβTg;βIRKO mice. Nuclear FOXO1 can
increase MAFA expression under metabolic stresses as seen in hyperglycaemia and high-
fat diet feeding (42,43), and MAFA up-regulation was required for insulin secretion (44-
46). These results may indicate that nuclear FOXO1 expression in c-KitβTg mice
develops initially as a response to metabolic stress but results in glucose intolerance due
to reduced insulin signalling; reduction of IR expression in ageing c-KitβTg;βIRKO mice
may delay the progression of glucose intolerance through MAFA up-regulation.

The present study demonstrated that long-term overexpression of c-Kit in beta cells does
not maintain the improved glucose tolerance seen in mice with short-term c-Kit activation
and resulted in altered beta cell function that reduced glucose-induced insulin exocytosis.
Examining the long-term activation of c-Kit in a mouse model has identified that
transient signalling through the receptor can improve beta cell function and the expansion
of the beta cell compartment, yet chronic activation of this pathway can result in the
development of beta cell dysfunction that led to compromised blood glucose control. The
use of RTK-based therapies to enhance beta cell function in transplanted murine, primate,
and human islets has been previously studied (47,48). However, the long-term effects of
chronic RTK signalling must be examined to determine their lasting efficacy and any
potential complications. Regulating the duration of c-Kit overexpression to prevent the
development of negative feedback in beta cells needs to be considered when examining
the potential use of RTK-based therapies for the treatment of diabetes in order to ensure
optimal beta cell function.
3.5 References


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Chapter 4

4 Postnatal knockout of beta cell insulin receptor impaired insulin secretion in mice exposed to high-fat diet stress

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3 Text and figures from this chapter have been adapted from the following manuscript:

** Results from ND mice in Figures 4.1, 4.2, 4.3, and 4.4 were analyzed and previously presented by L. Zhou (featured in the manuscript above)
4.1 Introduction

Optimal beta cell function requires activation of signalling pathways activated through different membrane receptor tyrosine kinases (RTKs). The RTK insulin receptor (IR) has high affinity for insulin and insulin binding leads to the activation of multiple signalling pathways in beta cells (1-3). The first report utilizing a beta cell-specific IR knockdown mouse model (βIRKO) demonstrated reduced first-phase insulin release, age-dependent loss of beta cell mass, and decreased GLUT2 expression, all of which contributed to the loss of glycemic control and development of diabetes (4). Subsequent studies have determined that phosphorylation of IR is associated with activation of the PI3K–Akt pathway, and that signalling through this axis is important for insulin synthesis and maintenance of functional beta cell mass through PDX-1 nuclear expression (1,5,6).

However, the role of autocrine insulin stimulation on insulin granule release from beta cells is unclear due to reports that have found both positive and negative regulatory roles for insulin signalling on insulin release (7-10). One pathway proposed to contribute to beta cell-specific insulin resistance is dysfunctional IR–IRS–Akt axis signalling (11).

The high-fat diet (HFD) mouse model exhibits peripheral insulin resistance and glucose intolerance, and promotes rapid weight gain and a steady increase in serum insulin levels (12). HFD-challenged mice demonstrated hyperinsulinemia after 8 weeks and a parallel decrease in beta cell PDX-1 and GLUT2 expression (13). HFD mice have also been shown to undergo a period of proliferative beta cell expansion in the weeks initially following the start of HFD as an adaptation to the abnormal glucose tolerance that developed (14,15). The βIRKO mouse line has been previously used to determine the role of beta cell IR signalling under HFD-induced metabolic stress (16). Unlike control HFD fed littermates that demonstrated a proliferative increase in beta cell mass, beta cell mass of βIRKO mice fed a HFD did not show compensatory proliferation and did not increase beta cell mass. This indicates that maintenance of IR signalling was required for beta cell response to HFD challenge.

Although the βIRKO mouse model, in which IR was deleted in beta cells from the onset of fetal islet development, has shown reduced beta cell mass and function postnatally
(4,17), the loss of IR in developed beta cells has not been examined. In order to
determine if IR signalling is required for beta cell maintenance and function postnatally,
this study investigated the effects of postnatal beta cell IR loss in mice fed a normal diet
(ND) and HFD diet (18-week duration).

4.2 Materials and methods

Generation and maintenance of MIP-βIRKO mice: MIP-CreER^{+/−};IR^{fl/+} mice were
generated as previously described (Section 2.1.3) and bred to acquire control (MIP-
CreER^{+};IR^{+/+} or MIP-CreER^{−};IR^{fl/fl}) and MIP-βIRKO (MIP-CreER^{+};IR^{fl/fl}) mice. Male
mice were used for all experiments. Tamoxifen was administered to all mice at 4 weeks
of age (4 mg/20 g bodyweight, i.p.) for 3 days. Control and MIP-βIRKO littermates were
divided into normal diet (ND) or high-fat diet (HFD; 20% protein, 60% fat, 20%
carbohydrates, 5.21 kcal/g; product # D12492; Research Diets Inc, New Brunswick, NJ)
groups at 6 weeks of age and maintained for 18 weeks (analysis at 24 weeks of age).

In vitro INS-1 cell experiments: INS-1 832/13 cells were serum-starved in RPMI 1640
with 1% BSA media overnight at 90% confluency. Cells were then treated with insulin
(100 nmol/L; Sigma-Aldrich) for 1 hour and harvested for immunofluorescence analysis
of SNARE proteins. Additional information can be found in Section 2.7.

Metabolic experiments: Control and MIP-βIRKO mice fed ND or HFD had their body
weights, fasting blood glucose levels, and fasting plasma insulin levels measured at 24
weeks of age. IPGTT, IPITT, and ex vivo GSIS were performed at this time-point using
the methods described in Sections 2.3 and 2.5. Islet insulin content was measured and
normalised to total islet protein (µg/mg).

Immunohistochemical staining: Pancreata from control and MIP-βIRKO mice and
harvested INS-1 cells were stained with select primary antibodies listed in Table 2.2
(from Chapter 2). Detection was performed using fluorophore- or peroxidase-linked
secondary antibodies. For chromagen staining, AEC substrate was used. Details are
presented in Section 2.8.
Western blot analyses: Islets from control and MIP-βIRKO mice were isolated and lysed as described in Section 2.4. Proteins were analyzed by western blot using primary antibodies (Table 2.2 from Chapter 2) for proteins of interest (IR, Cyclin D1, VAMP2, SNAP25, Syntaxin 1A, MUNC18-1) and normalised to a loading control (β-actin), or respective total protein for signalling proteins (p-AktS473, p-AktT308, p-P70S6K1T389, p-ERKT202/Y204) (Section 2.9).

Statistical analysis: All data presented are represented as mean ± SEM and were analyzed using unpaired Student’s t-tests. Statistical significance was obtained when p < 0.05.

4.3 Results

4.3.1 IR knockout demonstrated glucose intolerance when challenged with HFD

Islets isolated from MIP-βIRKO ND mice showed that the deletion efficiency of IR expression was approximately 50% compared to islets from control ND mice (Figure 4.1a). Knockout of IR was also confirmed using immunofluorescence staining of islets from control ND and MIP-βIRKO ND mice (Figure 4.1b). Under ND conditions, MIP-βIRKO mice examined at 24 weeks of age did not demonstrate a change in body weight or fasting blood glucose levels when compared to control littermates (Figure 4.2a). Following 18 weeks of HFD feeding (24 weeks of age), MIP-βIRKO mice still did not display a change in either body weight or fasting blood glucose when compared to control HFD mice (Figure 4.2b). However, both body weights and fasting glucose levels were increased in HFD mice groups compared to ND groups (Figure 4.2ab), indicating that HFD stress increased body weight and blood glucose levels in both control and MIP-βIRKO mice.

IPGTT results from MIP-βIRKO ND mice demonstrated similar glucose tolerance as control ND mice (Figure 4.2c). However, unlike MIP-βIRKO ND mice, MIP-βIRKO HFD mice displayed impaired glucose tolerance when compared to their control HFD counterparts (Figure 4.2d). To examine if increased glucose intolerance in MIP-βIRKO HFD mice was due to the development of peripheral insulin resistance, insulin tolerance tests were performed. IPITT results from both ND and HFD mice revealed that insulin
sensitivity was similar between control and MIP-βIRKO mice (Figure 4.3ab). Fasting plasma insulin was collected from both ND and HFD groups to examine basal insulin release. Although no change was detected between control and MIP-βIRKO ND mice, MIP-βIRKO HFD mice demonstrated a reduction in fasting plasma insulin levels when compared to control HFD mice (Figure 4.3c). Ex vivo GSIS results from ND mice revealed similar levels of insulin release under low and high glucose conditions, although variability was noted between groups (Figure 4.3d). Ex vivo GSIS performed on isolated HFD islets showed that MIP-βIRKO HFD mice had significantly reduced insulin release when exposed to low glucose, with no statistical significance in insulin release in response to high glucose (Figure 4.3e). The insulin content of islets from control and MIP-βIRKO HFD mice following GSIS was unchanged (Figure 4.3f). These results indicate that normal postnatal IR expression on beta cells is required for maintaining adult beta cell function in response to HFD-induced cell stress.
a

IR

β-actin

b

IR

IR/Ins/DAPI

Control

MIP-βRKO
Figure 4.1: Confirmation of postnatal IR knockout in MIP-βIRKO mice revealed a reduction of IR expression in beta cells.

(a) Representative western blot and quantification of IR expression in isolated islets from control and MIP-βIRKO normal diet (ND) mice demonstrated a 50% reduction in IR with knockout (n= 6). (b) Representative immunofluorescence images of IR (green) in insulin+ cells (red) showed reduced IR expression in islets from MIP-βIRKO ND mice. Nuclei were stained with DAPI (blue). Scale bar: 50 µm. Closed square, control ND; open square, MIP-βIRKO ND. Data are expressed as mean ± SEM. **p < 0.01 vs control; analyzed using unpaired Student’s t-test.
Figure 4.2: MIP-βIRKO mice on ND maintained a similar response to glucose challenge as diet-matched controls, but MIP-βIRKO mice developed glucose intolerance after HFD challenge.

(a) Body weight (n= 9 control ND and n=11 MIP-βIRKO ND) and fasted blood glucose (n= 8 control ND and n=10 MIP-βIRKO ND) measurements were similar for control and MIP-βIRKO mice at 24 weeks of age on ND. (b) Body weight and fasted blood glucose for 24-week control and MIP-βIRKO mice revealed no change after 18 weeks of high-fat diet (HFD) feeding (n= 11). (c) IPGTT (n=7 control ND and n=6 MIP-βIRKO ND) in control ND and MIP-βIRKO ND mice with corresponding area under curve (AUC) results demonstrated similar glucose tolerance levels. (d) IPGTT (n=7 control HFD and n=6 MIP-βIRKO HFD) for control HFD and MIP-βIRKO HFD mice with corresponding AUC showed increased glucose intolerance in MIP-βIRKO mice. Closed square, control ND; open square, MIP-βIRKO ND; Closed circle, control HFD; open circle, MIP-βIRKO HFD. Data are expressed as mean ± SEM. **p < 0.01 vs control; analyzed using unpaired Student’s t-test.
Figure 4.3: MIP-βIRKO HFD mice demonstrated decreased insulin release when compared to diet-matched controls.

(a) IPITT results in control ND and MIP-βIRKO ND mice with corresponding area under curve (AUC) results were unchanged between ND groups (n= 5). (b) Similar IPITT results for control HFD and MIP-βIRKO HFD mice were found when comparing the corresponding AUC (n=6 control HFD and n=8 MIP-βIRKO HFD). (c) Fasting (16-hour) plasma insulin levels from ND (n=4) and HFD (n=5) control and MIP-βIRKO mice revealed reduced insulin release in MIP-βIRKO HFD mice. (d) Ex vivo GSIS results from isolated islets of control ND and MIP-βIRKO ND mice were comparable when stimulated at low (2 mmol/L) and high (22 mmol/L) glucose (n= 3). (e) Ex vivo GSIS in islets from control HFD and MIP-βIRKO HFD mice demonstrated reduced insulin secretion at low glucose levels in MIP-βIRKO HFD mice. (f) Insulin content normalised to total islet protein in control HFD and MIP-βIRKO HFD mice (n= 4). Closed square, control ND; open square, MIP-βIRKO ND; Closed circle, control HFD; open circle, MIP-βIRKO HFD. Data are expressed as mean ± SEM. *p < 0.05 vs control; analyzed using unpaired Student’s t-test.
4.3.2 IR deficiency does not alter islet morphology or the expression of exocytotic proteins under normal diet

Histological examination of pancreata from control and MIP-βIRKO ND mice was performed in order to determine whether postnatal loss of IR affected islet morphology. Islet density, alpha cell and beta cell mass, and beta cell proliferation were unchanged between control and MIP-βIRKO ND mice (Figure 4.4a-d). Synaptosome-associated protein of 25 kDa (SNAP25) and Vesicle-associated membrane protein 2 (VAMP2) are two proteins involved in the exocytotic complex, referred to as the soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) complex. Both SNAP25 and VAMP2 were expressed at similar levels in islets when comparing control and MIP-βIRKO ND mice (Figure 4.4e). These findings implicate that the postnatal partial loss of beta cell IR does not influence beta cell morphology or exocytotic proteins in adult mice maintained under normal conditions.
Figure 4.4: Islet morphology and exocytotic protein expression were unaltered in MIP-βIRKO ND mice.

(a) Islet density, (b) alpha cell mass, and (c) beta cell mass measurements from control ND and MIP-βIRKO ND mice did not show any alterations (n= 4). (d) Quantification of the beta cell proliferation marker Ki67 in insulin+ cells was similar between control ND and MIP-βIRKO ND mice (n= 3). (e) Representative immunofluorescence images of VAMP2 and SNAP25 (green) in insulin+ cells (red) of islets from control ND and MIP-βIRKO ND mice demonstrated similar levels of expression. Nuclei were labeled with DAPI (blue). White asterisk, red blood cell autofluorescence. Scale bar: 50 μm. Closed square, control ND; open square, MIP-βIRKO ND. Data are expressed as mean ± SEM; analyzed using unpaired Student’s t-test.
4.3.3 HFD decreased exocytotic SNARE protein expression in IR knockout mice

Since lower levels of insulin secretion were detected in islets from MIP-βIRKO HFD mice, proteins that comprise the SNARE complex involved in vesicle exocytosis were analyzed using immunofluorescence analyses. VAMP2, SNAP25, and MUNC18-1 immunoreactivity was significantly decreased in islets of MIP-βIRKO HFD mice compared to control HFD mice, indicating dysfunction in insulin vesicle tethering and exocytosis (Figure 4.5). The expression of exocytotic SNARE proteins was also quantified by western blot analysis and islets from MIP-βIRKO HFD mice demonstrated a significant decrease in Syntaxin 1A and VAMP2 levels with modestly lowered SNAP25 expression ($p = 0.0667$) compared to control HFD mice (Figure 4.6).

To verify if beta cell IR activity modulated SNARE protein expression, the INS-1 cell line was treated with 100 nmol/L insulin for 1 hour and examined by immunofluorescence. INS-1 cells showed increased SNAP25 expression after insulin stimulation, but levels of VAMP2 and MUNC18-1 were not induced (Figure 4.7). These results implicate that IR signalling may be required to maintain SNARE protein expression in beta cells exposed to HFD stress.
Figure a shows comparative images of control versus MIP-βIRKO samples stained with various markers. Each image demonstrates differences in expression levels of VAMP2, SNAP25, and MUNC18-1 in control and MIP-βIRKO conditions.

Figure b presents a bar graph illustrating the percentage of VAMP2+ insulin+ cells in control and MIP-βIRKO groups. Figure c shows a similar graph for SNAP25+ insulin+ cells. Figure d depicts the percentage of MUNC18-1+ insulin+ cells.
Figure 4.5: SNARE exocytotic protein expression was reduced in the islets of MIP-βIRKO HFD mice.

(a) Representative double immunofluorescence images for SNARE exocytotic proteins (green) and insulin (red), and (b) quantification of exocytotic proteins VAMP2 (n=5 control HFD and n=4 MIP-βIRKO HFD), SNAP25 (n=4), and MUNC18-1 (n=5 control HFD and n=3 MIP-βIRKO HFD) demonstrated a consistent decrease in insulin+ cells from islets of MIP-βIRKO HFD mice compared to control HFD mice. DAPI, blue labeled nuclei. White asterisk mark red blood cell autofluorescence in vasculature. Scale bar: 50 µm. Closed circle: control HFD; open circle: MIP-βIRKO HFD. Data are expressed as mean ± SEM. *p < 0.05, **p < 0.01 vs control; analyzed using unpaired Student’s t-test.
Figure 4.6: SNARE exocytotic protein levels in islets from MIP-βIRKO HFD mice demonstrated reduced expression of Syntaxin 1A and VAMP2.

Representative western blot images and densitometry quantification of VAMP2, SNAP25, MUNC18-1, and Syntaxin 1A in isolated islets from control HFD and MIP-βIRKO HFD mice (n=4). VAMP2 and Syntaxin 1A expression were decreased in islets from MIP-βIRKO HFD mice compared to control HFD mice. Closed circle: control HFD; open circle: MIP-βIRKO HFD. Data are expressed as mean ± SEM. *p < 0.05, **p < 0.01 vs control; analyzed using unpaired Student’s t-test.
**Figure 4.7: Insulin treatment of INS-1 cells led to increased expression of SNAP25.**

Representative images of SNARE protein expression (green) with quantification of (a) VAMP2 (n=4 for 0 mins and n=3 for 60 mins), (b) SNAP25 (n=5), and (c) MUNC18-1 (n=3) in INS-1 cells before and after stimulation with 100 nmol/L insulin. SNAP25 expression was significantly increased after insulin treatment. Scale bar: 25 μm. DAPI, blue. Closed triangle: 0 mins; open triangle: 60 mins. Data are expressed as mean ± SEM. *p < 0.05 vs control; analyzed using unpaired Student’s t-test.
4.3.4 IR deficiency reduced Akt–P70S6K1 signalling after long-term HFD stress

To examine if Akt signalling was affected in islets of MIP-βIRKO HFD mice, pancreatic histological sections and isolated islets were examined. Akt$^{S473}$ phosphorylation was reduced in islets of MIP-βIRKO HFD mice as evident through immunohistochemistry staining (Figure 4.8a) and western blot analysis (Figure 4.8b). In contrast, levels of Akt$^{T308}$ phosphorylation was unchanged in MIP-βIRKO HFD mice compared to control HFD mice (Figure 4.8c). P70S6K1$^{T389}$ phosphorylation, which is downstream of the Akt–mTOR signalling axis, was also reduced in MIP-βIRKO HFD mice compared to control HFD mice (Figure 4.8d). Examination of the phosphorylated ERK showed unchanged signalling in islets of MIP-βIRKO HFD mice (Figure 4.8e). These results demonstrate that islets from MIP-βIRKO mice fed a HFD develop reduced Akt–P70S6K1 intracellular signalling.
Figure 4.8: Reduced levels of Akt and P70S6K1 phosphorylation in islets of MIP-βIRKO HFD mice.

(a) Representative immunohistochemistry image (left) and quantification (right) of Akt\textsuperscript{S473} phosphorylation shown in red (n= 4), with hematoxylin co-staining shown in blue, demonstrated reduced islet levels in MIP-βIRKO HFD mice. Scale bar: 50 μm. Representative western blot images and densitometry quantification of (b) Akt\textsuperscript{S473}, (c) Akt\textsuperscript{T308}, (d) P70S6K1\textsuperscript{T389}, and (e) ERK\textsuperscript{T202/Y204} phosphorylation in isolated islets of control and MIP-βIRKO HFD mice (n= 4). MIP-βIRKO HFD mice demonstrated decreased Akt\textsuperscript{S473} and P70S6K1\textsuperscript{T389} phosphorylation. Closed circle: control HFD; open circle: MIP-βIRKO HFD. Data are expressed as mean ± SEM. *p < 0.05 vs control; analyzed using unpaired Student’s t-test.
4.3.5 IR deficient mice demonstrated reduced GLUT2 expression with no change in beta cell mass, proliferation, and transcription factor expression upon HFD challenge

Immunofluorescence analyses was performed on pancreata collected from control HFD and MIP-βIRKO HFD mice to examine if postnatal loss of IR affected HFD-induced compensatory increase in islet mass. No morphological differences were found in islets of control HFD and MIP-βIRKO HFD mice (Figure 4.9a). Islet density and alpha cell and beta cell mass were similar between control and MIP-βIRKO HFD mice (Figure 4.9b-d). Beta cell replication was also found to be similar between control HFD and MIP-βIRKO HFD mice as determined by Ki67+/insulin+ double immunofluorescence staining (Figure 4.10a) and Cyclin D1 levels from western blot (Figure 4.10b). Beta cell expression of the glucose-sensing channel GLUT2 in MIP-βIRKO HFD mouse islets was significantly decreased when compared to control HFD mouse islets (Figure 4.11a). Beta cell expression of PDX-1 was modestly decreased in MIP-βIRKO HFD mouse islets compared to controls, although this difference was not statistically significant ($p = 0.0960$) (Figure 4.11b). Similar expression levels for NKX6.1 and MAFA were observed in islets from control HFD and MIP-βIRKO HFD mice (Figure 4.11cd). Collectively, these data indicate that postnatal IR signalling was required to maintain beta cell GLUT2 expression but was dispensable for pancreatic transcription factor expression.
Figure 4.9: Islet morphology was unchanged in islets of MIP-βIRKO mice maintained on HFD.

(a) Representative images of glucagon$^+$ (green) and insulin$^+$ (red) staining in islets from control HFD and MIP-βIRKO HFD mice. Nuclei were stained with DAPI (blue). Scale bar: 100 μm. (b) Islet density, (c) alpha cell mass, and (d) beta cell mass were similar in islets from control HFD and MIP-βIRKO HFD mice (n=5). Closed circle: control HFD; open circle: MIP-βIRKO HFD. Data are expressed as mean ± SEM and analyzed using unpaired Student’s t-test.
Figure 4.10: MIP-βIRKO HFD mice did not demonstrate a change in beta cell proliferation.

(a) Representative image (left) and quantification (right) of the proliferation marker Ki67 (green) in insulin+ cells (red). DAPI, blue. White arrows, proliferating beta cells; white asterisk, red blood cells (n=5). Scale bar: 50 μm. The frequency of proliferating beta cells was similar between islets from control HFD and MIP-βIRKO HFD mice. (b) Representative western blot (left) and densitometry analysis (right) showed similar expression of cell cycle protein Cyclin D1 in control HFD and MIP-βIRKO HFD mice (n=4). Closed circle: control HFD; open circle: MIP-βIRKO HFD. Data are expressed as mean ± SEM and analyzed using unpaired Student’s t-test.
Figure 4.11: Islet GLUT2 expression was decreased in MIP-βIRKO HFD mice.

Representative images (left) and quantification (right) of (a) glucose sensor GLUT2 and beta cell transcription factors (b) PDX-1, (c) NKX6.1, and (d) MAFA (n= 4). GLUT2 expression was decreased in islets from MIP-βIRKO HFD mice, but similar expression of beta cell transcription factors was found between control HFD and MIP-βIRKO HFD mice. GLUT2, PDX-1, NKX6.1: green; insulin: red; DAPI, blue. Scale bar: 50 μm. MAFA was stained using AEC (red) and counterstained with hematoxylin (blue). White asterisks mark red blood cells. Scale bar: 50 μm. Closed circle, control HFD; open circle, MIP-βIRKO HFD. Data are expressed as mean ± SEM. *p < 0.05 vs control; analyzed using unpaired Student’s t-test.
4.4 Discussion

This study used an inducible beta cell IR knockout mouse model to determine the role of IR signalling in adult beta cells. Under ND condition, MIP-βIRKO mice did not demonstrate metabolic or islet morphological changes. However, under long-term HFD stress, MIP-βIRKO mice demonstrated impaired glucose tolerance with reduced basal insulin release and exocytotic SNARE protein expression. Decreased expression of GLUT2 and phosphorylation of proteins in the Akt–mTOR–P70S6K1 pathway was also observed in islets from MIP-βIRKO HFD mice. The results from this study establish that beta cell IR signalling is required for maintaining adult beta cells function in response to HFD-induced metabolic challenge (Figure 4.12).
**βIRKO**
*Ref. (4,17)*
Reduced beta cell mass and function

**MIP-βIRKO**
*Ref. (18)*
Increased beta cell mass and vascularization

**MIP-βIRKO**
*Current study*
Normal beta cell mass and function

**βIRKO**
*Ref. (16)*
Loss of beta cell compensation, development of dysfunction

**MIP-βIRKO**
*Current study*
HFD beta cell compensation but loss of beta cell function
Figure 4.12: Summary of islet characterization using βIRKO and MIP-βIRKO mouse models.

A schematic summarizing previously reported studies on the βIRKO and MIP-βIRKO mouse models, as well as the findings from this chapter (4,17,18). The effects of beta cell IR loss on beta cell mass and function are dependent on the temporal time-window when IR deficit is created (black timeline, above). Solid green lines indicate the duration of beta cell IR knockout in mice under normal metabolic conditions. Broken red lines indicate beta cell IR knockout in mice subjected to HFD. In brief, βIRKO mice, which had beta cell IR knockout present when insulin⁺ cells first emerged during development, had reduced beta cell mass and defective insulin secretion in adult life that was exacerbated after HFD challenge (4,16,17). MIP-βIRKO mice with induced beta cell IR knockout at e13.5 showed increased beta cell mass and proliferation and islet vascularization due to compensatory signalling through the IGF-1R/IGF-2 signalling axis (18). MIP-βIRKO mice with beta cell IR knockout induced at 4 weeks postnatally demonstrated unchanged beta cell mass and proliferation under ND; however, MIP-βIRKO HFD mice developed insulin secretory defects due to impaired Akt signalling and reduced GLUT2 and SNARE protein expression in beta cells.
Postnatal IR knockout in beta cells did not influence glucose tolerance or islet architecture in ND mice, which is in contrast to the loss of first-phase insulin secretion and the reduction in beta cell mass and functional transcription factors previously reported in the βIRKO model (4,17). In the βIRKO mouse model, IR was deficient throughout embryonic development, postnatal remodelling, and in the adult beta cell compartment. Our laboratory’s previous study, which utilized the MIP-βIRKO model to examine the loss of beta cell IR during the second transition of murine pancreatic development, demonstrated that fetal MIP-βIRKO mice at embryonic day (e) 19.5 showed increased beta cell proliferation and vascularization linked to increased insulin-like growth factor-1 receptor (IGF-1R) and IGF-2 signalling in islets (18). These data, and the data collected in adult mice, suggest that the role of IR signalling in beta cells was dependent on the temporal window when IR knockout is initiated (Figure 4.12). It must also be taken into consideration that the incomplete knockout observed in this study’s inducible MIP-βIRKO model (approximately 50% islet reduction) was not sufficient to induce the development of glucose intolerance. It has been observed that using the MIP-CreERT mouse model to knock out GLI-similar 3 (Glis3) led to a 66% reduction in Glis3 mRNA in isolated islets, yet the Pdx1-CreERT model demonstrated a 90% reduction in Glis3 mRNA with the same treatment (19). Furthermore, the authors reported that while Pdx1-CreERT;Glis3<sup>0/0</sup> mice developed glucose intolerance, MIP-CreERT;Glis3<sup>0/0</sup> mice maintained normoglycemia, indicating that a high percentage of beta cell Glis3 knockout was required to produce a diabetic phenotype (19). This may reflect the results found in MIP-βIRKO ND mice and suggests that a partial loss of beta cell IR is able to maintain glucose homeostasis under the normal diet condition.

The current study also demonstrated that reduced IR signalling in adult mice affected postnatal beta cell function in MIP-βIRKO mice fed a long-term HFD. HFD feeding in C57BL/6 mice induced glucose intolerance, hyperinsulinemia, and the progressive loss of beta cell transcription factors expression (20,21). The maintenance of beta cell IR–insulin signalling is now identified as a critical factor in the prevention of beta cell insulin resistance. Sprague Dawley rats on a 60% HFD demonstrated increased islet expression of protein tyrosine phosphatase 1B (PTP1B), which decreased IR signalling, and
increased insulin secretion when PTP1B was silenced in isolated islets (22). These data parallel results in the MIP-βIRKO HFD mouse, where the reduction of IR signalling under HFD-induced stress produced insulin secretory defects. The results from these studies demonstrate that while the partial loss of postnatal beta cell IR signalling was not detrimental when mice were fed a normal diet, the maintenance of beta cell IR expression was required to prevent defects in beta cell insulin secretion when challenged with metabolic stresses.

MIP-βIRKO HFD mice displayed impaired fasting plasma insulin levels and ex vivo GSIS. The reduced insulin secretion in MIP-βIRKO HFD mice was linked to the decreased expression of the exocytotic proteins that compose the SNARE complex. The reduction of SNARE proteins was previously reported in both obese rodent models and in islets from patients with type 2 diabetes (23,24). Decreased SNARE protein expression in MIP-βIRKO HFD mice indicated that beta cell IR signalling may be required to recruit SNARE complex proteins, as observed in our in vitro study. Although the factors that regulate beta cell SNARE expression are not fully understood, mouse models have shown a link between reduced beta cell Akt phosphorylation and decreased SNARE protein expression (25,26), indicating that the Akt–P70S6K1 signalling axis may be responsible for the up-regulation of exocytotic proteins.

MIP-βIRKO HFD mice displayed decreased Akt–P70S6K1 signalling. Both βIRKO mice and IR knockout in the MIN6 cell line have previously demonstrated a loss of Akt phosphorylation (27-29), indicating that IR signalling is responsible for partially maintaining Akt–P70S6K1 signalling. Although phosphorylated AktT308 was unchanged between control and MIP-βIRKO HFD mice, phosphorylation of both AktT308 and AktS473 sites was shown to be required for maximum Akt signalling activity (30). Inhibition of phosphorylated Akt activity in beta cells has been shown to decrease first- and second-phase insulin secretion without affecting beta cell mass even after long-term (14 weeks) HFD feeding (31,32), which demonstrated that Akt signalling is important for maintaining insulin secretion but may be dispensable for regulating beta cell mass. Beta cell-specific mTOR knockout mice studies also demonstrated reduced insulin secretion, PDX-1 expression, and AktS473 phosphorylation with unchanged beta cell mass (32). It
should be noted that mice fed a long-term HFD developed chronic mTOR–P70S6K1 activation that resulted in reduced Akt phosphorylation, IRS-1 and IRS-2 expression, and GSIS (33,34), indicating that signalling through the IR–Akt–P70S6K1 pathway requires balanced regulation to maintain insulin release.

MIP-βIRKO ND and HFD mice did not show changes in beta cell mass or proliferation when compared to their control diet counterparts. One limitation that should be taken into consideration is the translation of the human growth hormone (hGH) transgene observed in MIP-Cre mouse models (35,36). Its production has been observed to affect beta cell proliferation. Interestingly, MIP-βIRKO HFD mice do demonstrate the decreased GLUT2 expression that has been previously reported in the βIRKO mouse model. Maintaining an appropriate level of beta cell GLUT2 has been reported to correct abnormal GSIS in mice with low beta cell GLUT2 expression (37,38), which, along with reduced SNARE expression, may account for the reduced insulin release in MIP-βIRKO HFD mice. The beta cell factors NKX6.1 and MAFA were unchanged in islets of MIP-βIRKO HFD mice when compared to control mice, indicating that beta cell identity was not altered with the partial loss of IR signalling. However, relatively lower PDX-1 levels were observed in islets of MIP-βIRKO HFD mice, linking the loss of IR with reduced PDX-1 and GLUT2 expression under HFD stress. Beta cell-specific PDX-1 knockout mouse models have demonstrated a loss of GLUT2 expression (39,40), which supports that PDX-1 expression is required to regulate GLUT2 in beta cells. Mice with the loss of both beta cell IR and IGF-1R also demonstrated a loss of PDX-1 and GLUT2 at 4 weeks of age (41). Taken together, these results suggest that IR signalling was required to maintain the glucose-sensing transporter GLUT2 through nuclear PDX-1 expression.

Overall, our findings propose that postnatal preservation of beta cell IR activity under HFD stress was required in order to maintain insulin secretion through activation of IR–Akt–P70S6K1 signalling and up-regulation of GLUT2 and SNARE proteins. These results determined a previously reported potential regulatory axis where reduced IR activation of Akt signalling contributes to decreased SNARE protein expression and defective insulin release (25,26). The loss of beta cell IR signalling under stress conditions that promote the development of insulin insensitivity (22) represent factors
that must be considered when examining beta cell dysfunction and developing potential therapeutics to restore IR signalling.
4.5 References


Chapter 5

5 c-Kit and IR co-stimulation does not enhance Akt signalling and pancreatic beta cell proliferation\(^4\)

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\(^4\) Text and figures from this chapter are currently in preparation for the following manuscript:

Oakie A, Li J, and Wang R. c-Kit and IR co-stimulation does not produce synergistic signalling or increase proliferation in the INS-1 cell line. Manuscript in preparation (2019).
5.1 Introduction

The activity of RTKs in the beta cell can regulate proliferation, survival, and insulin secretion. c-Kit and IR have been shown to activate overlapping signalling pathways to control beta cell function. Our group has previously demonstrated that SCF stimulation of INS-1 cells for 24 hours increased Akt signalling and up-regulated IR (1). IR activation in beta cell lines increased PI3K–Akt–P70S6K1 signalling and Insulin transcription, and the presence of IR on beta cells was required for glucose-stimulated Akt phosphorylation (2,3). The IR family member IGF-1R also increased proliferation of rat islet cells through the activation of the mTOR–P70S6K1 signalling pathway (4). c-Kit overexpression in beta cells of the 8-week c-KitβTg mouse model demonstrated an up-regulation of IR and IRS-1 and increased Akt phosphorylation when compared to wild-type mice (1). These findings indicate that combined c-Kit and IR signalling may be responsible for synergistic signalling through the PI3K–Akt pathway (5). However, the activation of the PI3K–Akt pathway has also been reported to induce negative regulation on IR–IRS signalling through inhibited phosphorylation of IRS-1 tyrosine sites and degradation of the IRS protein (6,7). The potential for negative feedback with increased PI3K–Akt activation must therefore be considered when examining co-activation of c-Kit and IR.

Co-activation of RTKs has been reported in various cell types and has demonstrated that receptor co-stimulation can be required to achieve synergistic intracellular signalling (8,9). FGFR2B has been shown to up-regulate c-Kit during salivary gland organogenesis, and cumulative Akt and MAPK signalling through the activation of both receptors was required for the initiation of transcriptional activity (10). In both MIN6 and INS-1 cell lines, the co-stimulation of growth hormone receptor (GHR) and IGF-1R demonstrated a synergistic increase in Akt phosphorylation 15 minutes after ligand stimulation and increased proliferation after 24 hours (11). Although these studies demonstrated synergistic signalling with receptor co-stimulation, it has not been established whether c-Kit and IR co-activation can achieve this effect through overlapping PI3K–Akt–P70S6K1 signalling. In this study, c-Kit and IR co-stimulation was examined in vitro to determine whether activation of both receptors produced a synergistic effect on beta cell PI3K–Akt–P70S6K1 signalling compared to single receptor activation.
5.2 Materials and methods

**INS-1 cell line culture and ligand treatments:** INS-1 832/13 cells were plated in 12-well plates and treated with ligands at 80-90% confluency. Before ligands were administered, cells were serum-starved in RPMI-1640 supplemented with 1% BSA media for either 16-24 hours (for short-term 15-minute or 1-hour stimulation time-points) or 3 hours (for long-term 24-hour stimulation time-points). Four experimental groups were examined in this study: [1] control with no ligand treatment; [2] stem cell factor alone (SCF, 50 ng/mL); [3] insulin alone (Ins, 0.2 nmol/L); and [4] SCF plus insulin (SCF+Ins) co-stimulation treatments. Three time-points were examined in this study: 15 minutes, 1 hour, and 24 hours. At the end of experiments, cells were processed for western blot analysis or were fixed for immunocytochemical labeling.

**Rapamycin treatment:** INS-1 cells were starved in RPMI-1640 with 1% BSA media overnight (16-24 hours) and then pre-incubated with or without 100 nmol/L rapamycin (LC Laboratories, Woburn, MA) for 1 hour in order to inhibit mTORC1 activity. Experimental groups were as follows: [1] no treatment (control), [2] 1-hour SCF and insulin co-stimulation (SCF+Ins), [3] 1-hour rapamycin pre-treatment, [4] 1-hour rapamycin pre-treatment with subsequent 1-hour SCF and insulin co-stimulation. Cells were harvested for western blot analysis following treatment.

**Immunofluorescence staining of cells:** INS-1 cells treated for 24 hours with ligands were harvested and fixed in 4% paraformaldehyde, embedded in 2% agarose gel, and processed to create paraffinized cell samples. Cell samples were sectioned and stained with antibodies against PCNA, C-peptide, and insulin (information can be found in Table 2.2). Representative images were captured using the Nikon Eclipse Ti2 confocal microscope (Nikon). Additional details can be found in Section 2.8.

**Western blot analysis:** INS-1 cells from the time-dependent study (15 mins, 1 and 24 hours) and from the rapamycin pre-treatment study were analyzed for phosphorylated (p-) c-KitY719, p-IRS-1Y606, p-IRY1146, p-AktS473, p-P70S6K1T389, p-ERK 1/2Y202/T204, p-IRS-1S612, and Cyclin D1 (further information can be found in Table 2.2). Bands were normalized to reference proteins α-tubulin or β-actin or to total protein for
phosphorylated signalling proteins Akt, P70S6K1, and ERK (detailed protocol can be found in section 2.9).

**Statistical analysis:** All data presented are represented as mean ± SEM and were analyzed using a one-way ANOVA test with a Tukey’s post hoc. Statistical significance was obtained when $p < 0.05$.

### 5.3 Results

#### 5.3.1 c-Kit and IR co-activation promoted phosphorylation of c-Kit$^{Y719}$, IRS-1$^{Y608}$, and IR$^{Y1146}$ sites

INS-1 cells stimulated for 15 minutes with SCF, insulin, or SCF+Ins were analyzed for phosphorylation of the c-Kit receptor at tyrosine site (Y) 719 and IRS-1/2 tyrosine site (Y) 608, both representing sites that associate with the p85 subunit of PI3K (7,12). SCF+Ins-treated cells demonstrated increased phosphorylation of c-Kit$^{Y719}$ compared to untreated controls (Figure 5.1a). However, no differences were noted between SCF+Ins and single ligand exposure. At 1 hour post-stimulation, SCF-treated cells demonstrated modestly elevated levels of c-Kit$^{Y719}$ phosphorylation but statistical significance was not reached due to high variation within treatment groups (Figure 5.1b). There was no significant difference in c-Kit phosphorylation between insulin- or SCF+Ins-treated cells and control cells. Phosphorylation of c-Kit$^{Y719}$ was also not enhanced by any ligand treatment after 24 hours of culture (Figure 5.1c). At 15 minutes following ligand treatments, IRS-1/2$^{Y608}$ phosphorylation was elevated in SCF+Ins-treated groups compared to untreated controls (Figure 5.2a). However, no change in p-IRS-1/2$^{Y608}$ levels for any of the treatment groups was detected at 1 hour or 24 hours following stimulation (Figure 5.2bc). Phosphorylation of IR at tyrosine (Y) 1146 was also analyzed at 24 hours of culture and demonstrated that that insulin-treated cells had increased phosphorylation of IR$^{Y1146}$ (Figure 5.2d). SCF+Ins-treated cells did not show increased IR phosphorylation (Figure 5.2d). These results show that SCF+Ins ligand treatment was able to induce c-Kit receptor and IRS-1 tyrosine phosphorylation shortly following stimulation, and that insulin only ligand stimulation induced long-term receptor activation in INS-1 cells.
Figure 5.1: c-Kit<sup>Y719</sup> phosphorylation was increased after short-term stimulation with SCF+Ins treatment.

(a) INS-1 cells co-stimulated for 15 minutes with SCF+Ins demonstrated increased p-c-Kit<sup>Y719</sup> phosphorylation compared to untreated control cells (n=4). c-Kit<sup>Y719</sup> phosphorylation was unchanged from control for all treatment groups at (b) 1 hour (n= 3) and (c) 24 hours (n= 4) following stimulation. Representative western blots and quantification by densitometry are shown. Untreated control (Ctrl): closed circles; SCF: open squares; Insulin (Ins): open triangles; SCF+Ins (S+I): open circles. Data were normalised to untreated controls and expressed as mean ± SEM. *p < 0.05 vs control; analyzed using a one-way ANOVA with a Tukey’s post hoc test.
Figure 5.2: IRS-1/2\textsuperscript{Y608} phosphorylation was increased after short-term treatment with SCF+Ins and IR\textsuperscript{Y1146} phosphorylation was increased only after long-term treatment with insulin.

(a) Representative western blots and quantification by densitometry for IRS-1 phosphorylation demonstrated an increase in IRS-1/2\textsuperscript{Y608} phosphorylation at 15 minutes in SCF+Ins-treated cells compared to untreated control cells (n=3). IRS-1/2\textsuperscript{Y608} phosphorylation was similar between treatment groups at (b) 1 hour (n=6) and (c) 24 hours (n=4) following stimulation. (d) Phosphorylation of IR\textsuperscript{Y1146} was increased in insulin-treated cells only at 24 hours compared to control cells (n=4). Untreated control (Ctrl): closed circles; SCF: open squares; Insulin (Ins): open triangles; SCF+Ins (S+I): open circles. Data were normalised to untreated controls and expressed as mean ± SEM. *p < 0.05, **p < 0.01 vs control; analyzed using a one-way ANOVA with a Tukey’s post hoc test.
5.3.2 SCF and insulin co-administration did not enhance Akt phosphorylation at 24 hours over single ligand treatments

To determine the effects of SCF and insulin co-stimulation downstream of receptor activation, intracellular signalling proteins Akt and P70S6K1, which are phosphorylated along the PI3K–Akt–mTOR–P70S6K1 pathway axis, were examined. At 1 hour following ligand stimulation, Akt phosphorylation at serine (S) 473 was not significantly different between untreated controls and any of the ligand-treated cell groups (Figure 5.3a). Interestingly, Akt$^{S473}$ phosphorylation levels were significantly increased in all ligand-treated groups when compared to control cells 24 hours following ligand stimulation (Figure 5.3b). Akt$^{S473}$ phosphorylation in the SCF+Ins-treated cells was similar to levels observed with SCF or insulin treatment alone, indicating that co-stimulation did not lead to a synergistic effect on intracellular Akt signalling. P70S6K1 phosphorylation of threonine (T) 389, which is downstream from mTOR activation, was increased in insulin-treated and SCF+Ins-treated cells at 15 minutes compared to untreated control cells (Figure 5.4a). However, P70S6K1$^{T389}$ phosphorylation levels were unchanged between control and treatment cell groups at 1 hour (Figure 5.4b). Although increased Akt phosphorylation was observed at 24 hours, p-P70S6K1$^{T389}$ levels were similar throughout all groups at this time-point (Figure 5.4c). MAPK–ERK signalling was examined in treated cells to determine if other intracellular signalling pathways were active. At 24 hours, ERK phosphorylation was similar between all treatment groups (Figure 5.4d). The findings indicate that phosphorylation of P70S6K1 was achieved in insulin-treated and SCF+Ins-treated cells shortly after ligand stimulation and Akt phosphorylation was achieved in all ligand stimulated groups after 24 hours compared to untreated controls. Importantly, SCF+Ins treatment did not enhance signalling compared to single ligand treatments.
Figure 5.3: Akt$^{S473}$ phosphorylation was increased 24 hours after SCF or insulin treatment.

(a) Akt$^{S473}$ phosphorylation in INS-1 cells after treatment with SCF, insulin, or SCF+Ins for 1 hour was not increased compared to untreated control cells (n=3). (b) Akt$^{S473}$ phosphorylation levels were increased for all treatments groups at 24 hours compared to untreated control cells. Co-stimulation with SCF and insulin did not increase phosphorylation compared to SCF or insulin alone (n=4). Representative western blot images are shown with quantification. Untreated control (Ctrl): closed circles; SCF: open squares; Insulin (Ins): open triangles; SCF+Ins (S+I): open circles. Data are normalised to untreated controls and expressed as mean ± SEM. *$p < 0.05$ vs control; analyzed using a one-way ANOVA with a Tukey’s post hoc test.
Figure 5.4: P70S6K1T389 phosphorylation was increased rapidly after insulin or SCF+Ins treatment, but phosphorylated P70S6K1T389 and ERK levels were unchanged at 24 hours.

(a) 15 minutes following stimulation, P70S6K1T389 phosphorylation was found to be elevated in insulin and SCF+Ins treatments compared to untreated control cells (n=4). P70S6K1T389 phosphorylation levels were unchanged between treatment groups and untreated control groups at (b) 1 hour and (c) 24 hours following stimulation (n=4). (d) ERK phosphorylation was unchanged between treatment groups and untreated control cells at 24 hours (n=4). Representative western blot images are shown with densitometry quantification. Control (Ctrl): closed circles; SCF: open squares; Insulin (Ins): open triangles; SCF+Ins (S+I): open circles. Data are normalised to untreated controls and expressed as mean ± SEM. *p < 0.05 vs control; analyzed using a one-way ANOVA with a Tukey’s post hoc test.
5.3.3 SCF and insulin co-administration increased inhibitory IRS-1<sup>S612</sup> phosphorylation at 1 hour post-treatment

The phosphorylation of serine residues on IRS-1 was examined to determine if SCF+Ins treatment induced serine phosphorylation at sites associated with intracellular negative feedback. At 15 minutes post-treatment, IRS-1<sup>S612</sup> phosphorylation was similar between all treatment groups and the untreated control cells (Figure 5.5a). However, the phosphorylation of IRS-1<sup>S612</sup> in SCF+Ins-treated cells was increased compared to untreated controls at 1 hour post-treatment (Figure 5.5b). Single ligand exposure failed to induce significant IRS-1<sup>S612</sup> phosphorylation. Increased IRS-1<sup>S612</sup> phosphorylation in SCF+Ins-treated cells was not observed after 24 hours of treatment, and IRS-1<sup>S612</sup> phosphorylation levels in all treatment groups were unchanged compared to untreated controls (Figure 5.5c). These results indicate that IRS-1<sup>S612</sup> phosphorylation occurred after short-term SCF+Ins co-treatment, suggesting the potential for negative feedback on the Akt−P70S6K1 signalling axis in co-stimulated cells.
Figure 5.5: IRS-1\textsuperscript{S612} phosphorylation was increased at 1 hour following SCF+Ins treatment.

(a) IRS-1\textsuperscript{S612} phosphorylation was unchanged in all treatment groups compared to untreated control cells 15 minutes following stimulation (n=4). (b) At 1 hour following stimulation, IRS-1\textsuperscript{S612} phosphorylation was increased in the SCF+Ins treatment compared to the untreated cells (n=6). (c) IRS-1\textsuperscript{S612} phosphorylation in all treatment groups was similar to untreated control cells 24 hours following treatment (n=4). Representative western blot images with densitometry are shown. Untreated control (Ctrl): closed circles; SCF: open squares; Insulin (Ins): open triangles; SCF+Ins (S+I): open circles. Data were normalised to untreated controls and expressed as mean ± SEM. *p < 0.05 vs control; analyzed using a one-way ANOVA with a Tukey’s post hoc test.
5.3.4 Rapamycin pre-treatment decreased IRS-1$^{S612}$ phosphorylation and increased Akt$^{S473}$ phosphorylation induced by SCF and insulin co-treatment

Since the potential inhibitory IRS-1$^{S612}$ phosphorylation residue was detected after SCF+Ins treatment, rapamycin, an inhibitor of mTORC1, was used to determine if phosphorylation of this IRS-1 serine site could be inhibited. Rapamycin pre-treatment did not significantly change IRS-1/2$^{Y608}$ phosphorylation at 1 hour in SCF+Ins-treated cells compared to untreated control cells (Figure 5.6a). Interestingly, SCF+Ins treatment showed an increase in IRS-1$^{S612}$ phosphorylation that was prevented with rapamycin pre-treatment (Figure 5.6b), implicating mTOR pathway activation in IRS-1$^{S612}$–induced negative feedback. Akt phosphorylation was examined to determine if rapamycin pre-treatment increased signalling through this axis. Pre-treatment with rapamycin was able to increase Akt$^{S473}$ phosphorylation in SCF+Ins-treated cells 1 hour following co-stimulation compared to untreated controls, although it did not reach statistical significance ($p = 0.0726$) (Figure 5.6c). ERK phosphorylation was also examined to determine if alternate pathways were affected by rapamycin pre-treatment. ERK phosphorylation was not changed with SCF+Ins treatment with or without rapamycin pre-treatment (Figure 5.6d). Findings from this study indicated that mTORC1 signalling increased IRS-1$^{S612}$ phosphorylation, and that inhibition of mTORC1 reduced IRS-1$^{S612}$ phosphorylation while potentially increasing Akt$^{S473}$ phosphorylation.
Figure 5.6: Rapamycin pre-treatment reduced IRS-1^{S612} phosphorylation by SCF+Ins treatment.

(a) At 1 hour, rapamycin pre-treatment had no effect on IRS-1/2^{Y608} phosphorylation (n=4). (b) IRS-1^{S612} phosphorylation induced by 1 hour SCF+Ins treatment was prevented with rapamycin pre-treatment (n=5). (c) Akt^{S473} phosphorylation induced by 1 hour SCF+Ins appeared to be increased with rapamycin pre-treatment (p = 0.0726 with one-way ANOVA) (n=3). (d) ERK phosphorylation was not changed with SCF+Ins treatment with or without rapamycin pre-treatment (n=4). Representative western blot images and densitometry are shown. Untreated control (Ctrl): closed circles; SCF+Ins (S+I): closed squares; Control with rapamycin pre-treatment (Ctrl+R): open circles; SCF+Ins with rapamycin pre-treatment (S+I+R): open squares. Data were normalised to untreated controls and expressed as mean ± SEM. *p < 0.05 vs control; analyzed using a one-way ANOVA with a Tukey’s post hoc test.
5.3.5 SCF and insulin co-administration did not stimulate INS-1 cell proliferation or C-peptide production

To examine if ligand stimulation was able to induce cell proliferation or insulin production, SCF, insulin, and SCF+Ins treatment groups were assessed for Cyclin D1 expression, proliferating cell nuclear antigen (PCNA), and C-peptide levels at 24 hours following ligand treatments. Cyclin D1 expression was unchanged between all treatment groups compared to untreated control cells (Figure 5.7a). Similar PCNA⁺ cell expression was observed in insulin⁺ cells from all treated groups when compared to untreated controls (Figure 5.7b). Finally, C-peptide levels were similar between all treatment groups and the untreated control groups after 24 hours (Figure 5.7c). Results from these experiments indicate that SCF+Ins co-treatment did not contribute to cell proliferation or C-peptide production after 24 hours of stimulation.
Figure 5.7: SCF+Ins treatment did not increase INS-1 cell proliferation or C-peptide levels at 24 hours following treatment.

(a) Cyclin D1 expression was similar at 24 hours for all treatment groups compared to untreated control cells (n=4). (b) Representative images of PCNA (green) in insulin+ (red) INS-1 cells showed similar PCNA+/insulin+ expression between cell treatments (n=3). (c) Representative immunofluorescence images of C-peptide (green) staining in insulin+ (red) cells of the INS-1 cell line demonstrated unchanged C-peptide levels between treatment groups and untreated control cells. Nuclei were stained with DAPI (blue). Scale bar: 25 μm. Untreated control (Ctrl): closed circles; SCF: open squares; Insulin (Ins): open triangles; SCF+Ins (S+I): open circles. Data were normalised to untreated control and expressed as mean ± SEM. Analyzed using a one-way ANOVA with a Tukey’s post hoc test.
5.4 Discussion

This study demonstrated that c-Kit$^{Y719}$ and IRS-1/2$^{Y608}$ phosphorylation was increased rapidly (15 minutes) following SCF+Ins treatment. This was accompanied by increased P70S6K1$^{T389}$ phosphorylation. After 1 hour of ligand stimulation, increased phosphorylation of IRS-1$^{S612}$ was observed in SCF+Ins-treated cells, suggesting that negative feedback on Akt–P70S6K1 signalling may have occurred. No synergistic increase in Akt$^{S473}$ phosphorylation was detected after SCF+Ins treatments at 24 hours when compared to single SCF or insulin treatments. INS-1 cell proliferation and C-peptide levels were unaltered after 24 hours of SCF+Ins treatment. This study demonstrated that c-Kit and IR co-stimulation did not produce sustained synergistic signalling, potentially due to short-term IRS-1$^{S612}$ phosphorylation and negative feedback on the Akt signalling pathway (Figure 5.8a-c). IRS-1$^{S612}$ phosphorylation was prevented through inhibition of mTORC1 activity with rapamycin pre-treatment, which led to increased Akt$^{S473}$ phosphorylation in SCF+Ins treatments compared to untreated controls (Figure 5.8d).
Figure 5.8: Proposed mechanism of SCF + Insulin co-stimulation in INS-1 cells.

(a) 15 minutes following SCF+Ins co-stimulation, c-Kit$^{Y719}$, IRS-1/2$^{Y608}$, and P70S6K1$^{T389}$ phosphorylation was rapidly increased in INS-1 cells. (b) At 1 hour, however, increased IRS-1$^{S612}$ phosphorylation from P70S6K1$^{T389}$ activation at 15 minutes led to reduced signalling through the Akt/P70S6K1 pathway. (c) 24 hours following co-stimulation, SCF+Ins-treated cells demonstrated Akt phosphorylation but no change in C-peptide production or cell proliferation. This may be due to the IRS-1$^{S612}$ phosphorylation that occurred following co-stimulation. (d) Rapamycin pre-treatment prevented phosphorylation of the negative feedback loop and IRS-1$^{S612}$ phosphorylation, leading to increased signalling via the Akt pathway. The red star denotes a hypothesized result of increased Akt phosphorylation with rapamycin phosphorylation where beta cell function would be increased. However, this needs to be further investigated and confirmed.
The INS-1 cell line was selected for this study due to its co-expression of the receptors c-Kit and IR (Appendix C). The dose of SCF required to stimulate beta cell proliferation and increased insulin production was validated previously in our laboratory using *in vitro* cell lines. The treatment of the PANC-1 cell line with 50 ng/mL SCF for one week induced cell proliferation (13). In addition, we have previously demonstrated that treatment of INS-1 cells with 50 ng/mL SCF for 24 hours increased Akt phosphorylation, indicating that this dosage was adequate for co-stimulation experiments (1). The concentration of insulin used for these experiments must be carefully considered due to potential detrimental effects observed using high insulin concentrations. A previous report examining both primary murine and human islets showed that culturing these cells in low insulin (0.2 nmol/L) and serum-free conditions resulted in decreased cleaved Caspase-3 levels and increased PDX-1 co-localization in insulin+ cells (14). However, long-term (7 days) exposure of islets to high insulin (200 nmol/L) increased cleaved Caspase-3 levels. Furthermore, INS-1E cells pre-treated with increasing insulin levels (from 10 nmol/L up to 1000 nmol/L) for prolonged periods (up to 48 hours) showed impaired AktS473 phosphorylation when re-stimulated with an additional 10 nmol/L insulin, suggesting that INS-1 cells can lose sensitivity to insulin stimulation if already exposed to a hyperinsulinemic environment (15). This may be reflected in the unchanged IR phosphorylation in SCF+Ins-treated cells, where prolonged stimulation of the receptors can reduce the impact of receptor co-activation and result in inhibitory signalling. It should be noted that there were variations within the INS-1 cell co-treatments when examining c-KitY719 and IRS-1Y608 phosphorylation. Additional cell lines, like the PANC-1 pancreatic epithelial cell line or the MIN6 mouse beta cell line, could be used to verify the mechanisms observed with c-Kit and IR stimulation provided that these cell lines also demonstrate c-Kit and IR expression.

Although SCF+Ins co-stimulation of INS-1 cells appeared to result in increased AktS473 phosphorylation at 24 hours, the levels were comparable to SCF or insulin treatments alone. Short-term analyses, selected at 15 minutes and 1 hour following ligand stimulation, were also investigated to determine whether co-stimulation rapidly altered intracellular signalling. Two interesting changes were noted after INS-1 co-stimulation: [1] increased c-Kit and IRS-1 tyrosine phosphorylation at 15 minutes were reduced 1
hour following ligand stimulation; and [2] IRS-1$^{S612}$ phosphorylation occurred in the SCF+Ins-treated cells at 1 hour post-stimulation. RTK transphosphorylation has been previously reported for receptor co-activation after ligand stimulation. FGFR3 and EphA4 receptors were able to phosphorylate tyrosine residues on the opposing receptor in HEK293 cells in vitro, while the RTKs EGFR and Axl have also demonstrated similar transphosphorylation after EGF stimulation in SNB-19 cells (16,17). Although no studies to date have examined c-Kit and IR co-stimulation in beta cells in vitro, there have been a number of studies that have analyzed the activity of platelet derived growth factor receptor (PDGFR) and insulin receptor co-activation. PDGFR is a member of the c-Kit-containing class III RTK family and shares both structural and functional homology with c-Kit (18). When stimulating NIH-3T3-IR fibroblast cells or C2C12 myoblast cells with PDGF and insulin, co-activation led to rapid dephosphorylation of PDGFR and reduced proliferation in these cells compared to PDGF treatment alone (19). It is possible that a similar mechanism of receptor dephosphorylation occurred with SCF and insulin co-stimulation in INS-1 cells since c-Kit phosphorylation, while increased at 15 minutes, was normalized at 1 hour following stimulation.

PDGFR studies have also examined the regulation of IRS-1 serine phosphorylation in insulin-sensitive cells. Studies conducted using 3T3-L1 adipocytes found that treating cells with both PDGF and insulin resulted in decreased IRS-1 tyrosine phosphorylation when compared to insulin alone (20). Increased serine phosphorylation of IRS-1 was also found in 3T3-L1 cells when stimulated with PDGF (21). Another key study exposed vascular smooth muscle cells to PDGF over a 48-hour period. In those cells, Akt and P70S6K1 phosphorylation were maintained when compared to untreated controls over a 48-hour period (22). In the current study, the early P70S6K1 phosphorylation seen at 15 minutes was not observed at 1 hour, which was likely due to the IRS-1$^{S612}$ phosphorylation seen in cells at 1 hour post-stimulation. The above study on vascular smooth muscle cells also found that IRS-1 serine phosphorylation was consistently increased in PDGF-treated cells throughout a 48-hour period (22). This is different than the findings in the current study, where IRS-1$^{S612}$ phosphorylation at 24 hours was unchanged between the treatment groups and untreated controls. This may explain why Akt phosphorylation was increased in ligand-stimulated groups at 24 hours, since IRS-
 phosphorylation was no longer present to inhibit this signalling pathway. However, this mechanism must be fully investigated to test this notion.

The PI3K–Akt–mTORC1–P70S6K1 signalling axis is an intracellular pathway that has been shown to contribute to beta cell function and insulin release. 8-week beta cell c-Kit overexpressing mice (c-KitβTg) demonstrated increased Akt$^{S473}$ phosphorylation and enhanced insulin secretion (1). However, as mentioned above, signalling through the Akt pathway has been shown to induce serine phosphorylation in adipocytes and vascular smooth muscle cells (21,22). Rapamycin administration, which inhibits mTORC1, was able to reduce IRS-1$^{S612}$ phosphorylation and partially restore signalling through Akt$^{S473}$ phosphorylation. This finding has been previously observed in other cell lines. IRS-1$^{S612}$ phosphorylation was present in 3T3-L1 cells after 1 hour of 100 nmol/L insulin stimulation, and the use of rapamycin pre-treatment in these cells inhibited serine phosphorylation at this residue (6). Rapamycin has also been shown to prevent the serine/threonine degradation of IRS-2 in the INS-1 cell line (23). The regulation of the Akt–mTORC1–P70S6K1 pathway is also of interest in maintaining insulin secretion and glucose homeostasis. The two complexes of mTOR, mTORC1 and mTORC2, were expressed at different levels in islets isolated from T2D patients. mTORC1 expression was elevated in T2D patients compared to non-diabetic controls, leading to enhanced P70S6K1$^{T389}$ phosphorylation (24). mTORC2 was also reduced in these islets from T2D patients, which resulted in reduced Akt$^{S473}$ phosphorylation, and insulin secretion was improved from islets of HFD-fed mice and T2D patients with in vitro inhibition of P70S6K1. Similar results were reported in INS-1 cells treated with siRNA against either mTORC1 or mTORC2 (Raptor and Rictor), where Raptor siRNA transfection increased GSIS (25). This suggested that short-term mTOR inhibition can improve insulin release from beta cells and that rapamycin treatment can prevent IRS-1 serine phosphorylation and increase insulin secretion from beta cells.

Overall, this study demonstrated that the activation of RTKs c-Kit or IR generated similar intracellular signalling, but co-stimulation of these pathways did not enhance or alter their signalling. Negative feedback during receptor co-stimulation resulted in IRS-1 serine phosphorylation. Although improved islet function observed after RTK stimulation
should still be considered for islet treatments, optimization of the temporal and dose-dependent stimulation range should be considered to avoid negative feedback signalling that limits improved beta cell function. By examining the effects of RTK co-activation on intracellular regulation of beta cells, RTK-based therapeutics can be designed to improve beta cell survival and function.
5.5 Reference


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Chapter 6

6 Summary, limitations, and future directions
6.1 Summary of Chapters

6.1.1 Review of overall rationale and main hypothesis

RTKs in the developing and mature beta cells are required to maintain cellular function under normal and diabetic conditions. Although c-KitβTg mice demonstrate an up-regulation of IR along with improved insulin secretion, it is unclear what role c-Kit and IR activation play in insulin release and if co-activation of the two receptors is required for beta cell function. The projects in this thesis work aimed to determine the role of these two RTKs on maintaining adult beta-cell function. We hypothesized that the c-Kit and IR signalling in beta cells under normal and metabolic stresses affected beta cell function and insulin release.

6.1.2 Prolonged c-Kit expression in beta cells impaired glucose tolerance due to reduced expression of exocytotic proteins and altered intracellular signalling activation

The examination of 8-week c-KitβTg mice had identified enhanced insulin secretion through increased beta cell mass and signalling through the PI3K–Akt pathway. The aim of Chapter 3 sought to determine the effects of prolonged beta cell c-Kit overexpression in c-KitβTg mice. Analysis of 60-week c-KitβTg mice showed an impairment in insulin release that resulted in glucose intolerance. Although c-KitβTg mice maintained increased beta cell mass and proliferation at 60 weeks, increased FOXO1 nuclear expression and cleaved PARP were present and indicated beta cell dysfunction and apoptosis. The exocytotic SNARE proteins Syntaxin 1A and MUNC18-1 were also reduced in 60-week c-KitβTg mice, potentially contributing to their loss of insulin secretion. The IR–IRS signalling axis was impaired in 60-week c-KitβTg mice and resulted in a loss of insulin-stimulated intracellular signalling. Generation of the c-KitβTg;βIRKO mouse model showed partially improved fasting glucose and glucose tolerance with challenge. 60-week c-KitβTg;βIRKO mice also demonstrated normalized beta cell mass similar in levels to control mice. In addition, c-KitβTg;βIRKO mice had increased nuclear MAFA expression in beta cells. The results from this chapter demonstrated that long-term beta cell c-Kit over-expression induced dysfunctional glucose regulation in mice through impaired IR–IRS signalling, increased beta cell
dysfunction and apoptosis, and reduced expression of proteins required for insulin vesicle exocytosis.

6.1.3 The loss of IR in postnatal beta cells led to reduced insulin release and PI3K–Akt–P70S6K1 intracellular signalling under high-fat diet conditions

Mice with a beta cell-specific loss of IR had been previously generated in our laboratory to examine the effects of IR signalling. This loss was induced during prenatal development when the immature beta cell first emerged. The aim of Chapter 4 was to examine the effects of postnatal beta cell IR loss in MIP-βIRKO mice after exposure to normal (ND) and high-fat diet (HFD). MIP-βIRKO ND mice maintained comparable fasting glucose levels, glucose tolerance, insulin release, and islet morphology similar to control ND mice. Although MIP-βIRKO HFD mice did not demonstrate a difference in body weight or fasting glucose levels compared to control HFD mice, MIP-βIRKO HFD mice displayed glucose intolerance when challenged with a glucose load. This was linked to reduced insulin release, decreased beta cell GLUT2 expression, and down-regulation of SNARE proteins. AktS473–P70S6K1T389 signalling was also compromised in MIP-βIRKO HFD mice. Although markers of insulin release were decreased in MIP-βIRKO HFD mice, beta cell mass and proliferation were unchanged between the two HFD groups. Taken together, these results indicated that normal postnatal beta cell IR expression was required in order to maintain GSIS when mice were exposed to high-fat diet stress.

6.1.4 Synergistic signalling was not achieved with c-Kit and IR co-stimulation

Although c-KitβTg mice demonstrated increased IR expression concurrent with improved insulin release, increased Akt signalling, and beta cell expansion, it is not clear if these receptors act in tandem to improve beta cell function. The aim of Chapter 5 was to explore if c-Kit and IR co-activation enhanced intracellular signalling that would lead to beta cell proliferation. INS-1 cells were used to model beta cells and were stimulated with both SCF and insulin. The results demonstrated increased c-Kit receptor activation with heightened IRS-1Y608 and P70S6K1T389 phosphorylation occurring rapidly after
stimulation (15 minutes). However, IRS-1$_{S612}$ phosphorylation was increased in SCF and insulin-treated cells at 1 hour, suggesting that there was negative feedback through the Akt–P70S6K1 signalling axis. At 24 hours, Akt$_{S473}$ phosphorylation was increased in all treatment groups but was not altered in the SCF+Ins co-treatment group. Rapamycin pre-treatment reduced SCF+Ins-treated IRS-1$_{S612}$ phosphorylation at 1 hour. INS-1 cell proliferation and C-peptide levels were unchanged at 24 hours between all treatment groups. The findings from these experiments indicated that c-Kit and IR co-stimulation did not alter signalling, possibly due to early negative feedback from IRS-1 serine phosphorylation. Inhibition of negative feedback through rapamycin may be a potential treatment to alleviate this axis.

6.2 Limitations

6.2.1 Chapter 3

The c-KitβTg mouse model is unique in that it is currently the only available model for c-Kit overexpression in beta cells. This mouse model was driven by the rat Insulin promoter (RIP), which transcribes Insulin 2. However, the use of RIP is associated with off-target gene transcription in other tissues that contain Insulin 2 transcription, notably in the hypothalamus (1,2). RIP-induced hypothalamic alterations have been associated with potentially inducing non-beta cell regulation of glucose homeostasis through influences on weight gain and feeding. However, studies that used a RIP-driven leptin receptor knockout found that food intake, weight gain, and body thermoregulation were not changed between control and RIP-driven mice (3,4). In our studies, there was no noticeable change in weight gain in c-KitβTg mice until 60 weeks of age. Even with these findings, other potential off-target effects from c-Kit expression in the hypothalamus should be considered.

The mouse Insulin promoter (MIP), which transcribes Insulin 1, was created to circumvent the off-target gene expression in non-beta cells seen with RIP mouse models (5). Early studies that examined the MIP-Cre and MIP-CreERT mouse models identified high recombination rates in their beta cells (80-90%) (5,6). However, one study that had utilized the MIP-CreERT line to induce excision of floxed GLI-similar 3 (GLIS3) found
that beta cell-specific MIP-CreERT recombination of Glis3 was lower in isolated islets (66%) when compared to the recombination achieved through another promoter that is mostly beta cell-specific, PDX-1-CreERT (90%), even though both lines received similar tamoxifen treatments (i.p. dose of 3 mg per day for 7 consecutive days in 8-week male mice) (7). This may explain the low Cre recombination observed in the c-KitβTg;βIRKO mouse model used in our study. There has also been research that has reported MIP-induced activity within the hypothalamus, which may result in the same confounding limitations presented with the RIP mouse model (8). This is still debated within the field but should be taken into consideration when interpreting results (6,9).

A decisive drawback with the RIP and MIP mouse models that has emerged within the last few years involves the apparent translation and autocrine feedback effect from the human growth hormone (hGH) insert within the transgene. hGH is located downstream from the promoter and Cre recombinase gene (10). The translated hGH has been found to bind to prolactin receptor on beta cells, which induced reduced GLUT2 expression on beta cells, decreased GSIS, and increased beta cell mass and proliferation (10,11). In addition, mice producing the hGH transgene demonstrated increased resistance to diabetes induction through high-fat diet and streptozotocin treatments (10,12). We pooled the data from the control group in the c-KitβTg;βIRKO study from both MIP+ and MIP− mice. When examining the IPGTT results using only MIP+ controls, a similar response was observed to the one presented in Chapter 3 (Figure 3.11). In light of this recent information, future experiments that use the c-KitβTg or c-KitβTg;βIRKO mouse lines should ensure that only Cre-positive controls be used to account for hGH-driven beta cell changes.

c-KitβTg mice demonstrated enhanced insulin release associated with receptor overexpression in beta cells. Although c-Kit overexpression in the beta cells of c-KitβTg mice was confirmed using western blot and immunofluorescence analysis, up-regulation of the receptor exclusively to the cell membrane of beta cells was not confirmed. It is possible that increased translation of c-Kit led to the accumulation of c-Kit in the ER, which promoted the unfolded protein response and ER stress in beta cells of c-KitβTg mice. Increased ER stress in diabetic rodent models has been linked to heightened beta
cell function and expansion as a method of beta cell compensation before the development of beta cell failure (13,14). Therefore, the effects of c-Kit overexpression in beta cells may not be solely due to receptor activation and can be due to off-target responses from the ER of beta cells.

Although enhancing the presence of RTKs during pancreatic development has shown initial improvements in maintaining normoglycemia, detrimental effects that may develop from chronic RTK over-expression must be examined. Pancreatic samples from patients with pancreatic adenocarcinoma demonstrated high levels of c-Kit+ islet cells, which was different from the islets of control pancreata (15). Careful consideration must be taken when utilizing RTK overexpression in islets to avoid the development of insulinomas.

6.2.2 Chapter 4

Similar to the limitations reported in the Chapter 3 section, the beta cell IR knockout in MIP-βIRKO mice is controlled through the MIP promoter-dependent expression of Cre recombinase. As mentioned above, MIP-driven knockout models have been reported to be less effective than the PDX-1-driven knockout mouse model (7). This can account for the partial (50%) reduction of beta cell IR observed in MIP-βIRKO mice. Since the loss of IR in beta cells is only partial, the remaining beta cell IR population may be sufficient to compensate for the reduced signalling in MIP-βIRKO ND mice. One alternate experiment that could be designed to strengthen the results from this section would be to cross the IR floxed mouse line with a tamoxifen-inducible PDX-1-Cre mouse line, confirm the percentage of IR knockout in the islets of these mice after tamoxifen-induced knockout at 4 weeks of age, and compare their insulin release and beta cell function to MIP-βIRKO mice. Examining a mouse model with near complete beta cell IR deletion would be beneficial to confirm that IR signalling does not play a major role in glucose metabolism in mice fed a normal diet.

The MIP-βIRKO mouse model is also subjected to potential confounding effects from hGH production. We had used both MIP-CreERT+ and MIP-CreERT− mice in our control groups for ND and HFD to increase our sample size. Although no change in proliferation was observed in our cohorts, it must be considered that hGH-driven proliferation may
mask any results seen between the two groups (10,11). Both control and MIP-βIRKO mice also received i.p. tamoxifen treatments to control for any effects from tamoxifen administration. However, tamoxifen administration has also been found to affect beta cell proliferation. C57BL/6 mice demonstrated reduced beta cell proliferation after tamoxifen administration (16,17). A separate report also noted improved glucose tolerance after tamoxifen administration in C57BL/6J mice (18). The potential estrogen receptor-promoting effects of tamoxifen may be similar to the 17β-estradiol protective effects observed in female mice on HFD, where both glucose tolerance and phosphorylated Akt levels in skeletal muscle were improved (19). The HFD feeding in both groups may therefore be subjected to hormone-mediated improvements in diet-induced obesity.

Many studies using the high-fat mouse model have reported ‘characteristics’ associated with high-fat diet-induced obesity, including peripheral insulin resistance, hyperinsulinemia, and beta cell dysfunction that leads to hyperglycemia (20,21). The degree of ‘diabetic’ progression in mice fed HFD has been shown to vary based on the mouse background, with C57BL/6 HFD mice demonstrating increased first-phases GSIS response compared to DBA/2 HFD mice (22). This is important to keep in mind since the floxed IR mouse line (B6.129S4(FVB)-Insr

Careful interpretation of the data from this chapter, like the recorded body weight measurements and fasting blood glucose levels, must be made when comparing these HFD-associated changes from both control and MIP-βIRKO HFD mice to other studies that use a different mouse background. In addition, there have been reports of individual mouse variability within the C57BL6/J line. After the same amount of time on HFD, a subset of mice were obese and hyperinsulinemic with reduced glucose clearance while lean body weight litter-mates, which demonstrated glucose levels similar to their obese counterparts, had normal insulin levels and glucose reuptake (23). This should be discussed when examining the variability in HFD studies since subsets of mice may demonstrate a more severe phenotype due to heightened weight gain and may account for the varying reports within the literature.
Although there are multiple classes of PI3Ks expressed in beta cells, the effects that each class has on insulin exocytosis is still not fully understood. INS-1 cells transfected with class II PI3K shRNA demonstrated a similar phenotype to mice with a loss of class I PI3K, where a defect in insulin secretion was noted without any change in insulin gene expression or content. However, these cells did not demonstrate a reduction in SNARE protein expression (24). These findings demonstrated that further research must be conducted to establish a connection between PI3K–Akt signalling and exocytotic protein expression in beta cells. Identifying the select PI3K classes affected by IR knockout in islets and examining the activity of all PI3K classes and SNARE protein expression in IR knockout islets following glucose stimulation would provide an additional connection between IR stimulation and exocytotic SNARE proteins.

6.2.3 Chapter 5

The INS-1 832/13 cell line provided a suitable *in vitro* model for examining c-Kit and IR cross-talk in beta cells since both are expressed within the cell line. However, this is a trait inherent in cancer-derived cell lines and is not reflective of normal mature beta cell physiology. As mentioned earlier on in this thesis, the subset of c-Kit+ beta cells composed a small percentage within the adult islet and was therefore not highly expressed in unmodified islet cells (25). In addition, it has been reported that INS-1 cells express both IR-A and IR-B isoforms (26). Isolated islet cells from human donors express both isoforms, but a higher percentage of beta cells expressed IR-B (64%) over IR-A (18%) (27). Different intracellular mechanisms have been reported for activation of each isoform in beta cells. The IR-A isoform is associated with insulin-induced beta cell proliferation, while the IR-B isoform has been shown to initiate signalling through class II PI3K-C2α and Akt-1 (serine 473) for glucokinase up-regulation and insulin secretion (28,29). Since it is not known which isoform is predominately expressed with SCF and insulin treatments, this work cannot make conclusions as to what isoform-specific pathway is being activated.

The INS-1 832/13 cell line is a rat insulinoma-derived line that is able to respond to glucose stimulation with insulin secretion, which is not present in all beta cell-derived
lines and provides a good model for studying beta cells *in vitro* (30,31). However, INS-1 cells are cancer-derived cells that may display signalling mechanisms inherent to neoplastic cells. Gastrointestinal stromal tumours expressed both c-Kit and FGFR3 (32). These cells were able to induce c-Kit phosphorylation when under receptor inhibition (imatinib treatment) due to FGF2-induced signalling and direct FGFR3–c-Kit interactions. Mammary tumour cells expressing IR and IGF-1R displayed a similar mechanism to maintain signalling; IGF-1R inhibition led to increased phosphorylation and downstream signalling through IR (33). Therefore, it may be that modifications to either or both c-Kit and IR receptors, through stimulation or inhibition, may induce pro-survival and pro-proliferative responses seen in other cancer cell experiments and not in primary beta cells. Although c-Kit expression in the adult mouse beta cell is low (10-20%), the isolation and stimulation of c-Kit+ primary beta cells from mouse islets with SCF and insulin co-treatment would be ideal to further confirm the results observed in INS-1 cells (25).

Rapamycin treatments of the INS-1 cell line should be taken into consideration due to the sensitive nature of mTOR inhibition in cells. As previously mentioned in Chapter 5, inhibition of the mTORC1–P70S6K1 signalling pathway has been shown to improve GSIS in both INS-1 cells and in islets from T2D patients (34,35). However, the use of mTOR inhibition through rapamycin treatment was also linked to the development of glucose intolerance. Although chronic Akt–mTOR–P70S6K1 signalling has been shown to lead to negative IRS-1 phosphorylation and the development of insulin resistance, the treatment of diabetic *Psammomys obesus* (desert gerbil) rodents with rapamycin worsened their hyperglycemia through reduced insulin release and beta cell mass through increased islet apoptosis (36). It has been found that maintaining transient mTOR signalling for a short period improved beta cell function while chronic mTOR activation reduced beta cell proliferation and led to high circulating glucose levels (37,38). *In vivo* rapamycin administration has also been reported to lead to both mTORC1 and mTORC2 inhibition, which differs from the mTORC1-selective inhibition observed in cell treatments, and led to the development of glucose intolerance and insulin resistance in mice (39). These findings indicate that rapamycin treatment may not be suitable for the *in
potentiation of beta cell RTK intracellular signalling and that decreasing the negative feedback effects from RTK over-activation requires another approach in order to avoid the development of diabetic complications.

6.3 Summarized conclusion

The results from the presented studies indicate that beta cell c-Kit and IR signalling pathways are required for maintaining intracellular signalling, beta cell function, and glucose regulation using *in vivo* and *in vitro* models. c-KitβTg mice progressively developed insulin secretory defects and impaired insulin-IR signalling that resulted in glucose intolerance by 60 weeks of age. Down-regulating beta IR in c-KitβTg mice partially restored glucose tolerance, which suggests that over-activation of the IR–IRS pathway due to c-Kit-induced increased insulin can result in long-term impaired beta cell function. Maintained signalling through the beta cell IR pathway regulated insulin secretion, intracellular signalling, and exocytotic proteins under prolonged high-fat diet-induced stress as seen in MIP-βIRKO mice. *In vitro* analyses of c-Kit and IR co-stimulation showed that the intracellular signalling interplay between the two receptors was not synergistic 24 hours after stimulation and may be regulated through negative feedback from PI3K–Akt–P70S6K1-induced IRS-1 serine phosphorylation. Overall, the results featured in this thesis have shown that beta cell RTKs are important for the regulation of insulin secretion, and this is an important consideration when designing potential RTK-based islet therapies.

6.4 Future directions

The progressive loss of c-Kit expression during normal pancreas development in rodent models and in humans has shown that c-Kit is perhaps not a major RTK in the adult islet but continues to be expressed in a small population of beta cells (25,40). The effects of islet c-Kit stimulation with the use of controlled, short-term SCF treatment on beta cell proliferation, survival, and insulin secretion should be examined to determine if regulated RTK activation in beta cells can improve islet function. Inducing islet proliferation and survival through activated RTKs has already been examined as a potential therapeutic for improving islet transplantation. Both hepatocyte growth factor and nerve growth factor
treatments have been shown to improve islet survival and were able to restore euglycemia in diabetic rodent models (41-44). Increasing VEGF-A production in islets has been shown to improve their vascularization, islet survival, beta cell volume, and glucose tolerance (45-48). Although these studies have produced promising effects on improving beta cell survival for transplantation, the activation of RTKs have also been linked to exacerbating glucose intolerance in diabetic patients (49). Since no study to date has reported c-Kit expression in islets from diabetic patients, examining islets from normal and diabetic patients for changes in c-Kit expression or signalling would be ideal to determine if increased activation of this receptor in diabetic patients is linked to poor glucose tolerance.

Many serine and threonine sites have been shown to be up-regulated after insulin treatment and through mTOR–P70S6K1 signalling (50,51). However, not all phosphorylated serine sites are linked to the development of intracellular negative feedback and insulin resistance. Although the phosphorylation of IRS-1S307 occurs with increased Akt–mTOR signalling, it has been found that this residue is required to prevent the development of glucose intolerance (52,53). Multiple reports have also found that the phosphorylation of different IRS serine and threonine sites are dependent on various pathways (mTOR, ERK1/2, c-Jun N-terminal kinase) and that this may differ based on the cell type analyzed (54-58). Screening islets from diabetic patients as well as from different experimental models of diabetes for phosphorylated serine/ threonine IRS residue would provide an appropriate reference for examining negative signalling on IRS proteins in the beta cell. Additional studies that perform residue-specific mutations would be useful to confirm their positive or negative regulatory roles on intracellular signalling.

The results from this study have demonstrated that there is a potential link between insulin-induced intracellular signalling and the maintenance of the proteins required for exocytosis. Alterations in glucose levels and intracellular signalling have been linked to SNARE regulation (59-62). Promoting beta cell differentiation has also been shown to up-regulate SNARE proteins (63). However, the pathways that are responsible for beta cell SNARE production, and the direct role that RTK activation plays in SNARE transcription, are not well understood. Further studies examining the relationship between
receptor signalling, intracellular pathway regulation, and transcription of exocytotic proteins must be conducted to determine how RTK-based therapies can prime insulin granules for exocytosis.
6.5 References


59. Do OH, Low JT, Gaisano HY, Thorn P. The secretory deficit in islets from db/db mice is mainly due to a loss of responding beta cells. Diabetologia. 2014;57(7):1400-1409.


Appendices

Appendix A: 2017 Animal Use Protocol (AUP)

AUP Number: 2017-007
PI Name: Wang, Rennian
AUP Title: Pancreatic Beta Cell Development: The Role Of The C-kit And Integrin Receptors

Approval Date: 01/16/2017
" has been APPROVED by the Animal Use Subcommittee of the University Council on Animal Care. This approval, although valid for four years, and is subject to annual Protocol Renewal.2017-007::1

1. This AUP number must be indicated when ordering animals for this project.
2. Animals for other projects may not be ordered under this AUP number.
3. Purchases of animals other than through this system must be cleared through the ACVS office. Health certificates will be required.

The holder of this Animal Use Protocol is responsible to ensure that all associated safety components (biosafety, radiation safety, general laboratory safety) comply with institutional safety standards and have received all necessary approvals. Please consult directly with your institutional safety officers.

Submitted by: Copeman, Laura
on behalf of the Animal Use Subcommittee
University Council on Animal Care

The University of Western Ontario
Animal Use Subcommittee / University Council on Animal Care
Health Sciences Centre, • London, Ontario • CANADA – N6A 5C1
Appendix B: Biosafety Approval Form

Dear Dr. Wang

Your biosafety approval number is **BSP-LHSC-0019**. This number is a requirement for your research grants, purchasing of biohazardous materials and Level 2 inspections. Please use this number on all correspondence with the Biosafety Officer (BSO).

This permit expires on **April 4, 2020**

Research Grants

Your study’s LBAPP number is required for any research grants involving biohazards. Please provide this number to Research Services when requested.

Purchasing Materials

Your LBAPP number must be included on purchase orders for all Risk Group 1 and Risk Group 2 pathogens and toxins. Please include your name as the Primary Investigator (PI) and your biosafety approval number on all purchase orders through HMMS or on all University of Western Ontario purchases.

Annual Inspections

Your Containment Level 2 laboratory will be inspected every year by the BSO and Lawson Safety Analyst.

This permit allows you to work with Risk Group 1 and Risk Group 2 biohazardous agents.

To maintain your Biosafety Permit, you will need to:

- Have a complete, up to date Biohazardous Agents inventory;
- Ensure that the employees, students and researchers working in your laboratory are trained in Biosafety;
- Ensure that your laboratory follows the requirements of the Lawson Biosafety Manual and mitigation strategies on your Biosafety / Biosecurity Risk Assessment, and
- Follow the guidance of the BSO and Lawson Safety Analyst on laboratory safety.

Please let me know if you have questions or comments.

Regards,

Charis Johnson-Antaran, MSc
Biosafety Officer
Lawson Health Research Institute
# BIOSAFETY PERMIT

## Permit Summary

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## Approved Microorganisms
- PaSC, Panc-1, HUVEC, HEK293, INS-1, MIN6, AR42J

## Approved Primary and Established Cell Lines
- Fetal pancreatic tissue, Fetal duodenum

## Approved Genetic Modifications (plasmids/vectors/rDNA)
- Mus musculus

## Approved Use of Animals
- Tamoxifen

## Approved Biological Toxins
- Approved Gene Therapy
- Approved Plants
- Approved Insects
Appendix C: Confirmation of c-Kit and IR expression in the INS-1 832/13 cell line

INS-1 832/13 cells were probed with rabbit anti-c-Kit (1:100, Santa Cruz Biotechnology) and rabbit anti-insulin receptor (1:50, Santa Cruz) antibodies (green) to confirm receptor expression within the cell line. Nuclei were counterstained with DAPI (blue). Scale bar, 10 µm. Staining was performed by R. Wang and J. Li.
Appendix D: License for Chapter 1

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Curriculum Vitae

Amanda Oakie

4.1 Education

**PhD Candidate:** Pathology and Laboratory Medicine, University of Western Ontario, 01/01/2015 – Present [In progress]

*Dissertation:* The role of c-Kit and insulin receptor tyrosine kinase in beta cell function and insulin secretion

**MSc:** Physiology and Pharmacology, University of Western Ontario, 09/01/2012 – 12/31/2014

*Dissertation:* Characterization of ALDH positive cells in the human fetal pancreas

**BSc:** Biomedical Sciences, University of Guelph, 09/01/2008 – 04/30/2012

4.2 Scholarships/ Awards

4.2.1 Scholarships

**Dr. Frederick Winnett Luney Graduate Scholarship,** Department of Pathology and Laboratory Medicine

Duration of Award: Awarded 04/13/2018

**Dr. P.C. Raju and Jyoti Shah Graduate Education Prize,** Department of Pathology and Laboratory Medicine

Duration of Award: Awarded 04/13/2018

**Lawson Internal Research Fund (Studentship),** Lawson Health Research Institute

Duration of Award: 01/01/2016 – 12/31/2016

**Western Graduate Research Scholarship,** Graduate and Postdoctoral Studies, University of Western Ontario

Duration of Award: 09/01/2012 – 08/31/2014 (MSc); 01/01/2015 – 12/31/2018 (PhD)

**EASD Travel Grant,** European Association for the Study of Diabetes

Duration of Award: awarded in 2015, 2017, 2018

**Schulich Graduate Scholarship,** Schulich School of Medicine and Dentistry, University of Western Ontario

Duration of Award: 01/01/2015 – 12/31/2018
4.2.2 Presentation Awards

**Second Place, Oral Presentation**, Diabetes Research Day, University of Western Ontario (Date of Award: 11/2015)

**Second Place, Poster Presentation**, Physiology and Pharmacology Research Day, University of Western Ontario (Date of Award: 11/2015)

**First Place, Poster Presentation**, Diabetes Research Day, University of Western Ontario (Date of Award: 11/2013)

4.3 Abstracts and Other Presentations

4.3.1 International Presentations

54th EASD (European Association for the Study of Diabetes) Annual Meeting Berlin, Germany, 10/02/2018 – 10/05/2018

**Title:** Postnatal loss of pancreatic beta cell insulin receptor affects insulin secretion observed under long-term high-fat diet

**Authors:** Oakie A, Zhou L, Rivers S, Cheung C, Li J, Wang R.

**Platform:** Poster Presentation

53rd EASD (European Association for the Study of Diabetes) Annual Meeting Lisbon, Portugal, 09/11/2017 – 09/15/2017

**Title:** Overexpression of c-Kit in aged mice stimulates beta cell proliferation, but leads to impaired beta cell function

**Authors:** Oakie A, Feng Z-C, Li J, Silverstein J, Wang R.

**Platform:** Oral Presentation

51st EASD (European Association for the Study of Diabetes) Annual Meeting Stockholm, Sweden 09/14/2015 – 09/18/2015

**Title:** Examination of sorted ALDHhi and ALDHhi/CD133+ cells in the developing human pancreas.

**Authors:** Oakie A, Li J, Grewal G, Fellows GF, Wang R.

**Platform:** Poster Presentation

4.3.2 Institutional Presentations

Collaboration of Practitioners and Researchers (CPR) Seminar Series, 11/29/2017

Guest speaker for seminar on Diabetes

London Health Research Day, 03/2014 – 04/2019

Pathology and Laboratory Medicine Research Day, 03/2015 – 03/2019


4.4 Publications


4.5 Graduate Teaching Assistantships

ANAT3309 Mammalian Histology, 09/01/2018 – 12/31/2018

MEDSCIENCE4900 Advanced IMS Laboratory, 09/01/2017 – 04/30/2018

PHYS3130 Physiology Laboratory, 09/01/2013 – 04/30/2014