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Role of C-Kit Receptor Tyrosine Kinase on INS-1 Cell VEGF-A Expression in Vitro

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Graduate Program in Physiology

A thesis submitted in partial fulfillment of the requirements for the degree in Master of Science

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ROLE OF C-KIT RECEPTOR TYROSINE KINASE ON INS-1 CELL VEGF-A EXPRESSION *IN VITRO*

(Thesis format: Monograph)

by

Alexei Popell

Graduate Program in Physiology and Pharmacology

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science

The School of Graduate and Postdoctoral Studies
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London, Ontario, Canada

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Abstract

Interactions between Stem Cell Factor (SCF) and c-Kit are critical in maintaining β-cell survival and function. VEGF-A, essential in facilitating islet vasculature formation, is secreted by β-cells. This work investigates the mechanisms by which c-Kit regulates VEGF-A production in β-cells, using a rat insulinoma (INS-1) cell line as an in vitro model. Cells were treated with recombinant hSCF or cultured with c-Kit (r) siRNA for 72 h. VEGF-A accumulation in the culture medium was measured by ELISA, while qRT-PCR, western blot, and immunofluorescence analyses were also performed. SCF augmented VEGF-A production in a time-dependent ($p < 0.05$ vs. control at 6 h and 24 h), and dose-dependent manner ($p < 0.01$ vs. control at 30-100 ng/mL). SCF stimulation increased phosphorylation of proteins along the PI3K/Akt/mTOR pathway ($p < 0.05$ vs. control groups), and decreased phosphorylation following siRNA treatment. Our results indicate that c-Kit exerts a regulatory effect on VEGF-A production in β-cells.

Keywords

SCF, c-Kit, INS-1, VEGF-A, β-cell, PI3K, Akt, mTOR
Co-Authorship Statement

All experiments were performed by Alexei Popell with the assistance of several lab members. Technical support, preparation of solutions, quantitative real-time RT-PCR analysis, and Real-Time PCR primer design, were provided by Jinming Li. Assistance with western blot analysis and VEGF ELISAs was provided by Zhi Chao Feng. This manuscript was reviewed and edited by Dr. Rennian Wang and Dr. Christopher Pin.
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<tbody>
<tr>
<td>μg</td>
<td>microgram</td>
</tr>
<tr>
<td>μL</td>
<td>microliter</td>
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<tr>
<td>μM</td>
<td>micromolar</td>
</tr>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>bp</td>
<td>base pairs</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary deoxyribonucleic acid</td>
</tr>
<tr>
<td>c-KitβTg</td>
<td>beta-cell specific overexpression of c-Kit</td>
</tr>
<tr>
<td>Ctrl</td>
<td>control</td>
</tr>
<tr>
<td>DAPI</td>
<td>4′-6′ diamidino-2-phenylindol</td>
</tr>
<tr>
<td>DM</td>
<td>diabetes mellitus</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>DSHB</td>
<td>developmental studies hybridoma bank</td>
</tr>
<tr>
<td>ECL</td>
<td>enhanced chemiluminescence</td>
</tr>
<tr>
<td>ERK</td>
<td>extracellular signal-regulated kinases</td>
</tr>
<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Glyceraldehyde 3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>GDM</td>
<td>gestational diabetes mellitus</td>
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<tr>
<td>GLUT</td>
<td>Glucose transporter</td>
</tr>
<tr>
<td>GNNK</td>
<td>Gly-Asn-Asn-Lys</td>
</tr>
<tr>
<td>Gsk3β</td>
<td>glycogen synthase kinase 3 β</td>
</tr>
<tr>
<td>h</td>
<td>hours</td>
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<tr>
<td>HEPES</td>
<td>4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid</td>
</tr>
<tr>
<td>Acronym</td>
<td>Definition</td>
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<tr>
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<tr>
<td>HIF-1</td>
<td>hypoxia inducible factor-1</td>
</tr>
<tr>
<td>HSCs</td>
<td>hematopoietic stem cells</td>
</tr>
<tr>
<td>INS-1</td>
<td>rat insulinoma cell line</td>
</tr>
<tr>
<td>JAK/STAT</td>
<td>janus kinase and signal transducers and activtors of transcription</td>
</tr>
<tr>
<td>kDa</td>
<td>kilodalton</td>
</tr>
<tr>
<td>MAP</td>
<td>mitogen-activated protein</td>
</tr>
<tr>
<td>mL</td>
<td>milliliter</td>
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<tr>
<td>mM</td>
<td>millimolar</td>
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<tr>
<td>mol</td>
<td>moles</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
</tr>
<tr>
<td>mSCF</td>
<td>membrane-bound SCF</td>
</tr>
<tr>
<td>mTOR</td>
<td>mammalian target of rapamycin</td>
</tr>
<tr>
<td>nM</td>
<td>nanomolar</td>
</tr>
<tr>
<td>NO</td>
<td>nitric oxide</td>
</tr>
<tr>
<td>NOD/SCID</td>
<td>non-obese diabetic/severe combined immune deficient</td>
</tr>
<tr>
<td>PANC-1</td>
<td>human pancreatic carcinoma epithelial-like cell lines</td>
</tr>
<tr>
<td>PARP</td>
<td>Poly ADP ribose polymerase</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate-buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PDGFR</td>
<td>Platelet-derived growth factor receptor</td>
</tr>
<tr>
<td>Pdx-1</td>
<td>pancreatic duodenal homeobox-1</td>
</tr>
<tr>
<td>PFA</td>
<td>paraformaldehyde</td>
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<tr>
<td>PI3K</td>
<td>phosphoinositide 3-kinase</td>
</tr>
<tr>
<td>PKA</td>
<td>protein kinase A</td>
</tr>
<tr>
<td>PKB</td>
<td>protein kinase B</td>
</tr>
<tr>
<td>PKC</td>
<td>protein kinase C</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>PLC-γ</td>
<td>phospholipase c-gamma</td>
</tr>
<tr>
<td>qRT-PCR</td>
<td>quantitative reverse transcriptase-polymerase chain reaction</td>
</tr>
<tr>
<td>RIP</td>
<td>rat insulin promoter</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>RT</td>
<td>reverse transcription</td>
</tr>
<tr>
<td>RTK</td>
<td>receptor tyrosine kinase</td>
</tr>
<tr>
<td>SD</td>
<td>standard deviation</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>sodium dodecyl sulfate-polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>sSCF</td>
<td>soluble SCF</td>
</tr>
<tr>
<td>Src</td>
<td>v-src avian sarcoma</td>
</tr>
<tr>
<td>TNF</td>
<td>tumor necrosis factor</td>
</tr>
<tr>
<td>T1DM</td>
<td>type 1 diabetes mellitus</td>
</tr>
<tr>
<td>T2DM</td>
<td>type 2 diabetes mellitus</td>
</tr>
<tr>
<td>VEGF</td>
<td>vascular endothelial growth factor</td>
</tr>
<tr>
<td>VEGFR</td>
<td>vascular endothelial growth factor receptor</td>
</tr>
<tr>
<td>vs.</td>
<td>versus</td>
</tr>
<tr>
<td>W&lt;sup&gt;v&lt;/sup&gt;</td>
<td>viable dominant spotting</td>
</tr>
</tbody>
</table>
Chapter 1

1 Introduction

1.1 Significance of the Study

Diabetes mellitus (DM) is a chronic condition characterized by loss of glucose homeostasis that results most commonly from autoimmune attack on β-cells in type 1 DM (~10% of DM cases), peripheral tissue insulin resistance in type 2 DM (~90% of DM cases), or insulin receptor dysfunction during pregnancy in gestational DM. Patients suffering from type 1 or 2 DM commonly develop co-morbidities including cardiovascular disease, peripheral neuropathy, and nephropathy. Current treatment of diabetes includes an array of medications that increase insulin secretion and sensitivity as well as injecting exogenous insulin. These methods of treatment, however, are limited in their capacity to properly manage glucose homeostasis when faced with the progression of insulin resistance characteristic of DM pathology. Advancements in medical science have led to a focus on islet transplantation as a promising approach in curing diabetics. Yet, this procedure is still riddled with issues, with a reported 10% of total patients remaining free of daily insulin supplementation after a period of five years (Ryan et al., 2005). One of the main obstacles surrounding islet transplantation is not only the limited supply of donor islets, but also the maintenance of islet health, including islet vasculature. Previous studies performed on cell types derived from a variety of tissue, including keloid fibroblasts, human mast cells, melanocytes, and small cell lung cancer cells, suggest that c-Kit receptor activation may be linked to regulation of hypoxia inducible factor-1α (HIF-1α), a transcription factor that is instrumental in the production of vascular endothelial growth factor-A (VEGF-A). VEGF-A is widely recognized as one of the most important proangiogenic factors in various murine models (Litz et al., 2006; Zhang et al., 2006; Monsel et al., 2010).

This work seeks to provide evidence supporting that activation of c-Kit by its ligand, stem cell factor (SCF), in vitro, stimulates HIF-1α-mediated production of VEGF-A via
the PI3K/Akt/mTOR pathway, previously identified to be activated both in vitro and in vivo by c-Kit ligand-binding (Li et al., 2006; Li et al., 2007; Feng et al., 2012b). This study aims to further explore a basis for c-Kit as a potential cell-based therapeutic target, particularly for pro-angiogenic signaling-aided reconstruction of a dense, fenestrated capillary network that islets require in maintaining function and survival. Previous studies in our laboratory have shown that SCF immunoreactivity in the developing human pancreas was found in the duct regions and scattered throughout the fetal pancreas, including endocrine cells (Li et al., 2006), and SCF mRNA and protein levels was detected in the isolated mouse islets (Feng et al., 2012).

1.2 The Pancreas

The pancreas is a “dual organ” consisting of two major compartments: exocrine and endocrine, composed primarily of exocrine acinar cells, endocrine cells, and ductal cells, as well as a high degree of vascularisation in the endocrine compartment (Moore and Dalley, 1999). Most studies on normal insulin secretion and pancreatic development have utilized rodent islets or clonal cell lines, which have been proven to develop and respond to insulin secretagogues similarly to human islets, thus providing an apt model for studies on endocrine cell function and structure (MacDonald et al., 2011). Conversely, clonal cell lines have their limitations, as they are susceptible to transformation and alteration of genotype over time of the culture and must be periodically assessed for function in order to rule out allelic imbalances and normal-cell contamination. In this case, recent studies in our laboratory have tested the INS-1 cells used for proper glucose-response (Riopel et al., 2013).

1.2.1 The Exocrine and Endocrine Pancreas

The exocrine components of the pancreas comprise most of the organ’s mass and work to produce and secrete a multitude of enzymes into the intestine, including proteases such as trypsin and chymotrypsin, pancreatic lipase, and amylase. This amalgamation of enzymes collectively works to aid in the digestion of macromolecules into more readily absorbable forms (Slack, 1995; Moore and Dalley, 1999). The endocrine component, constituting
only ~2% of pancreatic mass, but ~10% of pancreatic blood flow, is made up of functional units called the Islets of Langerhans (Jansson, 1994; Brunicardi et al., 1996). Islets are composed of five different cell types. This includes insulin-secreting β-cells, responsible for glucose uptake in body tissues, and glucagon-secreting α-cells that stimulate glycogen breakdown to provide peripheral tissue with energy (Slack, 1995; Moore and Dalley, 1999). Somatostatin-secreting δ-cells, pancreatic polypeptide-secreting PP cells, and ghrelin-secreting ε-cells are present in considerably fewer numbers (Andralojc et al., 2009). β-cells are of particular importance in current research due to their regulatory role with respect to carbohydrate metabolism, specifically in diabetes, a disease that affects multiple organ systems and stems from impairments of insulin secretion and defects in insulin action.

1.2.2 Pancreatic β-Cells

The most numerous islet cells, comprising 60-80% of the endocrine pancreas, are β-cells, responsible for secreting the hormone insulin, to reduce blood glucose levels upon elevated blood glucose concentration, measured by the passage of glucose through GLUT2 receptors (McCulloch et al., 2011). Once elevated blood glucose levels are detected by β-cells, insulin secretory response occurs as a biphasic process (Rorsman et al., 2000; Straub and Sharp, 2002). Transient stimulation of insulin secretion from granules in the readily releasable pool (<5% of total granule number), induced by elevation of blood glucose concentration, is followed by gradually developed secondary stimulation, thought to be an energy-dependent, active process requiring physically translocating or chemically modifying secretory granules (Rorsman et al., 2000). Insulin is then able to stimulate anabolic processes, such as glucose uptake by cells of the body and conversion of glucose to glycogen in the liver (Moore and Dalley, 1999). This effect is counterbalanced by α-cell-mediated glucagon release, stimulating glycogenolysis and gluconeogenesis.

β-cells produce a variety of other hormones and exert effects on the pancreatic microenvironment besides insulin detection and secretion. Under high concentrations of glucose or other environmental stressors, it has been found that β-cells begin to produce
proinflammatory cytokines, caused by accumulation of molecules such as tumor necrosis factor (TNF) and lymphotoxin α, which are important contributing factors to suppression of β-cell function and subsequent apoptosis in type 1 diabetics (Kagi et al., 1999; Eizirik et al., 2009). Besides cytokine-mediated pancreatic dysfunction, it is widely reported that β-cells, due to their dependency on islet vasculature, are influenced by a variety of proangiogenic factors, such as angiopoietin-2, and antiangiogenic factors, such as thrombospondin-1. These molecules are found in the microenvironment, and are able to provide positive and negative control for islet vasculature development, maintenance, and reconstruction. VEGF-A is secreted by β-cells, and is found to play a critical, non-redundant role in angiogenic switching, a process that is characterized by a change in the balance of proangiogenic and antiangiogenic factors that allows tumors to develop in size and metastatic potential, and pancreatic β-cell carcinogenesis (Folkman, 2002; Inoue et al., 2002). By modulation of these signaling molecules, mature β-cells are able to interact with intra-islet endothelial cells that create a supportive environment that is conducive to their survival and function. For example, recruited endothelial cells are able to synthesize the basement membrane laminin chains α4 and α5 to upregulate insulin gene expression and β-cell proliferation by interacting with laminin receptors containing β1-integrin (Nikolova et al., 2006). Additionally, β-cell-secreted angiogenic factors are required to maintain endothelial cell morphology which, in turn, allows for maintenance of their own structure and morphology (Figure 1.1) (Lammert et al., 2003).

1.3 The INS-1 Cell Line

Insulin-secreting cell lines (INS-1 and INS-2) were established using cells isolated from an x-ray-induced rat transplantable insulinoma. This cell line is unique, compared to those previously used, in that it retains features characteristic of physiological β-cells including morphology, insulin biosynthesis, and secretion. Unfortunately, similar to the phenotype observed in the parent tumor in vivo, these cells have a limited capacity for glucose-stimulated insulin secretion and are unable to synthesize and store normal levels of insulin. Compared to hamster beta cell lines, derived from hamster islet cells by SV40, and β-TC lines, derived from transgenic mice, INS-1 cells are able to retain glucose responsiveness and insulin content with increasing passage numbers (Asfari et al., 1992).
Figure 1.1 Schematic of inter-islet relationship between endothelial cell and β-cell.

Endothelial cells promote β-cell proliferation, survival and function by secreting hepatocyte growth factors (HGF), thrombospondin-1 (TSP-1), laminins, collagens. β-cells promote endothelial cell proliferation, vessel sprouting and angiogenic processes by secreting insulin, vascular endothelial growth factor-A (VEGF-A), and angiopoietin-1 (Ang-1) (Cao and Wang, 2014).
Several daughter cell lines were derived from the INS-1 and INS-2 cell lines, including INS-1 832, INS-1 832/2, and INS-1 832/13. The INS-1 832 and INS-1 832/2 cell lines exhibit higher insulin content than their parental cell lines. Due to low levels or insensitivity to cAMP, a molecule required in maintaining primary β-cells in a glucose competent state, these cell lines demonstrate relatively poor or unresponsive glucose-stimulated insulin secretion (Yang et al., 2004). INS-1 832/13 cells, used in this study, were found to be very sensitive to cAMP, as evidenced by a relatively enhanced level of insulin secretion compared to INS-1 832/2 cells following forskolin or isobutylmethylxanthine stimulation or decreased insulin secretion when treated with a protein kinase A (PKA) inhibitor (Yang et al., 2004). Furthermore, when the regulatory subunit of PKA was overexpressed in the 832/13 cells, insulin secretion and PKA activity were severely inhibited (Yang et al., 2004). The glucose effect on this cell line is potentiated by insulin secretory agents such as IBMX, GLP-1, free fatty acids and sulfonylurea tolbutamide, and inhibited by potassium channel openers, with responses similar to adult rodent β-cells (Hohmeier et al., 2000). Importantly, INS-1 832/13 cells express Glut2 receptors and uses glucokinase, as opposed to hexokinase, to phosphorylate intracellular glucose (Skelin et al., 2010). Finally, the INS-1 832/13 cell line is derived from an insulinoma, it has endogenous proangiogenic factor production to support tumor growth, though it lacks a microenvironment that would normally create a balance between positive and negative angiogenic signal regulation. For these reasons, despite some limitations, the following study made use of the INS-1 832/13 cell line, due to their physiological resemblance to normal adult β-cells, compared to other available cell lines. Thus, manipulation of this cell line allows for insight into the mechanism behind disease state resulting from impairment of β-cells.

1.4 Diabetes Mellitus (DM)

Diabetes mellitus, screened and diagnosed by standards based on the World Health Organization (WHO) and Canadian Diabetes Association (CDA) criteria, is defined as an illness of the endocrine pancreas characterized by hyperglycemia, insulin resistance, and relative insulin deficiency (CDA, 2008). It is estimated that in 2011, 347 million people around the world were diabetic and diabetes is on track to become the 7th leading cause of
death worldwide by 2030 (WHO, 2009; Danaei et al., 2011). In 2009, it was estimated by the CDA that over 2.3 million Canadians were living with diabetes, with 6 million others at elevated risk. It has been reported that, in 2009, the strain of diabetes on the Canadian healthcare system was $12.2 billion annually, projected to rise to $16.9 billion by 2020 (CDA, 2008; Gougeon et al., 2014).

Diabetes mellitus can be broken down into three subtypes: type 1 diabetes (insulin-dependent), type 2 diabetes (non-insulin dependent), and gestational diabetes (GDM) (WHO, 2009). In type 1 diabetes, which comprises ~10% of the total diabetic population, pancreatic β-cells are destroyed by an autoimmune attack, resulting in the inability to produce and secrete insulin. Onset of type 1 diabetes typically occurs in children and young adults and is treated by insulin injections to normalize blood glucose (American Diabetes Association, 2010; Florez, 2014). Type 2 diabetes, comprising ~90% of all diabetes, is believed to be caused by a combination of genetic factors, such as carrying TCF7L2 allele, and lifestyle factors, such as poor diet, lack of exercise, and obesity. Type 2 diabetes’ onset generally occurs toward middle age and can be characterized by insulin resistance in body tissues (primarily muscle, adipose, and liver) and often deficiency in insulin production and secretion, due to decreased β-cell mass (American Diabetes Association, 2010; Florez, 2014). Type 2 diabetes is a multifactorial illness, contributed to by the accumulation of many genetic and physiological factors exacerbated by oxidative stress caused by elevated glucose. This can result in an increase of mitochondrial reactive oxygen species (ROS) production, nonenzymatic glycation of proteins, and glucose autoxidation. Additionally, a variety of transcription factors and signaling pathways have been known to be activated by hyperglycemia, including NfκB, p38 MAPK and JNK/SAPK activation, all known to be activated by hyperglycemia, ROS, and accumulation of inflammatory cytokines (Evans et al., 2002). The simultaneous and additive exacerbation of these pathways is thought to play a part in the development of type 2 diabetes. GDM occurs during pregnancy, diagnosed in women that had not previously exhibited elevated blood glucose levels, and often resolves following delivery. Women are normally screened for high levels of glucose in order to provide treatment and management of GDM, typically with dietary adjustment, exogenous fast-acting insulin injection, or medications such as metformin and glyburide (McIntyre et al.,
Complications associated with diabetes include significantly increased risk of cardiovascular disease, diabetic retinopathy, diabetic neuropathy, and a slew of other comorbidities that result in a shortened lifespan with a lower quality of life (American Diabetes Association, 2010; Florez, 2014).

Currently, administration of exogenous insulin is the primary treatment for type 1 diabetes, and advanced cases of type 2 diabetes, but only mitigates the negative effects and, as such, is not considered a cure for the illness. Type 2 diabetics are typically managed with a variety of lifestyle and dietary changes, and pharmacological treatments including increasing insulin secretion and sensitivity, β-cell replication and survival (Turner et al., 1998; Knowler et al., 2002; Jos et al., 2005). An alternative to exogenous insulin has emerged recently, in the form of islet transplantation. The Edmonton protocol is a popular islet transplantation procedure that has allowed patients to remain insulin-independent after approximately a year. It involves isolation of islets from several cadaveric donor pancreata, and infusion into a patient’s hepatic portal vein, supplemented by a regimen of immunosuppressants in order to avoid autoimmune-mediated rejection (Shapiro et al., 2000). Unfortunately, a variety of issues exist with this procedure, since it moves islets into the hypoxic environment of the hepatic portal vein and there is a scarcity of donor tissue (Ryan et al., 2002).

As previously mentioned, pancreatic islets exhibit a high degree of vascularisation, which is important for their ability to quickly secrete insulin in response to changes in blood glucose and to effectively deliver insulin to peripheral tissues. It is known that the procedure of pancreatic islet isolation severs the connections between islet vasculature and systemic circulation. This renders transplanted islets avascular for several days following the procedure, with overall less islet vascularisation and lower oxygen tension, compared to native pancreatic islets, even once vascularization has completed (Brissova et al., 2014; Irving-Rodgers et al., 2014). This work examines the possibility of a cell-based therapeutic approach to combat significant islet cell death due to ischemia and compensate for impaired vasculogenesis following transplantation by targeting the receptor tyrosine kinase (RTK), c-Kit, which has already been shown to play an important role in β-cell survival, migration, and function in vivo. c-Kit signaling as a cell-
based therapy for enhancement of β-cell function has been previously demonstrated by transplantation of bone marrow-derived c-Kit-expressing cells that resulted in initiation of islet regeneration in streptozotocin-treated non-obese diabetic/severe combined immunodeficient (NOD/SCID) mice (Hess et al., 2003). Since one of the main issues with islet transplantation, as opposed to a regimen of exogenous insulin delivery, is the limitations of islet donors, it is crucial to ensure maximum islet survival and function in the long term by improving post-transplant vasculogenic function (Brissova, 2008). Thus, applying similar protocols following islet transplantation in order to aid in β-cell survival and revascularization is potentially a viable course of treatment.

1.5 c-Kit Receptor Tyrosine Kinase and Stem Cell Factor

1.5.1 c-Kit Receptor Tyrosine Kinase
c-Kit (CD-117), a class III receptor tyrosine kinase (RTK) structurally similar to the colony stimulating factor-1 receptor (CSF-1R) and the platelet-derived growth factor receptor (PDGFR), is encoded by the c-Kit proto-oncogene (Yarden et al., 1987). The c-kit gene has been mapped to the dominant white spotting (W) locus on mouse chromosome 5 and human chromosome 4q11-q21 with two or four reported isoforms in mice and humans, respectively, as a result of alternative splicing. These isoforms are characterized by the presence or absence the tetrapeptide sequence, Gly-Asn-Asn-Lys (GNNK), in the extracellular domain and, in humans, the presence or absence of a serine residue in the interkinase region of the cytoplasmic domain (Geissler et al., 1988). The c-Kit isoform lacking the tetrapeptide sequence has been shown to induce stronger but transient signals and have a higher transforming ability, compared to its GNNK-included counterpart (Caruana et al., 1999; Voytyuk et al., 2003).

c-Kit is a transmembrane protein containing an extracellular domain, a single transmembrane region, an inhibitory cytoplasmic juxtamembrane domain, and a split cytoplasmic kinase domain separated by a kinase insert segment (Figure 1.2) (Lennartsson and Ronnstrand, 2006). The extracellular region is composed of five immunoglobulin-like domains, the first three of which form a concave surface that serves
Figure 1.3 Schematic of c-Kit, a transmembrane receptor tyrosine kinase.

The extracellular region is composed of five immunoglobulin-like domains, the first three of which form a concave surface serving as a ligand-binding site for stem cell factor (SCF). The fourth and fifth domains play a role in receptor dimerization. The intracellular region is composed of a juxtamembrane domain with an ATP-binding region and a phosphotransferase region, containing 9 identified phosphorylation sites, split by a 77 amino acid kinase insert and a COOH tail (Ashman, 1999).
as a ligand binding site for Stem Cell Factor (SCF). The fourth and fifth domain, however, are thought to play a role in c-Kit dimerization (Lev et al., 1992; Blechman et al., 1995). Additionally, the fifth domain is thought to allow the c-Kit receptor to be cleaved by proteases to allow shedding from the membranes of hematopoietic, mast, and endothelial cells (Pandiella et al., 1992). The intracellular region of c-Kit is composed of a juxtamembrane domain with an ATP-binding region and a phosphotransferase region containing multiple autophosphorylation sites, split by a 77 amino acid kinase insert, and a COOH tail (Brannan et al., 1992; Linnekin, 1999).

1.5.2 Stem Cell Factor

SCF, c-Kit’s ligand, is a cytokine produced by keratinocytes, fibroblasts and endothelial cells as well as endocrine cells in either a soluble form or as a transmembrane protein after alternative splicing and proteolytic cleavage of 8 exons that, upon binding to c-Kit, is linked to growth and differentiation of melanocytes, hematopoietic, mast, germ, and pancreatic islet cells (Tajima et al., 1998; Li et al., 2006; Feng et al., 2012). It has been shown that several cancer cell lines are also able to produce low amounts of soluble SCF (Beck et al., 1995). SCF has been mapped to the Steel (Sl) locus on mouse chromosome 10 and human chromosome 12q22-24, expressed as four or six isoforms in mice and humans, respectively, following alternative splicing (Martin et al., 1990; Zsebo et al., 1990). Of these, two transcripts are mainly expressed, encoding membrane-bound SCF, that can be cleaved by the metalloproteinase MMP-9, to form a third soluble form, with an extracellular domain, transmembrane domain, and an intracellular region (Figure 1.3) (Anderson et al., 1990; Majumdar et al., 1994; Oriss et al., 2014). The first form of SCF is membrane-bound SCF248, a 45 kD glycoprotein, which can be cleaved by proteases at the sixth exon to produce soluble SCF165. The second isoform, SCF220, a 32 kD glycoprotein, lacks a sixth exon and thus remains bound to the membrane, however can be cleaved at a secondary cleavage site located, in mice, at the seventh exon to create a soluble form (Majumdar et al., 1994). Both the transmembrane and soluble forms of SCF have been found to be biologically active, but serve different functions in mediating the effects of c-Kit. For example, experiments performed with Sl/Sl^d mouse models that produce either only soluble SCF or only membrane-bound SCF, show that the
Two membrane-bound isoforms of SCF include SCF220 and SCF248, while SCF165 is soluble. SCF 248, a 45 kD glycoprotein can be cleaved at the sixth or seventh exon to form soluble SCF165. SCF220, a 32 kD glycoprotein, lacks a sixth exon, but can be cleaved at the seventh exon to create soluble SCF165. All forms of SCF are biologically active, however membrane-bound SCF is able to readily form dimers, and is responsible for persistent activation and a prolonged lifespan of c-Kit, whereas soluble SCF induces transient receptor activation and enhances c-Kit protein degradation (Ashman, 1999).
membrane-bound form was able to partially recover from anemia, bone marrow hypocellularity, and runting, while the soluble form restored myeloid progenitor cell numbers (Kapur et al., 1998). It has been shown that membrane-bound SCF is able to form dimers, which are more biologically active than monomers (Kurosawa et al., 1996). Soluble SCF, however, has been shown to exist primarily in monomer form, where it is less potent in activating c-Kit. Evidence has shown that membrane-bound SCF is responsible for persistent activation and a prolonged lifespan of c-Kit, whereas soluble SCF induces transient receptor activation and enhances c-Kit protein degradation (Philo et al., 1996; Lemmon et al., 1997).

1.5.3 c-Kit Receptor Tyrosine Kinase & Signaling Pathways

c-Kit signaling begins as SCF forms homodimers and binds to the three extracellular immunoglobulin domains located on the c-Kit receptor. This process is stimulated by binding of the SCF dimer to two receptor monomers (Lemmon et al., 1997). c-Kit dimerization results in subsequent autophosphorylation of tyrosine residues, located primarily outside the kinase domain (Blume-Jensen et al., 1995; Lemmon et al., 1997). These phosphorylated tyrosine residues are then able to mediate binding and activation of signaling molecules within the cytoplasm that have Src homology 2 (SH2) or phosphotyrosine binding (PTB) domains. In the c-Kit RTK, a total of nine potential phosphorylation residues have been identified (in rodents: Tyr566, 568, 701, 719, 728, 745, 821, 898, and 934) (Figure 1.2) that serve as high-affinity binding sites for signal transduction molecules (Ronnstrand, 2004). Phosphorylation of these sites can yield activation of several major intracellular signaling pathways. Phosphorylation at Y719 results in activation of the phosphatidylinositol-3 kinase (PI3K) pathway, which promotes cell survival, migration and proliferation (Young et al., 2006). Phosphorylation at Y701 and Y934 causes an association with the adapter protein Grb2, which links to the Ras/Erk pathway through association with SHP-2. This complex is then able to begin a signaling cascade, which results in the activation of mitogen-activated protein kinase (MAPK), a signaling molecule that can act on transcription factors to modulate gene expression, and is essential for cell survival and proliferation (Ronnstrand, 2004; Sun et al., 2007).
Phosphorylation at Y566, 568, and 934 results in activation of the v-src avian sarcoma (Src) pathway, which plays a role in adhesion, chemotaxis, survival, proliferation and protein trafficking (Price et al., 1997; Thommes et al., 1999; Wollberg et al., 2003). Furthermore, it has been shown that murine myeloid 32D cells and mouse models with mutated Y566 and Y568 sites resulted in not only inhibition of the Src pathway, but also reduced activation of the PI3K, Ras/Erk and Janus kinase/Signal Transducers and Activators of Transcription (JAK/STAT) pathways (Bondzi et al., 2000; Lennartsson et al., 2005). These effects were reversed following restoration of Y566 and Y568 to these mutant models, suggesting that Src, in part, converges with them (Hong et al., 2004; Ronnstrand, 2004). Phosphorylation at Y728 results in the recruitment of phospholipase C-γ (PLC-γ), which exists as two isoforms: PLC-γ1 and PLC-γ2. The former is ubiquitously expressed, while the latter is mainly expressed in the hematopoietic system. This molecule is responsible for controlling calcium release in cells, a process vital to a variety of cellular functions (Gommerman et al., 2000).

c-Kit activation is negatively regulated by several cellular mechanisms. Protein kinase C (PKC) are a family of serine/threonine kinases that serve as regulators for c-Kit and other RTKs, activated by increases in intracellular levels of diacylglycerol (DAG) (Blume-Jensen et al., 1994). In this case, DAG is accumulated by c-Kit-mediated PI3K activation. This, in turn, stimulates PKC to phosphorylate S741 and S746, serine residues located in the kinase region of c-Kit that serve to attenuate its downstream signaling capabilities (Blume-Jensen et al., 1995). Additionally, suppressors of cytokine signaling (SOCS) are another family of proteins that work to suppress RTKs such as c-Kit. SOCS-1 has been found to be induced by SCF stimulation of mast cells, binding to c-Kit by its SH2 domain (Sepulveda et al., 1999; Yasukawa et al., 2000). SOCS-1, specifically, has been shown to suppress c-Kit-stimulated mitogenesis, but not interfere with survival signals by occupying Grb2 adaptor proteins via their SH3 domains (Sepulveda et al., 1999; Rottapel et al., 2002).
1.5.4 Role of SCF/c-Kit in Islet Biology

Research, performed both in our lab and others, has demonstrated the significance of SCF/c-Kit interactions in the development and function of the islets of Langerhans, specifically in regard to β-cell proliferation, maturation and survival (Yashpal et al., 2004; Li et al., 2007; Rachdi et al., 2001; Oberg-Welsh et al., 1994; LeBras et al., 1998; Oberg-Welsh and Welsh, 1996). c-Kit mRNA is found at detectable levels by embryonic day 13 in the rat pancreatic epithelial cells (Oberg-Welsh et al., 1994; LeBras et al., 1998), as well as in fetal and adult rat islets, but not in acinar cells (Rachdi et al., 2001; Oberg-Welsh et al., 1994; LeBras et al., 1998; Oberg-Welsh and Welsh, 1996). Previous studies performed in our lab have demonstrated that c-Kit-expressing human and rat fetal pancreatic ductal epithelial cells exhibit high proliferation and SCF expression (Yashpal et al., 2004; Li et al., 2006; Li et al., 2007). Additionally, it was found that ductal cells of the ligated rat pancreas and islets of streptozotocin-induced diabetic rats had c-Kit expression, suggesting that c-Kit may play a role in β-cell regeneration (Peters et al., 2005; Tiemann et al., 2007). In vitro experiments with cultured fetal rat islets treated with SCF showed a significant increase in insulin and DNA content (Oberg-Welsh and Welsh, 1996). INS-1 cells treated with SCF were found to have an increased proliferative capacity (Rachdi et al., 2001). In contrast, siRNA-mediated c-Kit knockdown resulted in reduced islet cell differentiation, associated with a decrease in PDX-1 and insulin mRNA and protein levels, decreased cell proliferation, and increased cell death (Li et al., 2007). Taken together, these studies suggest that there is a profound correlation between c-Kit function and β-cell development and function.

Experiments at the W locus of laboratory mice have demonstrated pleiotropic effects upon embryonic development and adult hematopoiesis, with most homozygotic mutations resulting in extensive white-spotting, sterility, and severe anemia resulting in perinatal mortality (Geissler et al., 1999). To obtain definitive information on c-Kit function in the β-cell in vivo, our lab used mice with a semi-dominant c-Kit mutation (c-KitWv/+), which expresses a non-functional c-Kit receptor with a less severe phenotype than c-KitWv/Wv mice. It has been shown that these mice developed a significant increase in blood glucose levels by 8 and 16 weeks, lowered insulin levels following glucose stimulation, and islets
with impaired insulin secretion in response to high glucose. These findings were further supported by decreases in β-cell mass and insulin content (Krishnamurthy et al., 2007). These effects were thought to be largely mediated by PI3K and its downstream signaling molecules, which was confirmed when c-KitWv/+ mice had downregulation of the Fas pathway by an lpr mutation, or inhibition of Gsk3β by 1-azakenpaullone injection, which resulted in the reversal of the adverse effects in c-KitWv/+ mice (Feng et al., 2012A; Feng et al., 2013). Since this is a global mutation affecting every cell in the c-KitWv/+ mice and does not reveal whether c-Kit has a primary or secondary role in β-cell function, our lab generated a novel transgenic mouse with β-cell specific c-Kit overexpression (c-KitβTg). This overexpression not only leads to improved β-cell function, with an increase in Pdx1 and MafA transcription factor expression in normal and high fat diet-induced diabetic mice, but also prevents the early onset of diabetes observed in c-KitWv/+ mice after cross-breeding (Feng et al., 2012B). Interestingly, we noticed changes in β-cell HIF-1α and VEGF-A expression as well as an alteration of islet capillary mass in c-KitWv/+ mutant and c-KitβTg mice. Taken together, these in vitro and in vivo studies suggest that the interactions between SCF and c-Kit may play a role in normal vascularization of the islet through an induction of VEGF-A expression in the β-cell.

1.6 Islet Microvasculature and Vascular Endothelial Growth Factor

1.6.1 Islet Microvasculature

As mentioned previously, the pancreas is a dual organ, consisting of both endocrine and exocrine tissues. However, there is an asymmetry in the distribution of blood vessels amongst its two integrated components. While the endocrine tissue comprises ~2% of the volume of a mature pancreas, the islets of Langerhans scattered throughout the exocrine pancreas receive ~10% of the organ’s total bloodflow (Jansson and Hellerstrom, 1983). Blood is supplied to the pancreas by the celiac and superior mesenteric arteries, and drains into the portal vein (Bonner-Weir and Orci, 1982; Murakami et al., 1992). Developmental studies based on embryonic growth have determined that there is separate
vascularisation, ductal drainage, and varied composition of islets. This demonstrates that the head of the pancreas, composed of pancreatic polypeptide-rich and glucagon-poor islets, is supplied by the superior mesenteric artery, while the glucagon-rich pancreatic polypeptide-poor body and tail are supplied by the celiac via the splenic artery (Ohtani, 1983; Ohtani et al., 1986). Interlobular arteries and veins run parallel to major ducts in the several lobuli that compose the pancreas, and all pancreatic veins empty their hormone-infused blood into the portal vein (Bockman, 1992). Lymph vessels in the pancreas are generally not prominent under normal conditions, but a capacity for drainage of fluids becomes evident during pancreatitis (O’Morchoe, 1997).

Studies in pancreatic vascular flow utilizing ink- or dye-infused whole mounts or scanning electron microscopy of corrosion casts have determined that islets nest in a dense glomerular-like capillary network of arterioles such that no endocrine cell is more than a single cell away from arterial blood supply (Bonner-Weir, 1988). Endocrine capillaries are highly permeable, comparable to those of the small intestine, due to possessing relatively high levels of fenestration, reportedly 10 times that of their exocrine counterparts. Upon morphological analysis, it has been observed that endocrine capillaries also tend to have a greater diameter than those found in the exocrine pancreas (Henderson and Moss, 1985; Hart and Pino, 1986). The great density, size, and surface area of the endocrine capillary network highlights the importance of highly sensitive, thorough vascularisation that is essential to proper islet function. In the pancreas, it is thought that vasulature is regulated primarily by vascular endothelial growth factor-A (VEGF-A) binding to either vascular endothelial growth factor receptors 1 and 2 (VEGFR-1 and VEGFR-2) (Lammert et al., 2003).

1.6.2 VEGF and VEGFR

Vascular endothelial growth factor (VEGF) is a family of signaling proteins that consists of seven members: VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E, VEGF-F, and PIGF (Ortega et al., 1999; Carvalho et al., 2007). The monomeric form of these proteins have a common homology domain composed of a cystine knot motif, with eight cystine residues involved in inter- and intramolecular disulfide bonds at one end of a four-
stranded β-sheet, allowing them to dimerize in an antiparallel side-by-side orientation (Neufeld et al., 1999; Ortega et al., 1999).

VEGF-A, the primary vascular growth factor found in pancreatic β-cells, lung, kidney, heart, and adrenal gland, has been shown to exist in seven homodimeric isoforms with monomers consisting of 120, 144, 147, 164, 182, 188, or 205 amino acids in rodents, with majority appearing in the pancreas as the 120, 164, or 188 amino acid forms (Figure 1.4) (Qaum et al., 2001; Vasir et al., 2001). There is a single VEGF-A gene coding for eight exons (Poltorak et al., 1997; Neufeld et al., 1999), with conservation of exons 1 to 5 and 8 in all isoforms, except VEGF-A147, and alternative splicing of heparin-binding domain-encoding exons 6 and 7 (Ruhrberg et al., 2002; Hoeben et al., 2004). VEGF-A188, which contains exon 6, is bound tightly to cell surface heparin-containing proteoglycans in the extracellular matrix, VEGF-A164 contains exon 7 is moderately diffusible, and VEGF-A120, lacking exon 6 or 7, is highly diffusible (Ortega et al., 1999). Upon dimerization, VEGF proteins are able to bind to three types of RTKs, composed of a 750 amino-acid-residue extracellular domain organized into seven immunoglobulin-like domains, a transmembrane region, a juxtamembrane domain, a split tyrosine-kinase domain interrupted by a 70 amino acid kinase insert, and a C-terminal tail, named: the fms-like tyrosine kinase Flt-1 (VEGFR-1/Flt1), the kinase domain region (VEGFR-2/Flk-1), and Flt4 (VEGFR-3) (Christinger et al., 2004). In the context of pancreatic islets, VEGFR-1 and VEGFR-2 are expressed almost exclusively on endothelial cells, while functional endocrine cells, such as β-cells, lack VEGFR expression (Figure 1.4) (Ortega et al., 1999). VEGFR-2 is the primary receptor responsible for mediating mitosis and migration of endothelial cells to promote angiogenesis, vasculogenesis, and increased vascular permeability, by stimulating endothelial production of platelet-activating factor (PAF) (McMahon, 2000; Hudry-Clergeon et al., 2005). VEGFR-1, containing ligand-binding site Ig domain-2, exhibits much weaker kinase activity, and is found to have a greater affinity for VEGF-A, compared to VEGFR-2, containing Ig domain-3 to determine ligand-binding specificity, suggesting that it is responsible for negatively modulating pathological vascularisation by competing with VEGFR-2 for substrate (Davis-Smyth et al., 1996; Shibuya, 2006). However, it has been found that VEGFR-1 may serve a role in cell migration, chemotaxis, and a minor role in angiogenesis and vasculogenesis (Barleon
Figure 1.5 Schematic of three isoforms of VEGF-A and two VEGF receptors (VEGFR) predominantly expressed in rodent pancreas.

The single Vegf-A gene encodes all three isoforms, produced by β-cells, all of which express exons 1 to 5 and 8. VEGF-A^{188} contains exon 6 and is bound tightly to cell surface heparin-containing proteoglycans in the extracellular matrix. VEGF-A^{164} contains exon 7 and is moderately diffusible. VEGF-A^{120} lacks exon 6 and 7 and is highly diffusible. VEGF-A can bind to VEGFR-1 and VEGFR-2 in heterodimeric and homodimeric forms, expressed on endothelial cells in the pancreas (Hoeben et al., 2004).
et al., 1996; Tchaikovsky et al., 2008). The presence of VEGFR-3 in the body is limited to lymphatic endothelial cells where activation results in their mitosis, migration, differentiation, and survival (Achen et al., 1998).

1.6.3 VEGF Signaling

Of the two receptors, VEGFR-1 and VEGFR-2, available in the pancreas for binding by VEGF-A dimers, VEGFR-2 is responsible for most kinase activity, while VEGFR-1 serves primarily as a negative regulator due to a repressor sequence in the VEGFR-1 juxtamembrane domain. VEGFR-2 activation, however, can lead to autophosphorylation of a total of six identified tyrosine residues (Y949, Y994, Y1052, Y1057, Y1173, Y1212) that can recruit an array of important signaling molecules (Olsson et al., 2006). Phosphorylation of Y1173, a kinase domain residue, creates a binding site for the SH2 domain of PLCγ, which mediates activation of PKC, resulting in generation of intracellular calcium, and the mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase 1/2 (Erk1/2) pathway, increasing proliferative capacity of endothelial cells (Ito et al., 1998; Takahashi et al., 2001). Additionally, phosphorylated Y1173 creates a binding site for the adaptor molecule, Shb, in order to activate the PI3K/Akt pathway (Sakurai et al., 2005). Active Akt is able to regulate nitric oxide (NO) production by phosphorylation of endothelial NO synthase (eNOS), allowing for modulation of vascular permeability (Parenti et al., 1998; Bates and Harper, 2002). Phosphorylation of Y949 creates a binding site for VEGFR-associated protein (VRAP), shown to be important in regulating endothelial cell migration (Wu et al., 2000; Grunewald et al., 2010). Phosphorylation of Y1212 results in activation of the p38MAPK pathway, resulting in modulation of vascular permeability, and activation of HSP27, responsible for actin remodeling and cell migration (Holmes et al., 2007). Together, these effects allow for angiogenesis and vasculogenesis in tissue with increased VEGFR-2 activation.

Several avenues of research have already demonstrated the importance of VEGF signaling within the context of the pancreas. One study showed that Pdx-1 promoter-dependent overexpression of VEGF-A^{64} led to hypervascularization of the pancreas and
islet hyperplasia, as well as expression of insulin in cells of the posterior stomach epithelium (Lammert, 2001). RIP-Cre control of Vegf excision from the genome led to reduced islet endothelial cells and glucose-stimulated insulin secretion (Watada, 2010). Another study has linked VEGF-A expression to formation of endothelial cell fenestrations, an important feature in islet vascular structures (Brissova, 2006).

Developmental studies have shown that mesoderm-derived signals from embryonic blood vessels are instrumental in supporting pancreas development, preceding the differentiation of endocrine cells (Lammert, 2001). This observation was further confirmed in murine homozygous knockouts of VEGFR-2, where expression of Pdx-1 in the dorsal pancreas remained unchanged, compared to controls, but the bud never emerged, Pdx-1+ cells were decreased, and Ptf1a expression was lost (Yoshitomi, 2004). The ventral pancreas in these knockout mice was able to bud normally, but had severe defects in vasculature, further establishing the essential role that VEGF-A/VEGFR-2 signaling plays in the formation of vascular and pancreatic formation at the embryonic stage.

1.6.4 Transcription Factor HIF-1α

The primary stimulus for angiogenesis and vasculogenesis is hypoxia (Lin et al., 2004). The transcription factor monomer, hypoxia-inducible factor 1α (HIF-1α), stable under hypoxic conditions, is able to bind to the constitutively expressed hypoxia-inducible factor 1β (HIF-1β) in the cytosol to form an active dimeric form, HIF-1 (Salceda and Caro, 1997; Richard et al., 1999). VEGF-A expression is closely related to HIF-1 formation and, thus, HIF-1α production, since it has been shown to be induced when cells are subject to hypoxic or hypoglycemic conditions (Kamiyama et al., 2005; Jeon et al., 2007). HIF-1 is a heterodimer whose monomeric parts are both basic-helix-loop-helix per-aryl hydrocarbon receptor nuclear translocator-sim proteins (Forsythe et al., 1996; Ryan et al., 1998). HIF-1α prolyl hydroxylases (HPH)/prolyl hydroxylase domain proteins (PHD) 1-3 hydroxylate proline 402 and proline 564, while factor inhibiting HIF-1 (FIH-1) is able to hydroxylate asparagine 803 (Semenza, 2001; Semenza, 2007). These mechanisms are thought to be limited by the cellular presence of O2, allowing for
regulation of HIF-1 function. Following proline hydroxylation, HIF-1α is able to interact with von Hippel-Lindau tumor-suppressor protein (VHL), ubiquitinilating it and marking it for proteosomal degradation. Asparagine hydroxylation prevents HIF-1α interaction with co-activators CBP and p300, which also serves to inhibit HIF-1α function (Semenza, 2004). The functional HIF-1 imposes its effects on VEGF-A transcript production by acting in conjunction with coactivator proteins CBP, p300, SRC-1, and TIF-2 to bind to Hypoxia Regulated/Responsive Element/Enhancer sequences in the 5’ and 3’ regions of the VEGF-A gene (Ferrara and Davis-Smyth, 1997; Ryan et al., 1998; Dachs and Tozer, 2000; Tsuzuki et al., 2000; Semenza, 2001). In this way, HIF-1 is able to efficiently modulate vasculogenesis and angiogenesis in mammalian tissue.

1.6.5 Relationship between c-Kit/SCF and VEGF-A

Multiple studies have indicated a possible connection between SCF/c-Kit signaling and VEGF-A production and secretion in the pancreas. SCF treatment of an H526 small cell lung cancer cell line showed increased VEGF expression. This effect was shown to be at least partially dependent on HIF-1 formation and was inhibited by Ly294002 treatment (a PI3K inhibitor) (Litz and Krystal, 2006). Mammary tumor cell lines transfected with antisense SCF DNA showed tumors with significantly decreased numbers of mast cells and decreased microvascular density (Zhang et al., 2000). In adipose tissue-derived stem cells, VEGFR-2 antagonism and MAPK inhibition resulted in decreased c-Kit expression (Bai, 2007). Furthermore, c-Kit and VEGF were found to co-localize in human metastatic melanoma samples (Heissig, 2003). A model of pancreatic islet cancer treated by SU5416 (VEGFR inhibitor) and Gleevec (RTK inhibitor) showed repression of large-stage tumors (Bergers, 2003). Gleevec treatment, inhibiting c-Kit and PDGFR, showed significant reduction of VEGF expression in neuroblastoma cell lines, leading to decreased tumor volume following injection in SCID mice (Beppu, 2004). Another study found that mast cell infiltration and activation in tumors was mediated largely by tumor-derived SCF, indicating that even low concentrations of SCF were able to induce chemotactic migration of mast cells (Huang et al., 2008). It was reported that in hypoxic MCF-7 breast cancer cells, SCF expression was increased at both the mRNA and protein levels, and that HIF-1α knockout by RNA interference, SCF expression and c-Kit
phosphorylation were both significantly reduced (Han et al., 2008). Studies on VEGF-A have shown a relationship with metalloproteinase MMP-9, a protein that is responsible for cleavage of membrane-bound SCF as well as neutrophil-mediated activation of VEGF-A (Christoffersson et al., 2012). Taken together, these studies indicate a definite link between c-Kit activation and the effects of VEGF-A on mammalian tissues in both in vitro and in vivo models. However, the role of c-Kit-induced VEGF-A production from β-cells in islet vascularization and the associated molecular mechanisms are largely unknown. The purpose of my thesis is to dissect the role of SCF/c-Kit interactions in regulating β-cell VEGF-A production and associated signaling pathways.

1.7 Objectives, Hypothesis and Specific Questions

1.7.1 Objective

To investigate the functional role of c-Kit in the regulation of β-cell VEGF-A production and the associated intracellular signaling pathway in an in vitro model.

1.7.2 Hypothesis

An increase in c-Kit activation will stimulate VEGF-A production in β-cells via the PI3K/Akt/mTOR pathway.

1.7.3 Specific Questions

1. Can VEGF-A production and secretion be stimulated by exogenous activation of c-Kit in vitro?
2. Will siRNA-mediated knockdown of c-Kit attenuate VEGF-A production and secretion in vitro?
3. If c-Kit regulates VEGF-A production in β-cells, which intracellular signaling molecules are involved?
Chapter 2

2 Research Design and Methods

2.1 Cell Culture and Treatments

INS-1 (832/13) cells (a gift from Dr. Christopher Newgard, Duke University Medical Center, USA) were thawed in a 37°C waterbath and transferred to a 25 cm² tissue culture flask (Corning/VWR; Toronto, ON, Canada) cultured with 3-4 mL of RPMI-1640 medium with L-glutamine and 11.1 mmol/L glucose containing 10% fetal bovine serum (FBS; Invitrogen; Burlington, ON, Canada), 10 mmol/L HEPES (Sigma; Oakville, ON, Canada), 1 mmol/L sodium pyruvate (Invitrogen) and 50 μmol/L β-mercaptoethanol (Sigma) (Jensen et al., 2001; Pederson et al., 2007). Cells were cultured and expanded at 37°C in an incubator under humidified conditions with 95% air and 5% CO₂ to near (~80%) confluence. Cells were subcultured onto 12-well plates (Corning/VWR) at a density of 8x10⁴ cells/well and allowed to grow to confluence. Prior to SCF-treatment and inhibitory experiments, subcultured cells were starved overnight in serum-free 1% bovine serum albumin (BSA; Sigma) RPMI-1640 medium in order to eliminate the presence of medium-based growth factors. All experiments are performed at 5-10 passage of the culture.

2.1.1 Dose and Time of SCF Treatment

After overnight incubation in serum-free RPMI-1640 1% BSA medium, INS-1 cells were treated with human recombinant SCF (SCF; ID Labs; London, ON, Canada), where the extracellular domain of human SCF is fused to the N-terminus of the Fc region of a mutant human IgG1, at dosages of: 10, 20, 30, 50 and 100 ng/mL, or were used as a control, treated with an SCF vehicle (10 mM acetic acid), for 24 hours. To establish an optimal treatment time, INS-1 cells were treated with SCF with the optimal dose of SCF (50 ng/mL) based on the information from the dosage experiments compared to the SCF vehicle (10 mM acetic acid), and cultured for 1, 6, or 24 hours. At each dose and time-
point, cell culture medium was collected and stored at \(-20^\circ\text{C}\) for quantification of VEGF-A release using a VEGF-A ELISA.

### 2.1.2 SCF Treatment of INS-1 Cells

Based on the results from the dose and time study, the dosage of 50 ng/ml and 24 h of SCF treatment was chosen. Following subculture of INS-1 cells onto 12-well plates, cells were allowed to grow to \(\sim80\%\) confluence overnight in 10% FBS RPMI-1640 medium. Cell media was then replaced with serum-free 1% BSA RPMI-1640 medium to starve the cells overnight. Following overnight starving, media was replaced with 600 μL of fresh 1% BSA RPMI-1640 medium per well, and cells were treated with either SCF at a concentration of 50 ng/mL or an SCF vehicle (10 mM acetic acid) for 24 hours. At the end of experiments, INS-1 cells were harvested and washed with phosphate buffered saline (PBS) and immediately processed for either RNA or protein extraction, or cells were fixed and embedded in paraffin for immunofluorescence staining analyses. Samples from at least three to four cell passages per experimental group were collected.

### 2.1.3 PI3K or mTOR Inhibitory Study in SCF-treated INS-1 Cells

INS-1 cells were prepared and cultured as previously described, and treated with one of two inhibitors either with or without the presence of SCF. For the PI3K inhibitor study, INS-1 cells were incubated with Lys294002 (Promega Madison; Madison, WI, USA) at concentrations of 1, 10, or 100 μmol/L, or untreated with serum-free medium serving as a control, supplemented with either SCF at a concentration of 50 ng/mL or an SCF vehicle (10 mM acetic acid), for a period of 24 hours. Lys294002 is able to inhibit PI3K activation by competing with ATP for the active site of catalytic subunit p110 (Walker et al., 2000). For the mTOR inhibitor study, INS-1 cells were treated with rapamycin (LC Laboratories; Woburn, MA, USA) at concentrations of 5, 20, or 100 nmol/L or untreated with serum-free medium, serving as a control, for 30 minutes prior to the addition of either SCF at a concentration of 50 ng/mL or an SCF vehicle (10 mM acetic acid) for a period of 24 hours. Rapamycin is able to impose its effects on mTORC1 formation by binding to its immunophilin, FK binding protein (FKBP12), which then interacts with
mTOR to inhibit its function (Dutcher, 2004). In both cases, following the treatment period, 500 μL of cell media was collected and stored at -20°C for analysis of VEGF release by ELISA.

2.1.4 Transfection of INS-1 Cells with c-Kit siRNA

INS-1 cells were subcultured onto 12-well plates (Corning/VWR) at a density of 8x10⁴ cells/well and allowed to grow near confluence. Prepared c-Kit (r) siRNA (sc-63363) and control siRNA (sc-36869, proprietary sequence) (Santa Cruz Biotechnology Inc; Dallas, TX, USA) transfection mixture per well of 12-well plate: (a) 3.6 μL of siRNA duplex was added into 60 μL of siRNA Transfection Medium (sc-36868), and (b) 3.6 μL of siRNA Transfection Reagent (sc-29528) was added into 14.5 μL of siRNA Transfection Medium. Each mixture was incubated for 5 minutes at room temperature, the siRNA duplex solution (a) was added directly to the dilute Transfection Reagent (b), mixed gently, and incubated for 25 minutes at room temperature. As per manufacturer instructions, 600 μL of fresh 10% FBS DMEM/F12 medium was added to each well followed by either c-Kit siRNA or control siRNA transfection mixtures. Serum-free media was not used in accordance to manufacturer instructions, to preserve function and transfection efficiency that would otherwise be degraded in serum-free media. Cells were cultured for either 48 or 72 hours at 37°C in a tissue culture incubator. A pool of three sequences, recommended by the manufacturer to increase the knockdown effect by targeting three different potential binding sites, for rat c-Kit siRNA (Santa Cruz) were used: (1) 5’-CCA UGU GGA UAA AGU UGA Att-3’ (sense), 5’-UUC AAC UUU AUC CAC AUG Gtt-3’ (antisense); (2) 5’-GAU GGU UCU UGC CUA CAA Att-3’ (sense), 5’-UUUGUA GGC AAG AAC CAU Ctt-3’ (antisense); (3) 5’-GCA AGA AUA GAC UCG UAU Att-3’ (sense), 5’-AUA ACG AGU CUA UUC UUG Ctt-3’ (antisense). Following experimental procedures, culture medium was collected and stored at -20°C for VEGF ELISA, or cells were harvested and prepared for qRT-PCR, western blot, or immunofluorescence staining. Samples from at least three to four cell passages per experimental group were collected.
2.2 VEGF-A ELISA

VEGF-A release in treated INS-1 cell cultures was detected using a Rat VEGF Mini ELISA Development Kit (PeproTech Canada; Dollard Des Ormeaux, QC, Canada) according to manufacturer instructions. A total of 50 μg antigen-affinity purified polyclonal rabbit anti-rat VEGF capture antibody with D-mannitol was diluted in 0.5 mL of sterile water to a concentration of 100 μg/mL, then diluted in 1x PBS to a concentration of 0.5 μg/mL. 100 μL was added to coat each well of an ELISA microplate (Corning/VWR). The plate was sealed with manufacturer-provided sealing film, and incubated at room temperature overnight. Wells were aspirated and washed four times with 300 μL of wash buffer, containing 0.05% Tween-20 (Sigma) in 1x PBS, then the plate was inverted to remove residual buffer. 300 μL of blocking buffer containing 1% BSA (Sigma) in 1x PBS was added to each well and incubated for 1 hour at room temperature, followed by aspiration and four more washes, as described previously. The rat VEGF standard (PeproTech Canada), containing 1 μg of recombinant Rat VEGF, 2.2 mg of BSA, and 11.0 mg of D-mannitol, was diluted into 1 mL of sterile water, and serially diluted in diluent solution (0.05% Tween-20 [Sigma], 0.1% BSA [Sigma] in 1x PBS) into varying concentrations, ranging from 2 ng/mL to zero. 100 μL of standard or thawed treated INS-1 cell medium sample was added to each well in triplicate and incubated at room temperature for 2 hours. 50 μg of biotinylated antigen-affinity purified rabbit anti-rat VEGF detection antibody with 2.5 mg of D-mannitol (PeproTech Canada) was diluted in 0.5 mL of sterile water to a concentration of 100 μg/mL. The solution was diluted again in diluent solution to a concentration of 0.5 μg/mL. 100 μL was added, per well, and incubated at room temperature for 2 hours. Wells were aspirated and washed four times, as described previously, and 100 μL of dilute Avidin-HRP conjugate (1:2000)(# A-7419; Sigma) was added, per well, and incubated for 30 minutes at room temperature. Wells were aspirated and washed four times, then 100 μL of ABTS Liquid Substrate Solution (# A3219; Sigma) was added to each well, and incubated at room temperature for colour development. Readings were taken with Multiskan spectrum spectrophotometer (Thermo Scientific; Vantaa, Finland) at 405 nm with wavelength correction set to 650 nm. Data are expressed as rat VEGF content in pg/mL.
2.3 mRNA Extraction and qRT-PCR Analysis

To quantify relative gene expression levels of *Hif-1α* and *Vegf-A* in INS-1 cells following SCF [50 ng/mL] treatment or c-Kit (r) siRNA-mediated knockdown, mRNA was extracted from INS-1 cells cultured in 12-well culture plates (Fisher Scientific), 24 hours following SCF treatment, or 48 hours following c-Kit (r) siRNA-mediated knockdown, using a RNAqueous-4PCR kit (Invitrogen), according to manufacturer’s instructions (Wang et al., 2005; Krishnamurthy et al., 2007). To verify RNA quality, 1% agarose gel electrophoresis using ethidium bromide staining was performed for 18S and 28S ribosomal subunits, and images were captured under UV light.

For each RT reaction, 2 μg of DNA-free RNA was heated at 60°C for 5 minutes, then kept at 4°C for 10 minutes. Two microtubes per sample containing RNA with 1 μL Oligo-(dT) primers, 2 μL of random hexamers/oligodeoxythymidine primers, and diethylpyrocarbonate (DEPC) water were prepared added for a total volume of 10.5 μL and an additional microtube containing no RNA was prepared, serving as a negative control. Microtubes were vortexed and centrifuged briefly, then placed in the T-gradient Biometra PCR thermal cycler (Montreal Biotech; Kirkland, QC, Canada) for 10 minutes at 72°C and 5 minutes at 4°C. Microtubes were removed from the PCR machine, and centrifuged briefly, then 8.5 μL of RT Master Mix (4.0 μL of 5x cDNA synthesis buffer, 2.0 μL of DTT, 2.0 μL of 10 mM dNTPs, 0.5 μL of Rnasin) and was added to each tube and aspirated by pipette. A total of 0.8 μL of Superscript reverse transcriptase (Invitrogen) was added to each tube and left to sit for 1 minute. Microtubes were put into the PCR machine for 90 minutes at 42°C, 5 minutes at 94°C, and 60 minutes at 4°C. The tubes were put on ice and samples were collected, together, into a new tube with an equal amount of ddH2O.

For qRT-PCR, 1 μL of sample cDNA was loaded into PCR tubes (Low Tube Strips; BioRad Laboratories) along with 5 μL of iQ SYBR Green Supermix (BioRad Laboratories), 3 μL of sterile H2O, 0.5 μL of appropriate forward primer, and 0.5 μL of appropriate reverse primer. Negative controls were loaded by replacing primer amounts with sterile H2O. PCR primers used included *Vegf-A*: F, 5′-ACT GCC ATC CAA TCG
AGA CC-3’ and R, 5’-GAG GTT TGA TCC GCA TAA TC-3’ (183 bp); Hif-1α: F, 5’-CTT GAA GAT GTC CCG TTG TA-3’ and R, 5’-ACA TTG ACC ATA TCG CTG TCC-3’ (314 bp); c-Kit: F, 5’-AGC AAG AGT TAA CGA TTC C GG AG-3’ and R, 5’-CCA GAA AGG TGT AAG TGC C TTC CT-3’ (344 bp); and 18S: F, 5’-GTA ACC CGT TGA ACC CCA TTC-3’ and R, 5’-CCA TCC AAT CGG TAG TAG CG-3’ (151 bp). Tubes were analyzed in Chromo4 Real Time PCR (BioRad Laboratories), with at least three repeats per experimental group. Relative gene expression was determined by the arithmetic formula “2ΔΔCt” (Wang et al., 2005; Feng et al., 2013). Data was normalized to the internal gene, 18S rRNA, while controls were established by a lack of amplified fragments following omission of reverse transcriptase, cDNA, or DNA polymerase.

2.4 Protein Extraction and Western Blot Analysis

At the end of culture, media was aspirated and INS-1 cells were washed with 1x PBS. Cells were then harvested and incubated in Nonidet-P40 lysis buffer (Nonidet-40, phenylmethylsulfonyl fluoride, sodium orthovanadate [Sigma]; complete inhibitor cocktail tablet [Roche]) for 30 minutes on ice. Cells were dispersed by sonicating to extract protein. Samples were centrifuged at 12,000 rpm for 20 minutes at 4°C, and supernatant was collected and frozen at -80°C for protein assay and western blot analysis.

Protein concentration was measured by a protein assay with Bradford dye (BioRad Laboratories; Mississauga, ON, Canada), with standards prepared from bovine serum albumin (BSA) at concentrations of 0-0.5 mg/mL. 10 μL of each standard and 1 μL of each sample solution was loaded into micro titer plate wells in duplicate and mixed with the colorimetric dye. The plate was incubated at room temperature for 20 minutes, and readings were performed at 595 nm by a Multiskan spectrum spectrophotometer (Thermo Scientific). An equal amount (15 μg) of lysate protein samples, mixed with 6x sample buffer, from experimental groups and controls were separated by 5% (proteins greater than 180 kDa), 7.5% (proteins 100-180 kDa) and 10% (proteins 20-100 kDa) sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)(Table 2.1). A constant voltage (40V) was applied to samples in order to migrate them through the stacking gel, after which voltage was increased (80V) as protein entered the resolving gel, until the dye
front reached the bottom. Protein was then transferred to a nitrocellulose membrane by electroelution with BioRad’s Transblot system using transfer buffer containing glycine (192 mM), Tris (25 mM) and methanol (20% v/v), and transferred at a constant current (250 mA) for 2.5 hours at 4°C on a stir-plate. To check protein transferring, a Ponceau S stain was used, followed by washing in Tris buffer-saline (TBS) containing 0.1% Tween-20 (TBST). Membranes were incubated in 5% non-fat dry milk with TBST overnight at 4°C to block non-specific binding. Membranes were then incubated with primary antibodies at the appropriate dilution (Table 2.1) for 2 hours at room temperature, and then incubated with goat anti-rabbit IgG or anti-mouse IgG horseradish peroxidise-conjugated secondary antibody at appropriate dilution (Table 2.1) for 1 hour at room temperature. Membranes were washed again in TBST after secondary antibody incubation and treated with enhanced chemiluminescent (ECL) reagents for 1 minute (Western Lightning® Plus–ECL, Perkin Elmer; Wellesley, MA, USA) to visualize protein. Images of immunoreactive bands were acquired using the Versadock 5000MP imaging system (Bio-Rad Laboratories Mississauga, ON, Canada) with Quantity One software (Bio-Rad Laboratories; Mississauga, ON, Canada). Densitometric quantification was performed using Image Lab 3.0 software (Bio-Rad Laboratories; Mississauga, ON, Canada) and normalized to either total protein or a loading control (GAPDH).

2.5 Immunofluorescence Staining

Following harvesting, cells were fixed in 4% paraformaldehyde (PFA) (Fisher Scientific Company; Ottawa, ON, Canada) overnight at 4°C, then embedded in 2% agarose gel, and processed into paraffin blocks (Wu et al., 2010), using an automated tissue processing and embedding machine (Shandon CitadelTM Tissue Processor, Citadel 1000, Thermo Electron Corporation; Waltham, MA, USA). Blocks were cut into sections using a microtome (Leica RM2245, Vashaw Scientific Inc.; Nocross, Atlanta, USA) onto glass slides (Fisher Scientific).

Mounted samples were allowed to incubate at 37°C overnight, after which, they were deparaffinised by xylene for 10-minutes three times, and rehydrated with ethanol of varying concentrations, starting with 100% and concluding with 70%. Following
rehydration, slides were rinsed with deionized water, followed by 1x PBS washing. Nonspecific binding was blocked by applying 10% normal goat serum diluted in 1x PBS for 30 minutes at room temperature in a humidified container. Primary antibodies at appropriate dilutions, as listed in Table 2.2, were applied to sample sections and distributed evenly by application of a cover slip (Fisher Scientific). Sections were incubated overnight at 4°C in a humidified container. Following incubation with primary antibody, cover slips were removed and sections were rinsed with DI water, followed by 1x PBS. Appropriate dilutions of secondary antibodies conjugated with either fluorescein isothiocyanate (FITC) or tetramethyl rhodamine isothiocyanate (TRITC)(Jackson Immunoresearch Laboratories; West Grove, PA, USA) (Table 2.2) were applied to sections and distributed evenly by application of a cover slip (Fisher Scientific). Sections were left to incubate for 1 hr at room temperature within a humidified container. Following incubation, cover slips were removed, and sections were rinsed with DI water, followed by 1x PBS. Cell nuclei were counterstained with 4’-6’-diamidino-2-phenylindol (DAPI) (Sigma) for 5 minutes at room temperature within a humidified container, then rinsed in 1x PBS. Cover slips were adhered to slides by fluorescence mounting medium (Dako; Burlington, ON, Canada). Negative controls were obtained by omission of primary and secondary antibodies.

2.6 Morphometric Analyses

Sections were viewed through a Leica DMIRE 2 fluorescence microscope (Leica Microsystems; Bannockburn, IL, USA) and images were taken with a digital camera (Retiga 1300, High-sensitivity IEEE 1394 FireWire™ digital camera, QIMAGING; Burnaby, BC, Canada). Images were stored on a Windows computer, analyzed by ImagePro Plus imaging software (Media Cybernetics Inc; Rockville, MD, USA). The percentage of VEGF-A positive signaling in insulin-positive or insulin-negative cells, or insulin-positive with no VEGF-A staining was determined by cell counting. A minimum of 1000 cells per section per experimental group, with a minimum of three repeat experiments per group were counted. Co-localization of VEGF-A and insulin was determined as a percentage of total cells appearing in the section, characterized by DAPI counterstaining.
2.7 Statistical Analysis

Data are expressed as means ± SD. Statistical significance was determined by either unpaired Student’s t-test or one-way ANOVA with the post-hoc Fisher’s least significant difference (LSD) test. Differences were considered statistically significant when \( p < 0.05 \). GraphPad Prism 6 (GraphPad Software; La Jolla, CA, USA) was used to conduct analyses.
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Chapter 3

3 Results

3.1 c-Kit and VEGF-A co-localization in INS-1 Cells

To examine whether SCF/c-Kit interaction regulates VEGF-A production, co-expression of c-Kit and VEGF-A was analyzed. In order to clearly reveal the co-localization, we cultured INS-1 cells in serum-free medium containing 50 ng/mL SCF for 24 h. Cells were harvested and co-stained by double-immunofluorescence for c-Kit and VEGF-A. c-Kit-positive signals were observed near the cell membrane, while VEGF-A was found primarily in the cytoplasm of INS-1 cells (Figure 3.1).

3.2 Determining Sufficient Dosage and Time-points for VEGF-A Secretory Action in INS-1 cells

To examine the ramifications of exogenous SCF-stimulated c-Kit activation on VEGF-A release in vitro, an optimal dosage and time at which c-Kit signaling exerts a quantifiable effect on secreted VEGF-A was established. SCF stimulation at concentrations of 10, 20, 30, 50 and 100 ng/mL after 24 h in the INS-1 cell line (Baghestanian, 1997; Chung et al., 2003; Ren et al., 2003; Feng et al., 2013) were first examined. VEGF-A accumulation in culture medium was assessed at each dose by a rat VEGF mini ELISA development kit, and showed no change in VEGF-A release when the cells treated with 10 and 20 ng/mL of SCF, but release began to increase at 30 ng/mL \( (p < 0.01, \text{Figure 3.2}) \). Cells treated with 50 ng/mL of SCF showed a further increase in VEGF-A release when compared to controls, reaching a plateau at 100 ng/mL \( (p < 0.001, \text{Figure 3.2}) \). This suggests that 50 ng/mL at 24 hours was sufficient to elicit significant VEGF-A release response. To examine the time-dependent effect of SCF/c-Kit signaling on VEGF-A production, we treated INS-1 cells with SCF at a 50 ng/mL dosage for either 1, 6 or 24 hours followed by examination of VEGF-A release by ELISA. There were slight increases of VEGF-A levels in the culture medium at 1 hour, reaching statistical significance at 6 hours \( (p <
0.05, Figure 3.3), with greater significance at 24 hours of culture, when compared to time-matched controls (p < 0.01, Figure 3.3).

3.3 Expression of *Hif-1α* and *Vegf-A* mRNA and VEGF-A Protein in INS-1 Cells Following SCF Treatment

After establishing an optimal dose (50 ng/mL) at 24 h of SCF treatment in INS-1 cells, we examined changes in VEGF-A mRNA and protein levels. At the mRNA level, there was a significant increase in *Hif-1α* and *Vegf-A* mRNA levels 24 h following 50 ng/mL SCF treatment (p < 0.05, Figure 3.4A). Western blot analysis of VEGF-A protein levels in INS-1 cells showed a ~25% increase in VEGF-A protein levels in the SCF-treated group compared to controls, but did not reach statistical significance (Figure 3.4B).

3.4 Increased numbers of VEGF-A and Insulin Double-positive cells following SCF Treatment

Double immunofluorescence staining was performed to examine the co-expression pattern of VEGF-A and insulin in INS-1 cells 24 h following 50 ng/mL SCF treatment (Figure 3.5A). Increased numbers of VEGF-A and insulin double positive cells in the SCF-treated group (89.2 ± 0.5%) were observed compared to the control group (85.0 ± 1.5%) (p < 0.01, Figure 3.5B). However, the numbers of VEGF-A positive with no insulin staining (VEGF-A⁺ Insulin⁻) cells were reduced in SCF-treated cells (8.4 ± 0.5%) compared to controls (10.7 ± 0.9%) (p < 0.05, Figure 3.5C). Similarly, observation showed that insulin-positive cell populations without VEGF-A (VEGF-A⁻ Insulin⁺) were reduced in SCF-treated groups (2.5 ± 0.3%) compared to controls (4.3 ± 0.6%) (p < 0.01, Figure 3.5C). It is possible that, since this cell line has a high rate of proliferation, cells lacking insulin were in an early phase of growth, and had not yet acquired an insulin-producing phenotype.
Figure 3.1 Co-localization of c-Kit and VEGF-A in INS-1 cells.

Double immunofluorescence staining for c-Kit (green) with VEGF-A (red) in INS-1 cells cultured in serum-free medium plus 50 ng/mL SCF treatment for 24 h, shows co-localization of c-Kit with VEGF-A. A representative image of live cell staining on the coverslips is shown and bottom panel shows magnified images each corresponding to the section within the square. Nuclei were stained with DAPI (blue); scale bar: 10 μm.
Figure 3.2 Comparison of VEGF-A secretion of INS-1 cells treated with varying concentrations of SCF for 24 h.

INS-1 cells were cultured in 12-well plates and treated with varying concentrations of recombinant SCF [0, 10, 20, 30, 50, 100 ng/mL] for 24 hours to observe a dose-dependent VEGF-A secretory response. Culture medium was collected and analyzed for VEGF-A secretion. The Rat VEGF mini ELISA kit was used to quantify data. Data are expressed as picograms per milliliter in means ± SD (n=4; ** p < 0.01, *** p < 0.001 vs. 0 ng/mL group determined by one-way ANOVA with Fisher’s Least Significant Difference post-hoc test).
Figure 3.3 Comparison of VEGF-A accumulation in INS-1 cell culture medium following treatment with recombinant SCF for varying time-points.

INS-1 cells were cultured in 12-well plates and treated with recombinant SCF at a concentration of 50 ng/mL, determined to be sufficient in eliciting a VEGF-A secretory response, at time points of 1h, 6h, and 24 h. Culture medium was collected at all time-points, along with culture medium of untreated time-matched controls, and analyzed for VEGF-A content with the Rat VEGF mini ELISA kit. Data are expressed as picograms per milliliter in means ± SD (n=4; **p < 0.01, ***p < 0.001 vs. control group determined by one-way ANOVA with Fisher’s Least Significant Difference post-hoc test).
Figure 3.4 The mRNA levels of Hif-1α and Vegf-A, total protein level of VEGF-A incultured INS-1 cells following SCF treatment.

INS-1 cells were cultured in 12-well plates containing RPMI-1640 1% BSA medium overnight, and treated with human recombinant SCF at a concentration of 50 ng/mL for 24 hours. RNA and protein were harvested analyzed. (A) qRT-PCR analysis of total Vegf-A and Hif-1α mRNA levels in untreated controls and SCF-treated groups. Extracted RNA was purified by the RNAqueous-4PCR kit and analyzed using the iQ SYBR Green Supermix kit. Data are expressed as means ± SD (n=4; *p < 0.05 vs. controls determined by unpaired Student’s t-test) (B) Western blot analysis of VEGF-A protein level comparison between untreated controls and SCF-treated groups, normalized to GAPDH. Data are expressed as means ± SD (n=3).
A.

Relative gene expression (Fold vs. control)

![Graph showing gene expression](image)

B.

Relative VEGF-A expression (Fold vs. control)

![Image showing VEGF and GAPDH expression](image)
INS-1 cells were cultured in 12-well plates containing RPMI-1640 1% BSA medium overnight, and treated with human recombinant SCF at a concentration of 50 ng/mL for 24 hours. Cells were harvested and fixed in 4% paraformaldehyde (PFA), then embedded in 2% agarose gel, and processed into paraffin blocks, which were then cut into sections with a microtome onto glass slides. Sections were then immunolabeled with anti-VEGF-A (Cy2, green) and anti-insulin (Cy3, red). Nuclei were labeled with with 4',6-diamidino-2-phenylindole (DAPI, blue). (A) Representative images for VEGF-A and insulin co-localization are shown. Scale bar: 5 μm. (B) Morphometric analysis of the total number of INS-1 cells co-expressing VEGF-A and insulin 24 h following treatment by SCF [50 ng/mL]. Data are expressed as percentage of counted cells in means ± SD (n=3; ** p < 0.01 vs. controls determined by unpaired Student’s t-test). (C) Morphometric analysis of total number of INS-1 cells positive for VEGF-A and negative for insulin (left), and total number of INS-1 cells negative for VEGF-A and positive for insulin. Data are expressed as means ± SD (n=3; * p < 0.05, ** p < 0.01 vs. controls determined by unpaired Student’s t-test).
3.5 SCF/c-Kit Regulation of VEGF-A Production is Associated with PI3K/Akt/mTOR and Gsk3β/β-catenin Signaling

To investigate which intracellular signaling pathways are involved in regulating VEGF-A production, we examined signaling molecules downstream of c-Kit. INS-1 cells treated with 50 ng/mL SCF for 24 h showed significantly increased phosphorylation of c-Kit at tyrosine residue 719 when compared to controls (p < 0.05, Figure 3.6A). The PI3K/Akt/mTOR axis downstream of c-Kit was examined and it was found that levels of Akt phosphorylated at serine residue 473 were significantly increased in the SCF-treated cells compared to controls (p < 0.01, Figure 3.6B), and levels of mTOR phosphorylated at serine residue 2448 were also significantly increased in the SCF-treated cells, (p < 0.01, Figure 3.7A). mTOR activation was further confirmed by determining phosphorylation of its target, P70S6K, at threonine residue 389, which also exhibited increased activation when compared to controls (p < 0.05, Figure 3.7B). Activation of mTOR has been linked to activation of a variety of transcription factors, including NfκBp65 (Dan et al., 2008; Radhakrishnan et al., 2013). NfκBp65 itself has been implicated in the progression of a variety of cancers by its involvement in stimulation of angiogenic processes and, specifically, VEGF regulation at the mRNA level (Leibovich et al., 2002; Shibata et al., 2002). Thus, phosphorylation of NfκBp65 at serine residue 536 was assessed and shown to be significantly increased in SCF-treated groups, when compared to controls (p < 0.05, Figure 3.7C). These data demonstrate that exogenous SCF stimulation of c-Kit activation, results in activation of the PI3K/Akt/mTOR signaling pathway.

Previous studies in our laboratory showed that activation of c-Kit in β-cells leads to an increase in Gsk3β phosphorylation at serine residue 9 (Feng et al., 2012). Inactivating Gsk3β by phosphorylation allows for increased activation of β-catenin, a known inhibitory target of Gsk3β and a molecule with several consensus binding sites for the gene promoter of VEGF-A (Easwaran et al., 2003; Skurk et al., 2005). Gsk3β phosphorylation was assessed and, in comparison to controls, exhibited an increased level of phosphorylation following SCF stimulation (p < 0.01, Figure 3.8A). Total β-catenin
levels were also found to be significantly more abundant in SCF-treated group \( (p < 0.05, \textbf{Figure 3.8B}) \). These results suggest that SCF/c-Kit interactions increase Akt activation, leading to inactivation of Gsk3β and an increase in biologically active β-catenin, which is able to interact with VEGF promoters to produce VEGF-A. Taken together, these results suggest that, by targets downstream of the PI3K/Akt/mTOR pathway, SCF-mediated c-Kit activation resulted in increased production of VEGF-A.

### 3.6 SCF/c-Kit-mediated VEGF-A Production via Activation of Alternative Pathways

c-Kit activation has been found to stimulate increased activation of other pathways that converge onto the PI3K/Akt/mTOR axis and are able to synergistically enhance the downstream effects of this pathway (Ronnstrand, 2004). Two proteins in the MAPK signaling pathway, p38 and p44/42, are thought to be involved as a result of SCF/c-Kit interactions. Thus, we aimed to investigate whether this pathway might impose convergent action on stimulating VEGF production (Jin et al., 2005; Kuang et al., 2008). There was no change in p44/42 phosphorylation (\textbf{Figure 3.9A}), but levels of phosphorylated p38 at tyrosine residues 180 and 182 were increased in the SCF-treated group \( (p < 0.05, \textbf{Figure 3.9B}) \). These results suggest that activation of MAPK/p38 converges on targets in the PI3K/Akt/mTOR pathway, possibly eliciting synergistic stimulation of VEGF-A production.

### 3.7 Pharmacological Inhibition of PI3K and mTOR Attenuated the Release of VEGF-A Following SCF Treatment

To further demonstrate that VEGF-A production and release are dependent on the PI3K/Akt/mTOR pathway, VEGF-A release was measured following treatment with a combination of SCF 50 ng/mL, and various dosages of the PI3K inhibitor, Lys294002 (0-100 μmol/L), or the mTOR inhibitor, rapamycin (0-100 nmol/L). It was noted that INS-1 cells treated with Lys294002 or rapamycin at different dosages for 24 h showed no effect on cell loss (\textbf{Appendix I}), however, VEGF-A levels in culture medium were altered in a
dose-dependent fashion (Figure 3.10, 3.11). It was found that enhanced VEGF-A release stimulated by treatment with 50 ng/mL SCF was abolished when 10 μmol/L of Lys294002 was added (Figure 3.10C). Similarly, SCF-stimulated VEGF-A release was abolished when cells were treated with rapamycin at dosage of 20 nmol/L (Figure 3.11C). These results further verify that, despite SCF-stimulated activation of c-Kit, the inhibition of key targets along the PI3K/Akt/mTOR pathway resulted in a loss of VEGF-A accumulation in culture medium and, thus, SCF/c-Kit downstream PI3K/Akt/mTOR pathways are responsible for regulating VEGF-A production.

3.8 Knockdown of c-Kit Expression in the INS-1 Cell Line by c-Kit siRNA Transfection

INS-1 cells were transiently transfected with either c-Kit (r) siRNA or control siRNA to examine the effect of c-Kit down-regulation on VEGF-A production. Following siRNA treatment for 48 to 72 hours, the expression of c-Kit mRNA, protein and phosphorylation were determined. Following 48 h of transfection culture, c-Kit mRNA expression was reduced ~45%, as determined by qRT-PCR, but did not reach statistical significance (Figure 3.12A). There was a significant decrease in the amount of total c-Kit protein after 72 h of c-Kit (r) siRNA-transfection in INS-1 cells compared to those treated with control siRNA (p < 0.05, Figure 3.12B). We further examined the levels of phosphor-c-Kit at 72 h of c-Kit(r) siRNA-transfected cells and observed a reduction in c-Kit activation (Figure 3.12C). These data verified that siRNA treatment mediated c-Kit knockdown at both the mRNA and protein level, allowing for analysis of further downstream targets.

3.9 Expression of VEGF-A Production in INS-1 Cells Following siRNA-mediated c-Kit Knockdown

Forty-eight hours following c-Kit (r) siRNA-mediated knockdown in INS-1 cells, levels of Hif-1α and Vegf-A mRNA were assessed by qRT-PCR analysis. It was found that transcript levels of both Hif-1α and Vegf-A trended toward a decrease, though did not
reach significance (Figure 3.13A). After 72 hours of transfection, western blot analysis of intracellular VEGF-A content was reduced ~21% in the c-Kit (r) siRNA-treated group compared to control siRNA group, though was not significantly different (Figure 3.13B). VEGF-A release into the culture medium, analyzed by a rat VEGF mini ELISA kit, showed non-significant, minimal reduction compared to controls (Figure 3.13C), indicating that down-regulation of c-Kit expression by siRNA affects VEGF-A production. We further examined the number of VEGF-A-positive and insulin-positive cells in both experimental groups and found neither double-positive cell nor single-positive cell populations had changed (Figure 3.14).

3.10 Knockdown of c-Kit Inhibits the PI3K/Akt/mTOR Signaling Pathway

The downstream PI3K/Akt/mTOR axis was assessed in order to confirm down-regulation of signaling molecules downstream of c-Kit after a 72 h c-Kit siRNA transfection. Levels of Akt phosphorylated at serine residue 473 were significantly decreased in the knockdown cells, compared to controls ($p < 0.05$, Figure 3.15A), and levels of mTOR phosphorylated at serine residue 2448 were also significantly decreased in the c-Kit siRNA knockdown cells, compared to controls ($p < 0.05$, Figure 3.15B). mTOR activation was further confirmed by assaying phosphorylation of its well-established target, P70S6K, at threonine residue 389, which also exhibited decreased activation when compared to controls ($p < 0.01$, Figure 3.15C). Phosphorylation of NfκBp65 at serine residue 536 was found to be significantly decreased in c-Kit knockdown groups, when compared to controls ($p < 0.05$, Figure 3.15D). These analyses confirmed that knockdown of c-Kit expression resulted in decreased activation of molecules along the PI3K/Akt/mTOR pathway.
3.11 Inactivation of Downstream Targets of Akt and p38 inhibit VEGF-A Production and Release

Further signaling molecules downstream of the PI3K/Akt pathway were examined in order to provide a linkage specifically to decreased VEGF-A production in these cells, due to knockdown of c-Kit by siRNA transfection. Gsk3β phosphorylation was decreased, suggesting that its activation was increased, following knockdown of c-Kit compared to controls \((p < 0.05, \text{Figure 3.16A})\). Upon analysis, total β-catenin levels were shown to decrease significantly in abundance in the knockdown groups \((p < 0.05, \text{Figure 3.16B})\). As mentioned in section 3.8, p38 MAPK is thought to work synergistically to activate mTOR alongside the PI3K/Akt pathway and, ultimately, increase VEGF-A production. In the case of siRNA-mediated knockdown, it was found that p38 MAPK protein levels trended toward decrease, but did not reach significance \((\text{Figure 3.16C})\). Taken together, these results suggest that by inhibition of targets downstream of the PI3K/Akt/mTOR pathway, c-Kit knockdown results in the decreased production of VEGF-A.
**Figure 3.6 Protein analysis of c-Kit and Akt activation in cultured INS-1 cells treated with SCF.**

INS-1 (832/13) cells were cultured in 12-well plates containing RPMI-1640 1% BSA medium overnight, and treated with human recombinant SCF at a concentration of 50 ng/mL for 24 hours. Cells were incubated in Nonidet-P40 lysis buffer and dispersed by sonication to extract protein. c-Kit and Akt phosphorylation was compared by western blot analysis, with representative images of western blots shown on top of graphs. Proteins assayed include (A) Phosphorylated (Tyr719) c-Kit compared to total c-Kit, (B) phosphorylated (Ser473) Akt compared to total Akt. Data are normalized to total protein levels and expressed as fold versus control (means ± SD; n=3-4; * p < 0.05, ** p < 0.01 vs. controls analyzed by unpaired Student’s t-test).
Figure 3.7 Protein analysis of mTOR activation and its downstream targets, P70S6K and NfkBp65 in cultured INS-1 cells treated with SCF.

INS-1 cells were cultured in 12-well plates containing RPMI-1640 1% BSA medium overnight, and treated with human recombinant SCF at a concentration of 50 ng/mL for 24 hours. Cells were incubated in Nonidet-P40 lysis buffer and dispersed by sonication to extract protein. mTOR, P70S6K and NfkB phosphorylation was compared by western blot analysis, with representative images of western blots shown on top of graphs. Proteins assayed include (A) Phosphorylated (Ser2448) mTOR compared to total mTOR, (B) phosphorylated (Thr389) p70S6K compared to total p70S6K, (C) phosphorylated (Ser536) NfkBp65 compared to total NfkBp65. Data are normalized to total protein levels and expressed as fold versus control (means ± SD; n=3-4; * p < 0.05, ** p < 0.01 vs. controls analyzed by unpaired Student’s t-test).
**Figure 3.8 Protein analysis of activated Gsk3β and total β-catenin in cultured INS-1 cells treated with SCF.**

INS-1 (832/13) cells were cultured in 12-well plates containing RPMI-1640 1% BSA medium overnight, and treated with human recombinant SCF at a concentration of 50 ng/mL for 24 hours. Cells were incubated in Nonidet-P40 lysis buffer and dispersed by sonication to extract protein. Gsk3β phosphorylation and total β-catenin were compared by western blot analysis, with representative images of western blots shown on top of graphs. Proteins assayed include (A) phosphorylated (Ser9) Gsk3β compared to total Gsk3β, (B) total β-catenin compared to housekeeping protein GAPDH. Data are normalized to total protein levels or GAPDH and expressed as fold versus control (means ± SD; n=3-4; * p < 0.05, ** p < 0.01 vs. controls analyzed by unpaired Student’s t-test).
**A.**

<table>
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</tr>
<tr>
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<td>46</td>
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**B.**

<table>
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</tr>
<tr>
<td>GAPDH</td>
<td>37</td>
</tr>
</tbody>
</table>
Figure 3.9 Protein analysis of activated p42/44 MAPK and p38 MAPK in cultured INS-1 cells treated with SCF.

INS-1 cells were cultured in 12-well plates containing RPMI-1640 1% BSA medium overnight, and treated with human recombinant SCF at a concentration of 50 ng/mL for 24 hours. Cells were incubated in Nonidet-P40 lysis buffer and dispersed by sonication to extract protein. p42/44 MAPK and p38 MAPK phosphorylation was compared by western blot analysis, with representative images of western blots shown on top of graphs. Proteins assayed include (A) phosphorylated (Thr202/Tyr204) p42/44 MAPK compared to total p42/44 MAPK (B) phosphorylated (Thr180/Tyr182) p38 MAPK compared to total p38 MAPK. Data are normalized to total protein levels and expressed as fold versus control (means ± SD; n=3-4; * p < 0.05 vs. controls analyzed by unpaired Student’s t-test).
A. P-p42/44 MAPK$^{T202/Y204}$, 42, 44 kDa; T-p42/44 MAPK, 42, 44 kDa

B. p-p38 MAPK$^{T180/Y182}$, 40 kDa; t-p38 MAPK, 40 kDa

Relative P-p42/44 MAPK expression (Fold vs. control)

Relative P-P38 MAPK expression (Fold vs. control)
Figure 3.10 VEGF-A secretion in SCF-treated cultured INS-1 cells with and without varying concentrations of a PI3K inhibitor (Lys294002).

INS-1 cells were cultured in 12-well plates containing RPMI-1640 1% BSA medium overnight. Fresh medium was added to wells, and cells were cultured with Lys294002 in increasing concentrations [0, 1, 10, 100 umol/L], with human recombinant SCF [50 ng/mL] or an SCF vehicle for a period of 24 h. At the end of the culture period, culture medium was collected and analyzed for VEGF-A secretion. VEGF-A secretion analyses are shown for (A) control and SCF-treated, (B) Lys294002 1 umol/L with or without SCF, (C) Lys294002 10 umol/L with or without SCF, (D) Lys294002 100 umol/L with or without SCF. The Rat VEGF mini ELISA kit was used to quantify data. Data are normalized to total protein levels and expressed as fold versus control (means ± SD; n=3; ** p < 0.01 SCF vs control determined by unpaired Student’s t-test).
Figure 3.11 VEGF-A release in untreated and SCF-treated cultured INS-1 cells with and without varying concentrations of an mTOR inhibitor (rapamycin).

INS-1 cells were cultured in 12-well plates containing RPMI-1640 1% BSA medium overnight. Fresh medium was added to wells, and cells were pre-treated with increasing concentrations of rapamycin [0, 5, 20, 100 nmol/L] for 30 minutes prior to treatment with either human recombinant SCF [50 ng/mL] or an SCF vehicle for a period of 24 hours. At the end of the culture period, cell medium was collected and analyzed for VEGF-A secretion. VEGF-A secretion analyses are shown for (A) control and SCF-treated, (B) rapamycin 5 nmol/L with and without SCF, (C) rapamycin 20 nmol/L with and without SCF, (D) rapamycin 100 nmol/L with and without SCF. The Rat VEGF mini ELISA kit was used to quantify data. Data are expressed as means ± SD (n=3; * p < 0.05, *** p < 0.001 SCF vs. control determined by unpaired Student’s t-test).
Figure 3.12 c-Kit expression in cultured INS-1 cells following siRNA-mediated c-Kit knockdown.

INS-1 cells were cultured on 12-well plates and treated with either c-Kit (r) siRNA or control siRNA transfection mixtures. Transient transfection took place for either 48 h or 72 h. mRNA was harvested from cells after 48 h of transient transfection with the RNAqueous-4PCR kit. (A) RT-PCR images showed expression of c-Kit and 18S captured under UV light and qRT-PCR analysis of c-Kit mRNA at 48 h of culture. Data are expressed as fold-change vs. control siRNA group (n=3). Following 72 h of transient transfection, cells were incubated in Nonidet-P40 lysis buffer and dispersed by sonication to extract protein. Extracted protein was analyzed from both groups by western blot analysis, with representative images of western blots shown on top of graphs. (B) Total c-Kit levels were normalized to housekeeping protein GAPDH, (C) Phosphorylated (Tyr 719) c-Kit levels were normalized to housekeeping protein GAPDH. Data are expressed as fold versus control (means ± SD; n=3; * p < 0.05, ** p < 0.01 vs. control siRNA group determined by unpaired Student’s t-test).
A. 

Ctrl siRNA  
c-Kit siRNA  

18S  
c-Kit mRNA expression (Fold vs. Ctrl siRNA)  

B. 

c-Kit  
GAPDH  

107 kDa  
37 kDa  

C. 

P-c-Kit Tyr719  
GAPDH  

120 kDa  
37 kDa  

Relative c-Kit expression (Fold vs. control)  

Relative P-c-Kit expression (Fold vs. control)  

Ctrl siRNA  
c-Kit siRNA  

*  

**
Figure 3.13 Total mRNA levels of Hif-1α and Vegf-A, protein level of VEGF-A, and VEGF-A secretion of cultured INS-1 cells following c-Kit siRNA treatment.

INS-1 cells were cultured in 12-well plates containing RPMI-1640 10% FBS medium overnight, and treated with either c-Kit (r) siRNA or control siRNA for 48 h or 72 h. RNA was harvested at 48 h, while cellular protein and culture medium were collected after 72 h and analyzed. (A) qRT-PCR analysis of total Vegf-A and Hif-1α mRNA levels in controls and c-Kit siRNA-treated groups. Extracted RNA was purified by the RNAqueous-4PCR kit and analyzed using the iQ SYBR Green Supermix kit. Data are expressed as means ± SD (n=5) (B) Western blot analysis of VEGF-A protein level comparison between controls and c-Kit siRNA-treated groups. Data are normalized to GAPDH and expressed as fold versus control (means ± SD; n=3). (C) Analysis of VEGF-A secretion into medium after 72 h of siRNA-mediated c-Kit knockdown. The Rat VEGF mini ELISA kit was used to quantify data. Data are expressed as means ± SD (n=6).
A. Relative gene expression (Fold vs. control) for Hif-1α and Vegf-A under control siRNA (Ctrl siRNA) and c-Kit siRNA.

B. Western blot showing VEGF-A at 43 kDa and GAPDH at 37 kDa.

C. Bar graphs showing relative VEGF-A expression and secretion (Fold vs. control siRNA) for Ctrl siRNA and c-Kit siRNA.
Figure 3.14 The number of VEGF-A+ and insulin+ cells in c-Kit siRNA and control siRNA-treated INS-1 cells.

INS-1 cells were cultured in 12-well plates containing RPMI-1640 10% FBS medium overnight, and treated with either c-Kit (r) siRNA or control siRNA for a period of 72 h. Cells were harvested and fixed in 4% paraformaldehyde (PFA), then embedded in 2% agarose gel, and processed into paraffin blocks, which were then cut into sections with a microtome onto glass slides. Sections were then immunolabeled with anti-VEGF-A (Cy2, green) and anti-insulin (Cy3, red). Nuclei were labeled with 4’,6-diamidino-2—phenylindole (DAPI, blue). (A) Representative images for VEGF-A and insulin co-localization are shown. Scale bar: 5 μm. (B) Morphometric analysis of the total number of INS-1 cells co-expressing VEGF-A and insulin 72 h following treatment by c-Kit siRNA. (C) Morphometric analysis of total number of INS-1 cells positive for VEGF-A and negative for insulin (left), and total number of INS-1 cells negative for VEGF-A and positive for insulin. Data are expressed as means ± SD (n=3).
A.

Control  siRNA

B.

VEGF$^+$Insulin$^+$ cells (%)  

<table>
<thead>
<tr>
<th></th>
<th>Ctrl</th>
<th>siRNA</th>
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<tr>
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<td>82.3</td>
<td>82.7</td>
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C.

Positive cells (%)  

<table>
<thead>
<tr>
<th></th>
<th>Ctrl siRNA</th>
<th>c-Kit siRNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>VEGF-A$^+$Insulin$^+$</td>
<td>10.2</td>
<td>12.1</td>
</tr>
<tr>
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<td>5.6</td>
<td>8.1</td>
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INS-1 cells were cultured in 12-well plates containing RPMI-1640 10% FBS medium overnight, and treated with either c-Kit (r) siRNA or control siRNA for 72 h. Cells were incubated in Nonidet-P40 lysis buffer and dispersed by sonication to extract protein. Akt, mTOR, P70S6K, and NfkBp65 phosphorylation was compared by western blot analysis, with representative images of western blots shown on top of graphs. Proteins assayed include (A) phosphorylated (Ser473) Akt compared to total Akt, (B) phosphorylated (Ser2448) mTOR compared to total mTOR, (C) phosphorylated (Thr389) P70S6K compared to total P70S6K, (D) phosphorylated (Ser536) NfkBp65 compared to total NfkBp65. Data are normalized to total protein levels and expressed as fold versus control (means ± SD; n=3-4; * p < 0.05, ** p < 0.01 vs. control siRNA groups analyzed by unpaired Student’s t-test).
**Figure 3.16 Protein analysis of Gsk3β activation, total β-catenin, and p38 MAPK activation in cultured INS-1 cells treated with c-Kit siRNA.**

INS-1 cells were cultured in 12-well plates containing RPMI-1640 10% FBS medium overnight, and treated with either c-Kit (r) siRNA or control siRNA for 72 h. Cells were incubated in Nonidet-P40 lysis buffer and dispersed by sonication to extract protein. Gsk3β phosphorylation, total levels of β-catenin, and phosphorylation of p38 MAPK was compared by western blot analysis, with representative images of western blots shown on top of graphs. Proteins assayed include (A) Phosphorylated (Ser9) Gsk3β compared to total Gsk3β, (B) total β-catenin compared to housekeeping protein GAPDH, (C) phosphorylated (Thr180/Tyr182) p38 MAPK compared to total p38 MAPK. Data are normalized to total protein levels or GAPDH and expressed as fold versus control (means ± SD; n=3-4; * p < 0.05 vs. control siRNA analyzed by unpaired Student’s t-test).
Chapter 4

4 Discussion

The objective of this thesis was to investigate the functional role of c-Kit in the regulation of β-cell VEGF-A production and associated intracellular signaling pathway using the INS-1 cell line as an in vitro model. The hypothesis was that an increase in c-Kit activation could stimulate VEGF-A production in β-cells via the PI3K/Akt/mTOR pathway. The results presented in this study highlight that there is a significant increase in VEGF-A release, a factor required for islet vessel formation, in INS-1 cells following stimulation with exogenous SCF. This effect was mediated by upregulation of phosphorylated c-Kit and its downstream targets along the PI3K/Akt/mTOR signaling pathway. Cells treated with SCF plus either a PI3K inhibitor (Lys294002) or mTOR inhibitor (rapamycin) displayed an attenuation in VEGF-A levels in culture medium, providing further evidence that activation of c-Kit and its downstream PI3K/Akt/mTOR signaling pathway is essential for VEGF-A modulation (Figure 4.1). Conversely, INS-1 cells treated with c-Kit siRNA demonstrated that knockdown of c-Kit expression results in a decrease of phosphorylation along the Akt/mTOR pathway and, consequently, decreased production and release of VEGF-A. Taken together, these findings lay down the groundwork suggesting that the c-Kit receptor tyrosine kinase and downstream targets of the PI3K/Akt/mTOR axis exert a regulatory effect on VEGF-A production and release in β-cells in vitro. Further investigation of c-Kit-mediated VEGF-A production and function using exogenous SCF to stimulate isolated murine islets, and in vivo models with transgenic c-Kit over-expression (c-KitβTg) or c-Kit knockdown by point-mutation (Wy) would enhance our understanding of vascular defects in the diabetic state and provide insight in the process of restoring vasculature following islet transplantation in T1DM. This could be accomplished by assessing and comparing c-Kit knockdown and overexpression models’ vasculature and pancreatic function by protein and RNA analysis from isolated islets, as well as vasculature morphology through PECAM-1 or collagen IV staining, then examining whether crossing the two mouse models would result in recovery of vascular impairment.
Figure 4.1 Schematic of proposed model involving SCF-stimulated c-Kit activation through the convergence of the PI3K/Akt and p38 MAPK pathways to increase VEGF-A production.

SCF binding to the receptor tyrosine kinase c-Kit results in homodimerization of the receptor and subsequent autophosphorylation on tyrosine residue 719. This allows for recruitment of PI3K/Akt via its pleckstrin homology (PH) domain and phosphorylation of Akt at serine residue 473, resulting in its kinase activity. Akt is able to direct phosphorylation of mTOR on serine residue 2448, allowing it to bind to Raptor protein, forming the bioactive mTORC1 complex, and/or phosphorylate Gsk3β at serine residue 9 to inactivate it. The mTORC1 complex is then able to act upon NfkBp65 at serine residue 534, resulting in its translocation to the nucleus and increased transcription of HIF-1α, a transcription factor that promotes VEGF-A production and secretion. Inactivated Gsk3β is unable to degrade β-catenin which allows β-catenin to translocate into the nucleus and promote increased VEGF-A transcription. p38 MAPK is another molecule that is activated when bound via its SH2 domain to activated c-Kit. p38 MAPK is then phosphorylated at threonine residue 180 and tyrosine residue 182, allowing it to further stimulate mTOR phosphorylation and mTORC1’s downstream effects.
4.1 Do c-Kit and VEGF-A Co-localize in SCF-treated INS-1 Cells?

It has been reported that c-Kit is expressed in INS-1 cells (Rachdi et al., 2001), the cell line used in this work. Previous reports showed that SCF and VEGF are co-localized in cancer cells derived from human metastatic melanoma (Heissig et al., 2003). In order to confirm that c-Kit and VEGF-A are located in the same cell population, we first used double immunofluorescence staining to show the co-localization of VEGF-A and c-Kit in INS-1 cells. Our data from live cell staining on cover slips demonstrated that both c-Kit and VEGF-A co-localized in these cells, but distribution differed (Figure 3.1). c-Kit expression was located primarily near cell membranes, with some staining of internalized c-Kit receptors, while VEGF-A stained predominantly in the cytoplasm of cells. These data built a case which prompted us to examine whether there is a relationship between activation of the c-Kit receptor and modulation of VEGF-A production in the following experiments.

4.2 Can VEGF-A Production and Release be Stimulated by Exogenous SCF Activation of c-Kit in vitro?

Upon treatment with SCF at a variety of concentrations, a VEGF-A ELISA determined that there was, in fact, an increase in VEGF-A release in a dose- and time dependent fashion into cell culture medium after SCF treatment (Figure 3.2 and 3.3). A significant increase in culture medium VEGF-A levels was observed at 24 h of SCF-stimulation compared to controls (Figure 3.3). This delayed response was likely due to the fact that INS-1 is a cancer cell line and contains a high basal level of VEGF-A production and release, compared to that found in isolated islets or animal models. Despite the fact that cancer cell lines, such as INS-1, have a high basal VEGF-A secretion rate (Bellamy et al., 1999; Dankbar et al., 2000), previous studies have used this cell line to demonstrate quantifiable stimulation of VEGF-A production and secretion, specifically by the induction of HIF-1α by oxidative stress (Moritz et al., 2002; Gosh et al., 2010). A study reported that HIF-1α expression was upregulated following SCF stimulation in a
hematopoietic cell line, which yielded a transcriptionally active form of HIF-1α, able to modulate VEGF, induced through stimulation of c-Kit and subsequent activation of the PI3K and Ras/MEK/Erk pathways (Pedersen et al., 2008). Similarly, observations made in our study demonstrated that INS-1 cells treated with SCF exhibited significantly increased levels of Hif-1α and Vegf-A mRNA (Figure 3.4A) and VEGF-A release (Figure 3.2, 3.3). Intracellular VEGF-A content was shown to increase ~25%, but did not reach statistical significance (Figure 3.4B). One possible explanation for this observation is that increased VEGF-A production was secreted immediately in response to physiological stimuli, as demonstrated in cultured mouse islets responding to low glucose culture conditions (Xiao et al., 2013). Furthermore, performing this study under hypoxic conditions would likely exacerbate VEGF-A production and secretion due to a lack of VHL-mediated inhibition and degradation of factors such as HIF-1α that serve to promote angiogenic processes.

The mechanism by which VEGF-A is secreted from β-cells is not well-understood but it has been reported that, in bone marrow-derived mast cells, release of VEGF-A is largely mediated by hypoxia-triggered signaling pathways that depend on the Src family kinases and PI3K (Garcia-Roman et al., 2010). Furthermore, in a VEGF reporter study performed in Hep3B cells, it was demonstrated that the 5'-flanking region of the VEGF gene is able to functionally interact with HIF-1, composed of the HIF-1α and HIF-1β subunits, on at least two HIF-1 binding sites (Forsythe et al., 1996), further implicating HIF-1α as a key regulator of VEGF-A. We have previously shown, in vitro and in vivo, that c-Kit activation by SCF could target the PI3K/Akt signaling pathway (Li et al., 2006; Feng et al., 2011; Feng et al., 2012). It was thought that SCF-induced VEGF-A release in INS-1 cells might also be regulated by activation of c-Kit and downstream of the PI3K/Akt signaling pathway as discussed below.
4.3 Is the PI3K/Akt/mTOR Signaling Pathway Involved in VEGF-A Regulation?

4.3.1 c-Kit Receptor Phosphorylation

c-Kit is able to mediate a variety of effects on various cell types in a variety of cell lineages and cell types in murine and human models. Upon ligand-binding, the transmembrane receptor is phosphorylated on tyrosine residue 719 in the kinase insert region, allowing it to associate with the p85 subunit of PI3K, containing two SH2 domains, to induce its phosphorylation and activation of more downstream signaling molecules by activity of the 110 kDa catalytic subunit (p110) (Serve et al., 1994; Yee et al., 1994). Expectedly, 24 hours following SCF treatment, INS-1 cells had significantly increased phosphorylation of c-Kit at tyrosine residue 719, indicating that SCF and c-Kit interaction results in activation of the receptor tyrosine kinase (Figure 3.6A).

4.3.2 Akt Phosphorylation

Activation of PI3K results in the production of phosphatidylinositol-3,4,5-triphosphate (PIP3), which is able to recruit signaling proteins with pleckstrin homology (PH) domains, consisting of approximately 100 amino acids, to the membrane, such as Akt (Vara et al., 2004). Akt is expressed as three isoforms (Akt1/2/3) that exhibit highly overlapping functions in regulation of factors such as cellular growth, glucose homeostasis, and neuronal development, and feature many common phosphorylation sites due to their shared amino-terminal PH domain (Manning and Cantley, 2007; Gonzalez et al., 2009). Recruitment of Akt by PIP3, the lipid product of activated PI3K, results in conformational changes in Akt and exposure of two of its essential phosphorylation sites, threonine residue 308 and serine residue 473 (Andjelkovic et al., 1995; Kandel et al., 1999; Brazil and Hemmings, 2001). While phosphorylation of threonine residue 308, located within the activation loop of the kinase domain, is required for activation of Akt, phosphorylation of serine residue 473, located at the C-terminus, is thought to be required for full kinase activity (Kandel et al., 1999; Widenmaier et al., 2009). 3-phosphoinositide-dependent kinase (PDK1), a PIP3-dependent kinase, is responsible for
phosphorylation of threonine residue 308, while phosphorylation of serine residue 473 has been shown to be dependent on the mTOR complex 2 (mTORC2), containing mTOR, rictor, and mLST8 (Hresko et al., 2005; Sarbassov et al., 2006). PDK1 and mTORC2 activity play a role in a variety of cellular processes including cell death regulation, cell cycle progression, and cell growth (Juan et al., 2004). Our lab has previously determined that activation of c-Kit led to phosphorylation of Akt at serine residue 473, but not threonine residue 308 (Feng et al., 2011). Therefore, we examined phosphorylation of Akt on serine residue 473 after SCF stimulation, which showed a significant increase in the activity of Akt in association with VEGF-A release (Figure 3.6B). Lys294002 was used as a potent inhibitor of PI3K, and it was found that VEGF-A release was attenuated to control levels at a sufficient dosage (10 μmol/L) despite SCF stimulation (Figure 3.10C). This further confirmed the involvement of the Akt signaling pathway in SCF-stimulated VEGF-A release. However, whether PDK1 and mTORC2 activity is involved in this regulatory process was not examined in this work, thus it would be important to investigate in future research in order to verify whether SCF/c-Kit-induced VEGF-A release requires activation of multiple downstream kinases.

4.3.3 mTOR Signaling Pathway

The mammalian target of rapamycin (mTOR) is the most-examined downstream substrate of Akt. Akt exerts a number of mechanisms to regulate mTOR, either directly through phosphorylation of the substrate, or by modulation of other regulatory mechanisms. Akt is able to indirectly activate mTOR through phosphorylation of tuberous sclerosis complex 2 (TSC2) resulting in its inhibition and inability to heterodimerize with tuberous sclerosis complex 1 (TSC1) (Inoki et al., 2003; Tee et al., 2003). Once TSC2 is inhibited and unable to form its active complex with TSC1, it is unable to hydrolyze GTP to GDP in Ras homolog enriched in brain (Rheb). Without this negative regulation of Rheb, it is able to remain in its GTP-bound state, allowing for increased activation of mTOR (Inoki et al., 2003; Manning and Cantley, 2003). It has been proposed that for full TSC2 inactivation, Akt must play a role in regulation, through direct inhibition of TSC2 by phosphorylation, which it can also influence by increasing ATP levels (Hahn-Windgassen et al., 2005). In this work, mTOR activation was assayed
by examining phosphorylation of serine residue 2448, which has been demonstrated to be crucial in Akt-mediated mTOR activation (Nave et al., 1999; Inoki et al., 2002). As expected, due to increased activation of Akt, mTOR phosphorylation/activation was significantly increased following SCF treatment in INS-1 cells (Figure 3.7A). Inhibiting the mTOR pathway with rapamycin at a sufficient dosage (20 nmol/L) could block SCF-induced VEGF-A release in INS-1 cells (Figure 3.11C). Furthermore, it has been shown that insulin binding to its receptor increases Akt-mediated phosphorylation of TSC2 at serine residues 939 and 981, resulting in the dissociation of the TSC1/2 complex (Cai et al., 2006). Since INS-1 cells are able to produce greater amounts of insulin, and c-Kit activation has been linked to increased insulin production and expression (Rachdi et al., 2001), it is possible that mTOR activation was compounded by these factors, creating an even stronger response than hypothesized. Thus, it would be critical, for future study, to examine the effect of c-Kit activation on the TSC1/2 complex in this context. mTOR can bind to Raptor to form the mTORC1 complex, controlled by GTP-bound Rheb amongst other signaling molecules, or mTOR can bind to Rictor to form the mTORC2 complex, which is activated by a variety of RTKs and can interact with Akt to regulate it (Sarbassov et al., 2005). The exact function and mechanism by which mTORC2 is activated and exerts its effects is not well-known, but in this work, mTORC1 and its downstream effects are of particular relevance to VEGF-A regulation in INS-1 cells.

4.3.4 P70S6K and NfkB of the mTOR-targeted Signaling Pathway

mTOR is an important regulator of a variety of cellular functions, including cell growth and proliferation. It is located downstream of the PI3K/Akt pathway, and its most important substrate is p70S6K. Overall the pathway is implicated not only in cell proliferation, growth, differentiation and survival (Schmelzle and Hall, 2000), but also angiogenesis and tumor neovascularisation, making it a likely candidate for VEGF-A modulation in our current study (Maffucci et al., 2005). Once the mTORC1 complex is formed, mediated by Rheb due to the Akt-mediated inhibition of TSC2, it can act upon the hydrophobic motif of p70S6K, phosphorylating it at threonine residue 389, an effect that is confirmed to be attenuated in siRNA-mediated PI3K p110 catalytic subunit knockdown (Matheny and Adamo, 2009). In this work, phosphorylated p70S6K was
significantly increased following SCF treatment in INS-1 cells, further confirming the functional activation of mTOR (Figure 3.7B). It should be noted that P70S6K can also be regulated by phosphorylation of threonine residue 229 by PDK1, which also serves as a regulatory protein in PI3K signaling (Pullen et al., 1998). In future work, PDK1 activity should be analyzed to ensure changes in mTOR signaling are indeed modulated by c-Kit-mediated PI3K/Akt activation.

In addition to p70S6K, mTOR is also able to target the transcription factor NfκB, first identified as a nuclear factor that binds the κ light chain enhancer in B-cells, but thought to have a role in a variety of cellular functions including angiogenic processes and immune responses (Jung et al., 2003B; Shinojima et al., 2007). NfκB signal transduction is reliant on phosphorylation and degradation of the IκB protein by the IKK complex, consisting primarily of IKKα and IKKβ. Once the IKK complex phosphorylates the IκB protein, it is marked for proteasomal degradation, allowing for NfκB to migrate to the nucleus and bind to target sequences (Hayden and Gosh, 2004). It has been reported that Akt is able to activate NfκB transcriptional activity (Madrid et al., 2001; Sizemore et al., 2002) and direct its nuclear accumulation (Gustin et al., 2004). A study has demonstrated that insulin is important in mediating an interaction between mTOR and IKKα, which is essential for NfκB transcriptional modulation (Dan and Baldwin, 2008). NfκBp65 has been shown, in normoxic conditions, to respond to activation by the PI3K/Akt/mTOR axis and mediate HIF-1α transcription, thought to be a driving force in VEGF-A regulation (Jung et al., 2003a; Jung et al., 2003b; Bonello et al., 2007). It has been demonstrated that NfκBp65 is phosphorylated at serine residue 534, mediated by IκK kinase α and β, necessary for releasing NfκB as an active transcription factor, able to increase transcription of HIF-1α (Bonello et al., 2007; Bai et al., 2009). In this work, NfκBp65 phosphorylation in SCF-treated INS-1 cells was found to be increased and transcriptionally active, modulating HIF-1α expression and increasing VEGF-A production (Figure 3.7C).

Taken together, these results suggest that following SCF stimulation of c-Kit in INS-1 cells, the PI3K/Akt/mTOR axis is able to modulate a variety of downstream signaling
proteins as confirmed by an inhibitory study, in order to increase the production of angiogenic transcription factor HIF-1α and promote VEGF-A production and release.

4.4 Is the Akt/Gsk3β/β-catenin Signaling Pathway Involved in SCF-induced VEGF-A Production and Release in INS-1 Cells?

Our lab previously showed that the Akt/Gsk3β signaling pathway plays a key role in regulation of β-cell function and survival in both c-Kit mutation and over-expression mouse models (Feng et al., 2011; Feng et al., 2012), thus we have investigated whether this signaling pathway is also involved in SCF-mediated VEGF-A production in vitro. The serine/threonine kinase, glycogen synthase kinase 3 (Gsk3) consists of two highly homologous isoforms: Gsk3α and Gsk3β. These isoforms are similarly regulated and able to compensate for one another in some functions, sharing nearly identical sequences in their kinase domains (Shaw et al., 1997; Jope and Johnson, 2004; Thornton et al., 2008). However, studies have indicated that Gsk3β is a substrate that is inactivated by active Akt when phosphorylated at the N-terminal serine residue 9 (Shaw et al., 1997; Feng et al., 2012). Apart from active, unphosphorylated Gsk3β’s ability to regulate glycogen synthase, it has been implicated in a variety of cellular processes, mediating cell cycle, motility and apoptosis. Its dysregulation has been linked to several pathologies, including the onset of cancer and diabetes (Jope and Johnson, 2004). Studies on Gsk3β inhibition in isolated adult human and rat islets have indicated that Gsk3β pharmacological inactivation results in increased islet cell proliferation (Liu et al., 2009). In vivo, mice with a β-cell-specific Gsk3β overexpression displayed reduced β-cell mass and function, while mice with a heterozygous c-Kit point-mutation (c-Kit<sup>Wv/+</sup>) were treated with 1-AKP, a Gsk3β inhibitor, and were able to recover β-cell function, in terms of proliferative capacity and insulin secretion, as well as maintain normal glucose processing (Liu et al., 2008; Feng et al., 2012). Taken together, these studies indicate that Gsk3β inactivation plays a crucial role in β-cell function and survival, and is modulated by the PI3K/Akt pathway through c-Kit stimulation. We examined the level of Gsk3β phosphorylated at serine residue 9 in SCF-treated INS-1 cells and found that its phosphorylation was
significantly increased (Figure 3.8A), along with increased total β-catenin (Figure 3.8B), indicating that SCF/c-Kit-induced VEGF-A production is associated with Akt/Gsk3β phosphorylation.

It has been shown that modulation of the Gsk3β/β-catenin axis in a variety of cell types has resulted in the initiation of several angiogenic processes, including expression of VEGF-A/C, and increased phosphorylation of the VEGF receptor 2 (VEGFR-2) (Skurk et al., 2005; Leung et al., 2006). Furthermore, studies have demonstrated that Akt is able to directly phosphorylate β-catenin at serine residue 552, in vitro and in vivo, causing β-catenin to dissociate from cell-cell contacts, accumulating in both cytosol and the nucleus (Sharma et al., 2002; Fang et al., 2007). Phosphorylation of this site was not examined in this work, and would be beneficial to evaluate in future research.

4.5 Is the MAPK/Erk Signaling Pathway Involved in SCF-induced VEGF-A Production and Release in INS-1 Cells?

In addition to stimulation of the PI3K/Akt/mTOR axis by the mechanisms discussed previously, there are thought to be other converging pathways by which VEGF-A regulation is synergistically controlled from c-Kit activation. It has been reported that SCF stimulation in bone marrow stem cells and cardiac stem cells induced cell migration due to its ability to activate MAPKs, including ERK1/2, p38 MAPK, and JNK (Kuang et al., 2008). There are reports in human bone marrow mesenchymal stem cells and adipose progenitor cells are able to produce VEGF, HGF, and IGF-I by a p38 MAPK-dependent mechanism (Wang et al., 2006). Additionally, it has been established that p38 MAPK and Akt play a synergistic role in mTOR activation, necessary for VEGF-A modulation, in a variety of cell types, including the human osteosarcoma cell line SaOS, the human fibroblast HCA2 cell line and mouse embryonic fibroblast cell lines (Hernandez et al., 2011). Based on these reports, levels of p38 MAPK phosphorylation were examined, and found to have increased following SCF treatment in INS-1 cells (Figure 3.9B). However, the phosphor-Erk42/44 level in SCF-treated INS-1 cells showed no changes (Figure 3.9A), possibly due to inhibition by NfκB for which it may act as a compensatory mechanism (Frede et al., 2006). Additionally, some avenues of research have identified c-
Kit activation-dependent p42/44 MAPK signaling to peak shortly following c-Kit activation (<1 hour), and to attenuate over time in the absence of sustained c-Kit activation (Zheng et al., 2011). Moving forward, it would be interesting to examine its activity in comparison to the rest of the pathway at a much shorter time-point.

4.6 Does Down-regulation of c-Kit Expression Affect VEGF-A Production?

To further test whether down-regulation of c-Kit expression could influence VEGF-A production and release, a c-Kit siRNA study was performed in this study. In this work total c-Kit was found to be significantly reduced (Figure 3.12B) and activated c-Kit, phosphorylated at tyrosine residue 719 was significantly reduced (Figure 3.12C). RT-PCR analysis for RNA quality showed that there was a ~45% decrease in c-Kit RNA in c-Kit siRNA-treated groups, compared to control siRNA-treated groups, but did not reach statistical significance (Figure 3.12A). While this is indicative of inefficient transfection, likely confounded by growth factors found in the transfection medium and the use of 3 merged complexes of c-Kit siRNA that may have variable efficiency, it is still possible to see a significant effect on downstream pathways due to c-Kit activation’s profound effect on β-cell physiology, as demonstrated previously using c-Kit^{Wv/+} mice with a heterozygous point-mutation which knocked down ~50% of c-Kit function in these animals, but was still able to result in impaired β-cell growth and function even at 8 weeks of age (Krishnamurthy et al., 2007). As predicted, the minor reduction of c-Kit coincided with a small decrease in VEGF-A accumulation in culture medium (Figure 3.13C). Analysis of Hif-1α and Vegf-A transcript levels showed a trend toward decrease, but did not reach significance (Figure 3.13A). Taken together, these results suggest that there was transfection inefficiency, possibly due to the transient transfection technique utilized and, possibly, requirement of a longer time-point to see an effect on VEGF-A signaling. Similar to the SCF-treated conditions, it was found that siRNA-treated cells had slightly decreased levels of intracellular VEGF-A, however it did not reach statistical significance (Figure 3.13B), reflected in morphological analysis of VEGF-A and insulin double immunofluorescence staining (Figure 3.14BC). It has indeed been demonstrated
that, while VEGF-A is regulated by HIF-1α production, Von Hippel-Lindefau tumor suppressor (pVHL) is able to ubiquitinate and degrade HIF-1α by its E3 ubiquitin-protein ligase activity, an oxygen-dependent process (Manalo et al., 2005). Since c-Kit siRNA-treatment does not affect oxygen and, instead, suppresses HIF-1α production by c-Kit inactivation, it is possible that pVHL production is dysregulated and, thus, HIF-1α degradation is less profound under these conditions. It has also been shown that VHL-high hemangioblastomas are found to be positive for stem cell markers such as CD34 and CD133 (Chan et al., 2005). Since pVHL levels were not examined in this work, it would be beneficial to determine whether a reduction in c-Kit could affect pVHL production and be responsible for the minor change observed in regard to Hif-1α and Vegf-A transcript levels. It should be noted, however, that upon gross examination, both insulin and VEGF-A stain intensity seemed to be lower in the siRNA-treated group (Figure 3.14A), but this did not transfer to a significantly reduced level of VEGF-A protein. Levels of phosphorylated Akt, phosphorylated mTOR, phosphorylated p70S6K, and phosphorylated NfκB were all significantly reduced followed siRNA treatment (Figure 3.15). These changes were reflected further downstream with significant decreases in Gsk3β phosphorylation, and total β-catenin levels (Figure 3.16). Previous studies have noted decreases in many of these proteins in vivo, using the heterozygous c-Kit knockdown model (Krishnamurthy et al., 2007; Feng et al., 2012A). This further suggests that after extended siRNA knockdown time, it is possible that these changes would manifest as decreases in the final product, VEGF-A, or its key mediator, HIF-1α. p38 MAPK phosphorylation was not significantly different when compared to controls (Figure 3.16) suggesting again that the transfection time/efficiency was not sufficient to elicit an inhibitory response from c-Kit knockdown. It has been reported that p38 MAPK and p42/44 MAPK are involved in regulating intracellular calcium levels by PKC, which is largely related to secretion rate of angiogenic factors (Ito et al., 1998; Takahashi et al., 2001). Since p38 MAPK was reduced by ~54%, and p42/44 MAPK was not examined in the siRNA study, it’s possible that insufficiently reduced activation of these signaling molecules resulted in only minor changes to VEGF-A production and secretory capacity.
4.7 Limitations of the Study and Methodology

While this study highlights a correlation between c-Kit activity and modulation of the angiogenic factor VEGF-A, there are several drawbacks and limitations to this work. First, the INS-1 cell line used in this study is a good model for pancreatic β-cells, but are still physiologically different. This cell line is able to attain an 8-fold maximal insulin secretion response to glucose and has only marginal increases in cAMP levels in response to glucose (Yang et al., 2004). Furthermore, INS-1 cells have been shown to reach a plateau of insulin secretion once they reach a high level of confluence, suggesting that if cell density is affected by treatment, it may affect insulin secretion and cell health, confounding experimental results (Hectors et al., 2013). INS-1 cells, by their nature, lack endothelial cells to create a microenvironment, which results in a loss of feedback that β-cells would receive following changes in hormone secretion, especially since they lack receptors for VEGF-A, the hormone of interest (Inoue et al., 2002). In order to determine function of VEGF-A in vessel formation, it would be possible to co-culture the INS-1 cell line with an endothelial cell line, such as human microvascular endothelial cells (HMECs) or human umbilical cord endothelial cells (HUVECs), with or without exogenous SCF stimulation, and monitor their effect on endothelial cell organization, fenestration and caveolae formation, modulation of cell adhesion molecules, VEGF-A secretion changes, and antiangiogenic factor secretion (e.g. thrombospondin-1).

Secondly, while it was established that c-Kit and VEGF-A are both expressed in this cell population, an immunoprecipitation was not performed and thus, a direct link cannot be established between the two proteins, leaving room for the examination of other factors that may be responsible for the changes in VEGF-A levels observed. In particular, insulin-like growth factor 1 and 2 (IGF-1/2) signaling has been implicated in VEGF-A regulation and angiogenesis, specifically by HIF-1α induction, but were not examined in this work (Stoeltzing et al., 2003; Bjorndahl et al., 2005; Ma et al., 2006). Furthermore, insulin signaling has been shown to play a role in the modulation of VEGF-A in a variety of tissue types, and the insulin receptor is thought to crosstalk with c-Kit, thus, opening another potential avenue for research (Lu et al., 1999; Hale et al., 2013). It is of particular interest to examine the involvement of these signaling pathways to investigate possible cross-talk with c-Kit and more insight into how angiogenic signaling is controlled in β-
cells. Finally, the c-Kit siRNA transfection showed minimal knockdown of c-Kit, its downstream signaling molecules and VEGF-A, thus dosage and treatment time need to be reexamined to provide a more efficient knockdown and perhaps a more potent effect on VEGF-A production and release.

4.8 Conclusion and Future Direction

Understanding the mechanisms that regulate VEGF-A production and release are essential in reaching the goal of manipulating their activity to recover disrupted islet vasculature following trauma or transplant. This study has demonstrated that c-Kit activation, by SCF stimulation, is able to increase the proteins and targets of the PI3K/Akt/mTOR axis in order to have transcriptional effects on a functional model of β-cells in vitro. The results of this study suggest that siRNA-mediated knockdown of c-Kit is able to attenuate this effect to some extent, but further work with, perhaps, a modified transfection procedure would yield more conclusive results. More importantly, in order to confirm that SCF/c-Kit interaction plays an essential role in mediating VEGF-A production, combining siRNA-mediated c-Kit knockdown and SCF stimulation should be considered to determine whether knockdown following stimulation would attenuate the PI3K/Akt/mTOR-mediated VEGF-A production. Furthermore, this study was able to demonstrate that inhibition of c-Kit’s downstream targets was indeed able to attenuate VEGF-A production, but analysis of RNA and protein in these inhibition studies would be necessary to confirm that the pathway used to regulate VEGF-A expression is, in fact, PI3K/Akt/mTOR (Figure 4.1). Additionally, it should be noted that c-Kit is able to signal other pathways and, to fully understand its effect on VEGF-A modulation, changes in pathways such as Wnt signaling and MAPK pathways should be further examined. Wnt, specifically, is able to stimulate β-catenin nuclear translocation, and has been shown to play a role in blood cell formation in hematopoietic stem cell populations lacking c-Kit (Trowbridge et al., 2010). Thus, stimulation of cultured INS-1 cells by exogenous Wnt with and without c-Kit blockade would be an interesting avenue of research to pursue in the future. Finally, future work should take these concepts and apply them to primary islet cell culture studies and in vivo models in order to determine the effect of c-Kit activation and knockdown on a living system in regard to vascular changes, changes in
protein signaling, and possibly the effects of VEGF-mediated inflammatory responses. A thorough study of c-Kit-mediated VEGF modulation will allow researchers to work on developing cell-based therapies for revascularization of pancreatic islets.
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Appendices

Appendix I – Phase contrast Images Prior to and Following Inhibitor Treatment

Phase-contrast images demonstrating consistent cell confluence between controls and cells treated with (A) Lys294002 at varying concentrations with or without SCF, or (B) rapamycin at varying concentrations with or without SCF.
Appendix II – The Melting Curves and Derivative Melting Curves of qRT-PCR Products

(A) The melting curves and (B) derivative melting curves of *Hif-1α*, *VEGF-A*, and *18S* show that qRT-PCR assays have amplified the target sequences with high specificity.
Appendix III – Classification 2 Laboratory Permit Summary

### University of Western Ontario Permit Summary

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#### Organism
- Human (primary), fetal pancreas and duodenum, Rodent (primary), mouse pancreatic islets, human (established), PANC-1, HEK293, Rodent (established), INS-1, AR42J
- Organs and tissues (unpreserved), organs and tissues (preserved)

#### Gene Therapy

#### GMO

#### Animals
- B6 mice

#### Toxin
- Streptozotocin, Tamoxifen

#### Plant/Insect

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### Permit Summary

**Permit Holder**: Wang, Rennian  
**Permit #**: BIO-LHR-0046  
**Classification**: 2  
**Department**: Physiology  
**Phone**: 5196858500  
**Ext.**: 55098  
**Email**: nwang@uwo.ca  
**Approval Date**: Apr 25, 2014  
**Expiration Date**: Apr 24, 2017

### Permit Conditions

1. **INTERNAL PERMIT HOLDER RESPONSIBILITIES**
   - Comply with UWO BioSafety Safety Policies and Standard Operating Procedures. Ensure that the Health Canada Biosafety Guidelines, relevant regulations and safe laboratory practices are followed.
   - Receive adequate biosafety training from the institution. Permit Holders are responsible for the provision of specific training and instruction in biohazard agent handling that is necessary for the safe use of this material in their own laboratories. Supervisors must ensure that workers understand the health and safety hazards of the work or task (due diligence).
   - Ensure that the UWO Biosafety Manual is available to all lab personnel under the permit.
   - Report incidents of loss or theft of any biohazardous material immediately to the Biosafety Coordinator.

2. **WORKER RESPONSIBILITIES**
   - Be familiar with the UWO BioSafety Manual, attend all required safety training sessions and obey all safety regulations required by the UWO Biosafety Committee.
   - Report to the Permit Holder any incident involving known or suspected exposure, personal contamination or a spill involving a biohazardous agent.

I accept the above responsibilities as a Internal Permit Holder and I am accountable for following UWO BioSafety Guidelines and Procedures Manual for Containment Level 1 and 2 Laboratories.

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<tr>
<th>Permit Holder Name</th>
<th>Signed</th>
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April-28-14
Appendix IV – Biosafety Approval

April 28, 2014

Dear Dr. Wang:

Please note your biosafety approval number listed above. This number is very useful to you as a researcher working with biohazards. It is a requirement for your research grants, purchasing of biohazardous materials and Level 2 inspections.

Research Grants:
- This number is required information for any research grants involving biohazards. Please provide this number to Research Services when requested.

Purchasing Materials:
- This number must be included on purchase orders for Level 1 or Level 2 biohazards. When you order biohazardous material, use the on-line purchase ordering system (www.uwo.ca/finance/people/). In the “Comments to Purchasing” tab, include your name as the Researcher and your biosafety approval number.

Annual Inspections:
- If you have a Level 2 laboratory on campus, you are inspected every year. This is your permit number to allow you to work with Level 2 biohazards.

To maintain your Biosafety Approval, you need to:
- Ensure that you update your Biohazardous Agents Registry Form at least every three years, or when there are changes to the biohazards you are working with.
- Ensure that the people working in your laboratory are trained in Biosafety.
- Ensure that your laboratory follows the University of Western Ontario Biosafety Guidelines and Procedures Manual for Containment Level 1 & 2 Laboratories.
- For more information, please see: www.uwo.ca/hr/safety/biosafety/.

Please let me know if you have questions or comments.

Regards,

Tony Hammoud
Biosecurity Coordinator for Western
Support Services Building 4190
Phone: 519-661-2111 X88730
Fax: 519-661-3420
Curriculum Vitae

ALEXEI Popell

EDUCATION

**MASTER OF SCIENCE**  
Physiology and Pharmacology – Western University  
London, ON  
2012-Present

**BACHELOR OF MEDICAL SCIENCE**  
Medical Science – University of Western Ontario  
London, ON  
2007-2011

HONOURS AND AWARDS

Western Graduate Research Scholarship  
Faculty of Graduate Studies  
Western University  
September 2012-Present

PRESENTATIONS AND POSTERS

**Popell A**, Feng ZC, Li J, Wang R. “SCF/c-Kit interaction regulates VEGF-A production via concurrent Akt/mTOR and p38 MAPK pathway in β-cells”

Accepted for presentation at:

- London Health Research Day  
  March 2014
- Physiology and Pharmacology Research Day  
  November 2013
- Diabetes Research Day  
  November 2013