

THE ROLE OF VASCULAR ENDOTHELIAL GROWTH FACTOR-A (VEGF-A) IN
PANCREATIC ISLET FUNCTION IN ADULTS

By

Jeannelle Alexandra Kantz

Thesis

Submitted to the Faculty of the
Graduate School of Vanderbilt University
in partial fulfillment of the requirements
for the degree of

MASTER OF SCIENCE

in

Molecular Physiology and Biophysics

December, 2009

Nashville, Tennessee

Approved:

Owen P. McGuinness

Maureen Gannon

To my amazing family, friends, and my beloved fiancé David, all of whom have been infinitely supportive

And

A special thanks to my mentors Al and Marcela, for all your technical and professional coaching

ACKNOWLEDGEMENTS

The RIP-Cre^{ER} mice were graciously provided by Dr. Doug Melton at Harvard University; the Pdx1^{PB}-Cre^{ER} were graciously provided by Drs. Chris Wright and Maureen Gannon. These studies was supported by grants to Dr. Alvin Powers from the Juvenile Diabetes Research Foundation International, the VA Research Service, the National Institutes of Health (DK68764, DK66636), and the Vanderbilt Diabetes Research and Training Center (DK20593). Part of Jeannelle Kantz's graduate training was supported by the Molecular Endocrinology Training Grant from the NIH (T32DK007563).

TABLE OF CONTENTS

DEDICATION	ii
ACKNOWLEDGMENTS	iii
LIST OF FIGURES	vi
LIST OF ABBREVIATIONS	vii
Chapter	
I. INTRODUCTION	1
Current Therapies for Type 1 Diabetes	3
Challenges for Islet Transplantation	4
Pancreatic Islet Vascularization	5
Angiogenesis and Vasculogenesis	6
Role of VEGF-A in Angiogenesis and Vasculogenesis	7
Role of VEGF-A in Islet Vascularization	8
Limitations in Understanding of Islet Vascularization	9
Rationale for These Studies	11
II. CHARACTERIZATION OF INDUCIBLE CRE SYSTEM WITH R26R AND Z/AP REPORTER STRAINS.....	12
Introduction	13
Methods and Materials.....	15
Results	18
Discussion	21
III. INACTIVATION OF VEGF-A IN ADULT ISLETS.....	24
Methods and Materials.....	25
Results	28
Discussion	30
SUMMARY	32

FUTURE STUDIES	35
REFERENCES	36

LIST OF FIGURES

Figure 1. Procedure for Human Islet Transplantation	41
Figure 2. Expression of Angiogenic Factors and Receptors	42
Figure 3. Cre ^{ER} LoxP System.....	43
Figure 4. Reporter Mice Constructs	44
Figure 5. Characterization of Recombination in RIP-Cre ^{ER} ;R26R mice	45
Figure 6. Analysis of RIP-Cre ^{ER} ;Z/AP mice.....	46
Figure 7. Analysis of Recombination in Pdx1 ^{PB} -Cre ^{ER} ;R26R	47
Figure 8. Comparison of Cre ^{ER} production by RIP-Cre ^{ER} and Pdx1 ^{PB} -Cre ^{ER}	48
Figure 9. VEGF Expression Pre- and Post-Tamoxifen	49
Figure 10. Measurements of Vascular Density and Vascular Mass.....	50
Figure 11. Changes in Glucose Tolerance Post Tamoxifen Treatment	51
Figure 12. Summary of Glucose Tolerance Tests in Pdx1 ^{PB} -Cre ^{ER} VEGF ^{fl/fl}	52

ABBREVIATIONS

(RIP)	Rat insulin promoter
(<i>pdx1</i>)	Pancreatic-duodenal homeobox factor-1
(Pdx1 ^{PB})	Fragment of <i>pdx1</i> promoter
(PBS)	Phosphate-buffered saline
(FBS)	Fetal bovine serum
(VEGF-A)	Vascular endothelial growth factor-A
(R26R)	Rosa26R mice
(ER)	Estrogen receptor
(tm)	Tamoxifen
(Cre)	Cre recombinase
(fl)	Floxed
(mo)	Month
(E)	Embryonic day
(VEGFR)	Vascular endothelial growth factor receptor
(TBE)	Tris-borate-ethylenediaminetetraacetic acid
(RIA)	Radioimmunoassay
(ELISA)	Enzyme-linked immunosorbent assay
(hsp90)	Heat shock protein 90

CHAPTER I

INTRODUCTION

The mammalian pancreas has three main cell types: exocrine acini, pancreatic ducts, and endocrine islets. Pancreatic β -cells comprise approximately 80% of the pancreatic islet in rodents. The other cell types in the islet are glucagon-producing alpha cells, somatostatin-producing delta cells, pancreatic polypeptide-producing PP cells, and ghrelin-producing epsilon cells (Prado et al, 2004). Although the islets only represent a small portion of the volume of the pancreas, they are highly vascularized and receive a large portion (6-20%) of the total pancreatic blood flow (Lifson, N, 1980 and Lifson, N, 1985). The molecular mechanisms responsible for the vascularization of islets is the focus of this document and this will be discussed after a review of diabetes and glucose homeostasis.

Diabetes Mellitus and Impact on Health

Diabetes mellitus is a complex disease; it is a group of metabolic disorders wherein the cells of the body do not properly uptake/absorb and metabolize carbohydrates, fats, and amino acids (Thai,1993). High blood glucose levels (hyperglycemia) is a diagnostic characteristic of diabetes mellitus, and eventually can cause damage to the retina, kidney, nerves, heart, and blood vessels. There are four general categories of diabetes mellitus: type 1, type 2, "other specific types", and gestational diabetes (ADA, 2009).

In type 1 diabetes the dysregulation of glucose homeostasis results from an autoimmune attack against the insulin-secreting pancreatic β -cells (ADA, 2009; Mathis, 2001), usually leading to absolute insulin deficiency. The majority of the persons with

type 1 diabetes mellitus develop the disease before the age of twenty-five, with an equal incidence in both males and females.

Type 2 diabetes mellitus is characterized by insulin resistance in peripheral tissues and an insulin secretory defect of the β -cells (Costa, 2002). This is the most common form of diabetes mellitus (90%) and is highly associated with genetics, obesity, inactivity, and older age. In type 2 diabetes, insulin resistance and hyperinsulinemia eventually lead to impaired glucose tolerance. Current thinking postulates that defective, “over-worked” β -cells eventually become exhausted, which further impairs insulin secretion, fuels the glucose intolerance, and leads to hyperglycemia (Costa, 2002). The etiology of type 2 diabetes is multi-factorial and probably genetically based, but it also has strong behavioral, nutritional, and environmental components.

Other types of diabetes mellitus are less common and are grouped together in a class called “other specific types” (ADA, 2009). This group includes persons with genetic defects of β -cell function (formerly called MODY or maturity-onset diabetes in youth) or with defects of insulin action (ADA, 2009). Persons with diseases of the exocrine pancreas, such as pancreatitis or cystic fibrosis, and persons with pancreatic dysfunction caused by drugs, chemicals or infections are also included in this category of diabetes (ADA, 2009).

Gestational diabetes mellitus refers to women who develop diabetes mellitus during pregnancy. Most women with gestational diabetes have normal glucose homeostasis during the first half of the pregnancy but have relative insulin deficiency in response to the insulin resistance of pregnancy and this leads to hyperglycemia during the last half of the pregnancy. The hyperglycemia resolves in most women after delivery, but places them at increased risk of developing type 2 diabetes later in life (Daneman, 2006).

The significant morbidity and mortality of both type 1 and type 2 diabetes mellitus results predominantly from its complications, retinopathy, neuropathy, and nephropathy. Diabetes is a leading cause of blindness, renal failure, amputations, strokes, and cardiovascular events (Devendra, 2004; Mathis, 2001). Hyperglycemia and dyslipidemia are metabolic abnormalities of diabetes and lead to widespread cellular damage (Devendra, D. 2004).

Current Therapies for Type 1 Diabetes

Current management of type 1 diabetes involves daily blood sugar testing, insulin replacement, and careful meal planning. Insulin replacement is by either subcutaneous injection or subcutaneous infusion of insulin by an insulin pump. There have been significant advances in the options/approaches available to assist patients in controlling and monitoring their blood glucose levels. The current options include a repertoire of 'designer' insulins with varying rates of pharmacokinetics and programmable insulin pumps with a selection of delivery patterns and insulin-dosing algorithms (Powers, 2008). However, all current insulin replacement methods are imperfect and many patients have treatment-related hypoglycemia and develop diabetes-related complications in the long term. Precisely matching insulin need and insulin delivery is one of the major limitations with current therapies. Another therapeutic option is insulin replacement by transplantation of insulin-producing cells (cell-replacement therapy) (Robertson. 2004). Ideally, this therapy would mimic the biologically based glucose-sensing mechanism tightly coupled to insulin exocytosis, like that in the β -cell. One such approach is islet transplantation (Fig.1), and this is a potentially effective therapy for insulin-deficient diabetes (Shapiro, 2000; Ryan, 2001; Robertson. 2004) (reviewed in Fig.1). In this procedure, islets are isolated from the pancreas of a cadaver donor by intraductal delivery of the enzyme collagenase to the pancreas (Lakey, 1999). The collagenase

solution is slowly warmed to 35°C, transferred to a dissociation chamber and mechanically dissociated. The selective islet cleavage is achieved by the use of highly purified collagenase/thermolysin blend such as Liberase HI (Roche, Indianapolis, IN); the islets are separated by gentle enzymatic and mechanical dissociation. Ficoll gradients are used to purify the islets from the crude cellular sample (Sutton, 1984; reviewed by Fontaine, 2003). For the transplant procedure, the surgeon uses ultrasound to guide placement of a small plastic catheter through the upper abdomen and into the portal vein (Rossini, 1999). The islets are then injected through the catheter into the liver. Unfortunately, though the initial reports for islet transplants were promising, the long-term results have been disappointing since most of the patients must resume insulin injections within 3-5 years (Robertson, 2004, NEJM).

Challenges for Islet Transplantation

Several substantial challenges must be overcome before islet transplantation can be considered for widespread application. One issue is the limited supply of suitable islets for transplant; there is an insufficient number of pancreatic donors compared with the number of type 1 diabetics (Robertson, 2004; Lakey et al, 2003; Ricordi and Strom, 2004). Another issue is that patients need to be on potent immunosuppressive medications to prevent rejection of the transplanted islets. These medications weaken the immune system and increase the risk of infections.

Another major challenge for islet transplantation is that the current duration of the islet allograft function is inadequate. Several factors may impact the viability of transplanted islets and the success of islet transplantations, these include: (a) isolation procedure for the islets (Lakey JTR, 1999), (b) islet culture conditions (Gaber, 2001; Fraga, 1998), and (c) the islet vascular supply during culture and after transplantation (Menger, 1998; Parr, 1980).

One major issue affecting the function and survival of the islet allograft is that revascularization of islets after transplantation is required for successful engraftment (Carlsson, 2002; Davalli, 1996; Jansson, 1994). However, the islet isolation process disrupts the vasculature of islets, resulting in a lack of blood supply to the islet graft in the first few days following the transplantation thus leading to hypoxia and death of islet cells (Mattsson, 2002; Davalii, 1996; Vajkoczy, 1995). Thus, immediately after transplantation, the pancreatic islets are supplied with oxygen and nutrients solely by diffusion from surrounding tissues. Therefore, the speed and the degree of revascularization of transplanted islets may be a major determinant of the survival and function of transplanted islets. Although the revascularization process is initiated immediately, it is generally thought to require 2-3 weeks post-transplantation before it is complete (Brissova, 2004; Mattsson, 2002; Menger, 1989; Sandberg, 1995). Even after revascularization is complete, oxygen tension and blood flow are reduced in islet grafts, independent of the site of transplantation (Carlsson, 2001). The endothelium lining the newly formed blood vessels within islet grafts has been shown to be of both host and donor origin (Brissova, 2004; Schneider, 2000; Vajkoczy, 1995). This revascularization process is thought to be mediated by angiogenesis, and is most likely mediated by intraislet and/or localized release of angiogenic factors.

Pancreatic Islet Vascularization

Pancreatic islets are highly vascularized mini-organs. In addition to islets being more densely vascularized than the surrounding acinar tissue, the morphology of islet vasculature is distinctly different from that of the surrounding exocrine pancreas. Islet vessels are thicker and more torturous than the vessels of the acinar tissue (Brissova, 2006; Brunicardi, 1996). Additionally, the endothelial cells of islet vessels are fenestrated (Brissova, 2006, Brunicardi, 1996). Vascular fenestrations, an important

structural component for vascular permeability, are specialized plasma membrane microdomains in endothelial cells that appear as circular discontinuities of ~60 nm in diameter (Palade, 1979; Esser , 1998; Roberts, 1995; Brissova, 2006). Islets also have higher pO₂ (31-37 mmHg) than the exocrine pancreas (20-23 mmHg) (Carlsson, 1998; Carlsson, 2000, Carlsson, 2001).

Increasing evidence supports the concept that the intra-islet vascular network is important for islet health and islet function. A better understanding of molecular mechanisms underlying the development and maintenance of islet vasculature are of particular interest because of the benefits it could have on islet transplantation.

Angiogenesis and Vasculogenesis

Two different processes are involved in embryonic and extraembryonic blood vessel formation: 1) vasculogenesis, the de novo differentiation of endothelial cells from mesoderm and the organization of endothelial progenitors into a primitive vascular plexus; and 2) angiogenesis, the budding and branching of vessels from pre-existing vascular beds (Breier, 2000; Risau, 1995; Risau, 1997). Both these processes occur during organ development and differentiation in the embryo (Breier, 2000); angiogenesis is the primary mechanism by which new vessels form in the adult organism. In islets, angiogenesis is critical for normal islet formation during embryonic and early postnatal development (Ballian, 2007, Johansson, 2006, Brissova, 2006). In the adult, angiogenesis is also required for tumor growth, wound healing, reproductive functions, and neoplasia (Johansson, M 2006, reviewed in Veikkola, 2000 and Ferrara, 2004; Folkman J, 1995). Revascularization of the islet graft tissue into the donor tissue is thought to be mediated by angiogenesis (Jones, 2001).

Role of VEGF-A in Angiogenesis and Vasculogenesis

Vascular endothelial growth factor-A (VEGF-A), is a critical angiogenic factor in vascular development, ischemia-induced angiogenesis, and tumor-related angiogenesis (reviewed by Ferrara, 2003; Folkman, 1987). VEGF-A is the founder member of a family of growth factors, including VEGF-A, B, C, D, E, and placental growth factor (reviewed by Ferrara, 2003). VEGF-A induces endothelial proliferation, promotes cellular migration, and inhibits apoptosis (reviewed by Ferrara, 2003). While the other members of the family, VEGF-B, VEGF-C, and VEGF-D, have roles in selective areas, VEGF-A is thought to be the predominant member of the VEGF family (Ferrara, 2003), and is the subject of my thesis. It is known that VEGF-A is crucial for embryonic development, as targeted inactivation of even a single VEGF-A allele results in embryonic lethality (Ferrara, 1996; Carmeliet, 1996). Studies have shown that VEGF-A is critical during early stages of vascular development, and is required for survival in early postnatal life when the endothelium is still proliferating (Gerber, 1999). VEGF-A has also been shown to exert a pivotal role in angiogenesis under physiological and pathological conditions (Ferrara, 2003; Shibuya, 2001). Additionally, VEGF-A has been widely studied in vascular disease, ischemic injuries, and in other transplanted areas (Byun 2001, Kim 2004, Lee 2004), and we postulate that it plays an important role in the revascularization of islets after transplantation.

The VEGF proteins mediate angiogenic signals to the vascular endothelium by binding to high affinity receptor tyrosine kinases (RTKs), termed VEGFR-1 (flt-1), VEGFR-2 (flk-1), and VEGFR-3 (FLT-4) (Neufeld, 1999; Ferrara, 2003). All three receptors are expressed on endothelial cells and a limited subset of other cell types such as neurons (Neufeld, 1999; Lee, 1996; Ferrara, 2003). In adults, VEGFR-1 and VEGFR-2 are expressed primarily in the blood vessel endothelium, whereas VEGFR-3 is restricted largely to the lymphatic endothelium (Neufeld, 1999, Ferrara, 2003). The

VEGFR-2 is thought to be the receptor that largely mediates the actions of VEGF-A as an endothelial cell mitogen and vascular permeability factor (Shibuya, M 2001).

Targeted inactivation of either VEGF-A or VEGFR-2 interferes with angioblast differentiation and consequently with vasculogenesis (Shalaby, 1995; Carmeliet, 1996; Ferrara, 1997). The effects on vasculogenesis, large vessel formation, and capillary sprouting in both the VEGF-A knockout and VEGFR-2 knockout embryos are very similar (Carmeliet, 1996). In both cases, embryos die between embryonic (E) day E8.5 and E10 (Shalaby, 1995; Carmeliet, 1996). The mouse knockout of VEGFR-1 also dies around E9 and displays unique vascular complications. In contrast to VEGF-A and VEGFR-2 knockouts, mice lacking the VEGFR-1 gene display an increased number of endothelial progenitors and vascular disorganization (Fong, 1999, Fong 1995).

Role of VEGF-A in Islet Vascularization

Pancreatic islets express high levels of VEGF mRNA and proteins (Fig. 2), and the capillary network is about five times more dense than the capillary network of the surrounding exocrine cells (Brissova, 2006, Lammert, 2001, Christofori, 1995; Gannon, 2002). Additionally, VEGFR-2 is not expressed on pancreatic β -cells, but is expressed in the microvasculature of islets, exocrine tissues, and in the periductal capillary plexus (Brissova, 2006, Lammert, 2001). Data from our lab has also shown that the level of VEGFR-2 is greater in islet vessels than in exocrine capillaries (Fig 2). The localization and levels of VEGF-A in pancreatic islets suggests a paracrine mechanism of action by which VEGF-A secreted by islet cells modulates angiogenesis and vascular permeability in the capillary network of the islet-exocrine portal system (Fig 2).

Inactivation of a floxed VEGF-A gene in mouse islets with different promoters driving Cre has resulted in the discovery that paracrine signaling from endocrine cells to the adjacent endothelial cells induces the formation of a dense network of fenestrated

capillaries in islets (Lammert, Cell 2003; Brissova, 2006). Lammert and colleagues used the *pdx1* (Pancreas-duodenum homeobox gene 1) promoter to delete VEGF-A in the mouse islets. Studies in our lab inactivated VEGF-A in β -cells using RIP to drive Cre expression (Brissova, 2006). Both studies showed that deletion of VEGF-A did not prevent or grossly disrupt endocrine pancreatic development. Adult mice null for VEGF-A in β -cells had normal pancreatic weight, insulin content, islet morphology, and islet architecture (Brissova, 2006). In the absence of VEGF-A, the development of islet capillaries was severely reduced (Brissova, 2006, Lammert 2003). The few remaining capillaries found in the VEGF-A-deficient islets were poorly fenestrated and contain an unusual number of caveolae (Brissova, 2006, Lammert 2003). Additionally, VEGF-A^{-/-} islets appeared to develop adjacent to pancreatic blood vessels (Lammert, 2003). These studies also found that islet mass was not changed in the pancreas of -/- mice (Brissova, 2006), and that islets could reach a normal size, although the number of small islets appeared to be increased (Lammert, 2003).

Interestingly, RIP-Cre; VEGF^{fl/fl} mice cleared glucose following intraperitoneal glucose at a slower rate than wild type mice (Brissova, 2006). Plasma insulin levels, normalized for blood glucose after glucose administration, were also significantly lower in RIP-Cre; VEGF^{fl/fl} mice as compared to wild type controls. These results indicated that VEGF-A is important for the normal development of islet vasculature, and that islet function is affected by the abnormalities in islet microvasculature due to reduced VEGF-A expression in β -cells.

Limitations in Understanding of Islet Vascularization

While much information has been gained from the RIP-Cre; VEGF^{fl/fl} and Pdx1-Cre; VEGF^{fl/fl} models, these models have limitations. RIP-Cre is a well-characterized

transgenic mouse strain (Postic, 1999) in which Cre recombinase is under transcriptional control of the rat insulin promoter (RIP-Cre). In both the RIP-Cre; VEGF^{fl/fl} and Pdx1-Cre; VEGF^{fl/fl} mice, VEGF-A expression is reduced during embryogenesis (around E9.5-E13.5). Therefore, one was unable to discern the role of VEGF-A specifically in adult islet function and intra-islet vasculature maintenance. Additionally, by reducing VEGF-A expression during islet development, there may be compensatory changes in other angiogenic factors in the islet, such as other members of the VEGF family or Ang-1. A better understanding of the role of VEGF-A in the adult pancreas and mature islets would allow therapeutic manipulation of VEGF-A during islet isolation and perhaps post-islet transplantation.

Rationale for These Studies

I hypothesize VEGF-A plays an important role in adult islets and is involved in maintaining the health and morphology of intra-islet vasculature. I predict that VEGF-A plays a key role in the maintenance of a fenestrated endothelial cell (EC) phenotype in adult islets, and that the preservation of the EC phenotype is needed for normal islet function. Therefore to address the role that VEGF-A plays in adult islets, we conditionally and temporally inactivated VEGF-A expression in adult pancreatic islets. To accomplish this, we used an inducible version of the Cre/LoxP system, the CreERTM recombinase (Hayashi, 2002). With this model, VEGF-A levels are unaltered during embryogenesis, so islets and islet vasculature develop normally. This allowed us to examine the role of VEGF-A in adult islets.

This document presents experimental data using two individual experimental approaches. The first describes the characterization of two islet-cell-specific CreTM lines (RIP-Cre^{ER} and Pdx1^{PB}-Cre^{ER}), and demonstrates the advantages and disadvantages of each strain. In the second, we present results from studies on the effect(s) of down-regulating VEGF-A in adult islets on islet function and islet vasculature.

CHAPTER II

CHARACTERIZATION OF INDUCIBLE CRE SYSTEM WITH R26R AND Z/AP REPORTER STRAINS

Synopsis

For the purpose of reducing VEGF-A expression in islets, we used islet-specific Cre^{ER} transgenic lines: RIP-Cre^{ER} and Pdx1^{PB}-Cre^{ER}. The RIP fragment directs expression primarily in β -cells of the islet (Hanahan, D 1985). Pdx1^{PB} is a 1-kb fragment of the pancreatic duodenum homobox-1 promoter that is transcriptionally active in islet cells, but not in pancreatic exocrine cells, the stomach, or duodenum (Wu, 1997; Gannon, 2001). The Pdx1^{PB}-Cre^{ER} transgene is expressed in all the endocrine cell types of the islet (Zhang, 2005).

Both transgenic strains were bred with Rosa26R (R26R) reporter mice (Soriano, 1999) in order to determine the optimal method to administer tamoxifen and to test the recombination efficiency of the system. By immunohistochemistry, tamoxifen-induced recombination in both the RIP-Cre^{ER} and the Pdx1^{PB}-Cre^{ER} systems was observed. Unexpectedly, in the RIP-Cre^{ER} bigenic mice, basal recombination was noted in the absence of tamoxifen administration. We also studied Z/AP reporter mice using RIP-Cre^{ER}. We found that Cre-mediated recombination was partially dependent on the reporter gene used.

Introduction

The Cre/loxP system is a tool that allows for tissue-specific inactivation of genes. This is especially useful for genes like VEGF-A where inactivation cannot be investigated in differentiated tissues because of embryonic lethality in mice with a conventional knock-out. Recent advances in technology have modified this system so that it also allows for temporal inactivation of such genes (Sauer, 1998; Utomo, 1999). The CreERTM (Cre^{ER}) recombinase is a fusion protein comprised of the catalytic domain of the Cre recombinase and the ligand-binding domain of a modified/mutated estrogen receptor (Hayashi, 2002). This modification prevents the binding of endogenous estrogen at normal physiological concentrations, but renders the ER ligand-binding domain responsive to tamoxifen (Metzger, 1995). The CreERTM protein is sequestered in the cytoplasm of the cell by heat shock protein 90 (Hsp90) (Metzger, 1995; Bockcamp, 2002). Heat shock proteins are expressed by cells under conditions of stress, but they also function under normal physiological conditions (Hendrick and Hartl, 1993). Hsp90, named for the approximate molecular mass of the protein, is a molecular chaperone which functions include assisting in cell signaling, protein folding, and controlled switching of proteins between active and inactive conformational states (Hendrick and Hartl, 1993). Binding of tamoxifen disrupts this interaction with Hsp90, therefore allowing Cre^{ER} to translocate to the nucleus and initiate loxP-mediated recombination (Fig. 3).

R26R is a line of mice in which the β -gal reporter has been inserted into the Rosa26 locus and LacZ expression occurs only following the removal of an intervening stop sequence/segment by Cre recombinase (Soriano, 1999) (Fig.4). This strain is commonly used for the monitoring the efficiency and pattern of Cre-mediated recombination.

Another reporter strain used to characterize Cre activity is Z/AP mice (Fig.4). This transgenic strain constitutively and ubiquitously expresses LacZ under the control of the CMV enhancer/chicken actin promoter (Lobe, 1999). Expression is widespread, with a few exceptions (erythrocytes, chondrocytes, and adipocytes), and can be observed throughout all embryonic and adult stages. When crossed with a Cre recombinase-expressing strain, LacZ expression is replaced with human placental alkaline phosphatase (hPLAP) expression in tissues expressing Cre (Lobe, 1999). Recent lineage tracing studies crossed the RIP-Cre^{ER} line with Z/AP mice (Dor, 2004). They reported that there was no Cre activity until the administration of the ligand, tamoxifen (Dor, 2004).

Methods and Materials

Mouse models: Mice heterozygous for the tissue specific inducible transgenic (RIP-Cre^{ER} or Pdx1^{PB}-Cre^{ER}) (Dor, 2004 and Zhang, 2005) were bred with R26R homozygotic mice (Soriano, 1999) or Z/AP mice (Lobe 1999). Mice heterozygous for Cre^{ER} and R26R or Z/AP represent experimental mice, while mice with only R26R or Z/AP serve as wild type controls. Genotyping of mice was performed by PCR and Southern blot (Brissova, 2006). For PCR, mouse tail DNA was amplified in a thermal cycler using the following primers, 5'-CCA GGT TAC GGA TAT AGT TCA TG-3', and 5'-TGC CAC GAC CAA GTG ACA GC -3' (from Integrated DNA Technologies, Inc). The size of the amplified product is approximately 213 bp. For Southern blot analysis, tail DNA was digested using EcoRI, then run on a TBE agarose gel overnight. Gels were then transferred onto a nitrocellulose membrane overnight. Membranes were treated with 2% something SOC for 10 minutes. The membrane was then dried in a vacuum oven for 1 hour at 65°C. The membrane was then washed with warm Erase Buffer for 15 minutes. After which, the membranes were pre-hybridized between 4 hours to overnight at 65°C. Membranes were then probed in hybridization buffer containing Cre cDNA labeled with ³²P, and then exposed for autoradiography.

Methods of Tamoxifen Injections: Tamoxifen is a selective estrogen receptor modulator. It is part of a drug class termed anti-estrogens because it binds to the estrogen receptor and inhibits the binding of endogenous estrogen. This property has made tamoxifen a widely used treatment for estrogen-sensitive breast cancer. In humans, tamoxifen is typically taken orally in the form of a pill. Due to its chemical structure, tamoxifen has a low solubility in water. Therefore, for the purpose of these

studies, tamoxifen was dissolved in either an aqueous solution containing ethanol or in corn oil. For each route of tamoxifen administration, mice were given three doses of 8mg of tamoxifen over the course of 5 days (every 48 hours). Several methods of administering tamoxifen have been reported in the literature (Dor, 2004). To determine which procedure was optimal for our studies, we compared the following three approaches:

- (1) Orally: tamoxifen (Sigma) was suspended in aqueous solution of 3% ethanol and 0.5% methylcellulose at a concentration of 20mg/mL, and administered via oral gavage.
- (2) Intraperitoneally (IP): tamoxifen was dissolved in corn oil (Sigma) overnight at a concentration of 20mg/mL and stored at 4°C. Intraperitoneal injections of 8mg were administered via sterile syringe into the visceral cavity.
- (3) Subcutaneously (SQ): tamoxifen was dissolved in corn oil overnight at a concentration of 20mg/mL and stored at 4°C. Subcutaneous injections of 8mg were administered to the scapular region of each mouse.

Immunocytochemistry and X-Gal Staining: Pancreatic tissue was rinsed in ice-cold phosphate-buffered saline (PBS) and then fixed in 4.0% paraformaldehyde/0.1M sodium phosphate buffer for 1.5 h on ice. After washing with PBS several times, pancreata were incubated with 30% sucrose and embedded in OCT compound (Tissue-Tek). After fixation, 10 µm cryosections were prepared and assessed by either X-gal staining or immunostaining. For immunohistochemistry, sections were fixed by immersion in 1% paraformaldehyde, washed with PBS three times, and then blocked in PBS with 5% normal donkey serum as previously described (Brissova, 2006). The primary and secondary antibodies were diluted in PBS containing 1% bovine serum albumin and

0.1% Triton-X-100. The studies used the following: Guinea pig anti-human insulin IgG (1:1,000) from Linco Research, rabbit anti- β -galactosidase IgG (1:5,000) from ICN Pharmaceuticals, rabbit anti-Cre (1:5000) from Novagen. β -galactosidase activity was also detected by postfixing with 0.2% glutaraldehyde/1%paraformaldehyde for 15 minutes at room temperature. The sections were then washed three times for 5 minutes with PBS, and permeabilized for 10 minutes at room temperature. Sections were incubated with X-gal staining solution in a humidified chamber at 37°C, rinsed three times with PBS, and mounted with AquaPoly/Mount (Polysciences, Warrington, PA). Cells expressing hPLAP+ were identified by incubating slides with alkaline phosphatase substrate as described (Gu, 2002).

Results

Subcutaneous administration of tamoxifen results in optimal recombination with less morbidity.

Seven days after the last administration of tamoxifen (oral, IP, SQ), the pancreas of RIP-Cre^{ER};R26R mice was removed, fixed, and stained. By immunohistochemistry and X-gal staining, we found that oral administration of tamoxifen induced recombination in less than 10% of islet cells in bigenic mice (Cre transgene + R26R reporter insertion; data not shown). Both intraperitoneal and subcutaneous injections of tamoxifen resulted in 75%-90% recombination in the islet cells of bigenic mice. However, mice receiving intraperitoneal injections of tamoxifen or corn oil (n=4), lost weight and overall displayed diminished health, and in some cases died. Three subcutaneous doses of 8mg tamoxifen induced sufficient recombination without affecting the health of the animal, and therefore was the dose and route of administration used for subsequent studies.

Cre-Mediated Recombination Induced by Tamoxifen in RIP-Cre^{ER};R26R Mice

The characterization studies of Cre-mediated recombination were performed on 16 week RIP-Cre^{ER};R26R mice and R26R control mice. To evaluate the efficiency of recombination in these mice, mice were injected with three doses of 8mg of tamoxifen and the pancreata was dissected one week after the last injection of tamoxifen. As a control for any indirect effects of corn oil, a group of bigenic mice were injected three times with an equivalent volume of autoclaved corn oil. Sections of the pancreas of RIP-Cre^{ER}; R26R mice were stained with antibodies against β -gal and Cre (Fig 5). Costaining of sections with anti-insulin provided assessment of the β -cell population. Immunofluorescent imaging and MetaMorph analysis of Cre staining in bigenic mice demonstrated ~90% of β -cells per islet were positive for Cre (Fig. 5). Non- β -cells of the

islet (those not expressing insulin) were Cre negative. No β -gal or X-gal staining was detected in mice lacking the Cre transgene (data not shown). At this dosage of tamoxifen, recombination was detected in 75-80% of the β -cells per islet. However, to our surprise, a significant amount of recombination was observed in bigenic mice not treated with tamoxifen (Fig. 5). Recombination in bigenic islets independent of tamoxifen was between 5-20% of β -cells per islet. These results were unanticipated because previously published studies reported no recombination in RIP-Cre^{ER} mice not treated with tamoxifen (Dor, 2004).

A difference with our studies is that Dor & colleagues used the Z/AP reporter strain for genetic lineage tracing to investigate β -cell renewal and regeneration. To investigate these differences, RIP-Cre^{ER} mice were crossed with Z/AP reporter mice. RIP-Cre^{ER}; Z/AP mice and Z/AP littermates were treated with tamoxifen or corn oil. hPLAP activity was assayed in pancreatic sections from RIP-Cre^{ER}; Z/AP mice, treated and not treated with tamoxifen. Recombination as indicated by hPLAP activity was noted only in islets of mice treated with tamoxifen (3X8mg). No recombination was observed in the islets of bigenic mice not injected with tamoxifen (Fig. 6). These results indicate that the reporter gene construct in R26R mice and Z/AP mice explains the difference in the current work and work by Dor and colleagues.

Efficient and Tamoxifen Dependent, Cre-Mediated Recombination in Pdx1^{PB}-Cre^{ER};R26R Mice

Because the RIP-Cre^{ER} system had spontaneous recombination in the absence of tamoxifen, we also evaluated Pdx1^{PB}-Cre^{ER};R26R mice. Analysis of Pdx1^{PB}-Cre^{ER};R26R pancreas sections by immunostaining indicated that Cre was expressed in a majority of the cells of the islet (data not shown). Tamoxifen-induced recombination was observed in approximately ~80% of the endocrine cells per islet, as seen by

immunofluorescent staining for β -gal and X-gal staining (Fig. 7). Little or no recombination was detected in the islets of bigenic mice not treated with tamoxifen (Fig 7).

Level of Cre^{ER} Expression Differs in Pdx1^{PB}-Cre^{ER} and RIP-Cre^{ER} lines

To investigate possible reasons for the tamoxifen-independent recombination occurring in our RIP-Cre^{ER}; R26R mice, we compared the level of Cre expression in each line by quantitating relative fluorescence intensity of Cre antibody staining using MetaMorph. For this analysis, we stained the slides at the same time, under the same conditions, and then conducted image analysis them under identical settings. This analysis indicated that Cre expression in RIP-Cre^{ER};R26R was ~4X higher than Cre expression observed in Pdx1^{PB}-Cre^{ER};R26R (Fig 8).

Discussion

To characterize the tamoxifen-induced, Cre-mediated activity/efficiency of islet specific CreERTM lines, Pdx1^{PB}-Cre^{ER} and RIP-Cre^{ER}, we crossed both transgenic lines with Rosa26R mice. We found that both transgenes produced significant tamoxifen-induced recombination in the islets of bigenic mice. We also found that Cre protein expression was substantially greater in RIP-Cre^{ER};R26R compared to Pdx1^{PB}-Cre^{ER};R26R. Surprisingly, we saw a significant amount of Cre-mediated recombination in islets of RIP-Cre^{ER};R26R mice not treated with tamoxifen. Conversely, little to no recombination was observed in the islets of Pdx1^{PB}Cre^{ER} mice not treated with tamoxifen.

What is the likely explanation for the difference between Cre-mediated recombination directed by the RIP and Pdx1 promoters? Cre recombinase-mediated excision is a stochastic event influenced by the level of the Cre enzyme and the availability of the loxP-flanked DNA sequence to the enzyme. In this system, Cre expression is determined by the characteristics of the promoters, RIP and Pdx1. The insulin promoter is an extremely strong promoter and is the reason β -cells synthesize such high levels of insulin. The RIP promoter has been widely used to engineer a large number of transgenic mice and a feature of these mice is the high level of protein expression in β -cells. Conversely, Pdx1 is a transcription factor that plays a critical role in the pancreatic and islet development; inactivation of Pdx1 leads to pancreatic agenesis (Jonsson et al, 1994). Pdx1 is also an important regulator of insulin gene transcription (Ohlsson et al, 1993). As would be expected by the function of insulin (a secreted hormone) and the function of Pdx1 (transcription factor), the level of mRNA of the two genes is markedly different with the insulin mRNA being the most abundant mRNA in β -cells while pdx-1 mRNA is in low abundance. Thus, the markedly different level of Cre protein in the two transgenic lines likely reflects the strength of the two

different promoters in β -cells. While other factors such as the insertion site of the transgene and the number of transgenes inserted may also influence transgene expression, it appears that the greater activity of the insulin promoter in β -cells is the most likely explanation for the greater level of Cre expression in RIP-Cre^{ER} islets. A limitation of the current study is that the absolute level of Cre protein was not quantified, but the relative expression by fluorescence intensity clearly indicates greater Cre expression in the RIP-Cre^{ER} line. Immunoblotting of protein extracts from the two mouse lines is needed to more precisely quantify the level of Cre expression.

Why is there a difference between the R26R and Z/AP reporter lines when crossed with the same Cre transgene? With RIP-Cre^{ER} producing a very high concentration of Cre protein, the probability of recombination at target loxP sites increases. We speculate that the RIP promoter may produce high levels of Cre protein that exceed the amount of Hsp90 in the cell, and thus, not all Cre molecules are sequestered in the cytoplasm. Conversely, the Pdx1^{PB} promoter, being less robust, would not drive such high expression thus all Cre^{ER} protein would be bound by Hsp90. Therefore, in this instance the Cre^{ER} protein would be retained in the cytoplasm. Another possible contributing factor is the availability of the targeted floxed allele to the Cre line varies between mouse lines or gene loci. Therefore, if a Cre protein molecule does enter the nucleus independent of tamoxifen, the chances of recombination are dependent on the accessibility of the loxP site. The mechanisms that influence the accessibility are incompletely defined but factors such as chromatin structure and packaging are likely involved. Conversely, if an allele with a loxP site is less accessible to the Cre enzyme and the level of intranuclear Cre protein is low, then recombination is less likely to occur. This likely explains the absence of basal Cre-recombination in RIP-Cre^{ER};Z/AP mice, but basal recombination in RIP-Cre^{ER};R26R mice.

The amount of basal Cre-mediated recombination observed in RIP-Cre^{ER};R26R islets may be sufficient enough to impact studies aimed at temporally inactivating VEGF-A in adult islets. Given our interests in adult islets, we concluded that our VEGF-A inactivation experiments would use the Pdx1^{PB}-Cre^{ER} mice.

CHAPTER III

INACTIVATION OF VEGF-A IN ADULT ISLETS

Synopsis

Vascular endothelial growth factor-A (VEGF-A) is a well known angiogenic factor in vascular development, ischemia-induced angiogenesis, and tumor-related angiogenesis. Prior studies have reduced VEGF-A expression in mouse islets using Cre recombinase under the transcriptional control of rat insulin promoter (RIP-Cre) (Brissova, 2006) and pancreas-duodenum homeobox gene-1 promoter (Pdx1) (Lammart, 2003). Both of these models demonstrated that VEGF-A is required for the development of normal endothelial cell morphology in pancreatic islets. However, a limitation of these models is that VEGF-A is inactivated in islets during embryogenesis. Thus, the role of VEGF-A in adult islets is still not known. Here we report on the temporal and conditional deletion of VEGF-A in pancreatic islets and the subsequent effects this reduction has on islet function and intra-islet vasculature.

For these studies, we used Pdx1^{PB}-Cre^{ER} transgenic mice crossed with VEGF^{fl/fl} mice (Pdx1^{PB}-Cre^{ER}; VEGF^{fl/fl}). We show that in this model, administration of tamoxifen effectively reduces VEGF-A in pancreatic islets and reduced islet vascularization. Functional analysis of mice with islet inactivation of VEGF-A showed decreased glucose tolerance at one-week post tamoxifen injections. However, this decreased glucose clearance was not observed in mice one-month after tamoxifen injections.

Methods and Materials

Mouse models: Mice heterozygous for the tissue-specific inducible transgenic ($Pdx1^{PB}$ - Cre^{ER}) (Zhang, 2005) were bred with homozygotic mice (mice with VEGF-A gene floxed on both alleles; $VEGF^{fl/fl}$) (Gerber, 1999). Subsequent littermates were bred to generate transgenic, homozygotic floxed mice ($Pdx1^{PB}$ - Cre^{ER} ; $VEGF^{fl/fl}$) and non-transgenic, homozygotic floxed mice ($VEGF^{fl/fl}$).

Glucose tolerance testing: Intraperitoneal glucose tolerance testing (2g/kg body wt) was performed on mice (13-18 wks of age) after a 14-16 hr fast (Brissova, 2006). Plasma glucose was measured in whole blood by use of a Roche Accu-check glucose meter. Blood glucose was measured before administration of glucose and at 15 mins, 30 mins, 60 mins, 90 mins, and 120 mins after glucose injection.

Tamoxifen injections: Tamoxifen was dissolved in corn oil overnight at a concentration of 20/mg/mL and stored at 4°C. Three subcutaneous injections of 8/mg were administered as described in Chapter II.

Tissue collection and histological assessment of pancreatic islets:

Mice were infused with fluorescein isothiocyanate conjugated tomato lectin (*Lycopersicon Esculentum*; 1mg/mL; Vector Laboratories, Burlingame, CA) via the jugular vein (Brissova, 2006). This lectin binds specifically to endothelial cells. Lectin was allowed to circulate for 7 minutes, the pancreas was then removed and fixed in 4% PFA/0.1M PBS for 1.5 hours on ice. Afterward, tissues were washed several times with 10 mM PBS and then cryoprotected with 30% sucrose/0.1M PBS and embedded in OCT compound (Tissue-Tek). After fixation, 10 μ m cryosections were stained with antibodies for insulin,

Cre and lectin were visualized and imaged by fluorescence microscope (MagnaFire Digital Camera, Optronics connected to an Olympus BX-41 fluorescence). Primary antibodies used were: Guinea pig anti-human insulin IgG (1:1,000) from Linco Research and rabbit anti-Cre (1:5000) from Novagen. Antigens were visualized using secondary antibodies conjugated with Cy2, Cy3, and Cy5 fluorophors from Jackson ImmunoResearch Laboratories (West Grove, PA) at concentrations recommended by the manufacturer. Primary and secondary antibodies were diluted in phosphate-buffered saline containing (PBS) 1% bovine serum albumin and 0.1% Triton-X-100. The cryosections were blocked with 5% normal donkey serum (Jackson ImmunoResearch Laboratories) and then incubated with the primary antibodies overnight at 4°C.

For evaluation of intra-islet vascular density, non-overlapping images of an entire pancreatic section were taken under the 20X objective to visualize the antibody and lectin binding. Using MetaMorph v6.1 software (Universal Imagine, Downingtown, PA.), integrated morphometry of 10-20 islets per section (5-6 sections/mouse) was used to calculate islet area and the area of the islet vasculature. To systematically quantify endocrine cell mass, 10 μm pancreatic sections spaced by 100 μm from 3 different layers of the pancreatic tissue block (2 pancreata per genotype) were examined.

Islet isolation and Enzyme-linked immunosorbent assay (ELISA): Islets were isolated from Pdx1^{PB}-Cre^{ER}; VEGF^{fl/fl} and wild type mice by dissection of the splenic portion of the pancreas followed by digestion with Collagenase P (Roche Molecular Biochemicals) (Brissova, 2006). Groups of two pancreata were digested in 2 ml of collagenase/pancreas in Hanks buffered saline (GIBCO) for 6-9 mins at 37°C using a wrist-action shaker. Islets were handpicked under microscopic guidance and separated from exocrine tissue by serial transfers/picks into clean dishes. Islets (n=70 per well)

were cultured in 500ul of RPMI 1640 (11mM glucose) for 48 hours at 37°C. Culture media and islets were collected at 48 hours. Media and islets were centrifuged at 4°C at 2000/rpm, then 450ul of media was collected into a separate eppendorf tube. The islets were then washed four times with cold PBS, and then stored at -80°C. VEGF-A levels were measured in the media by ELISA (R&D Systems).

Radioimmunoassay (RIA): Mouse plasma insulin was determined by a heterospecies-specific RIA (Brissova, 2006) with guinea pig anti-rat insulin serum, rat insulin reference standard, normal guinea pig serum, goat anti-guinea pig gammaglobulin serum (Linco Research), and 125 I-human insulin (Diagnostic Products). The RIA has a sensitivity (ED90) of 7.5 pg of insulin/ml, which converts to 150 pg of insulin/ml when using 5 µl of mouse plasma/assay tube.

Statistical analysis: To compare the outcomes in the different genotypes, we used unpaired t-test and one-way analysis of variance with Newman-Keuls multiple comparison. The data were expressed as mean \pm SE.

Results

Administration of Tamoxifen to Pdx1^{PB}-Cre^{ER}; VEGF^{fl/fl} mice effectively reduces VEGF-A levels

To determine the efficiency of Cre-mediated recombination in pancreatic islets, VEGF-A protein secreted by isolated islets from tamoxifen-treated and control Pdx1^{PB}-Cre^{ER}; VEGF^{fl/fl} and VEGF^{fl/fl} mice was quantified by ELISA. Islets were isolated from Pdx1^{PB}-Cre^{ER}; VEGF^{fl/fl} (n=4) and VEGF^{fl/fl} (n=4) mice at the following time points: (A) pre-tamoxifen (B) 7 days after the last tamoxifen injection (C) 30 days after the last tamoxifen injection. Isolated islets (70 islets per well, n=4) were cultured for 48 hours. Before tamoxifen treatment, VEGF-A secretion, by islets from Pdx1^{PB}-Cre^{ER}; VEGF^{fl/fl} and VEGF^{fl/fl} mice was similar, measuring ~2.5-3.0 pg/islet/48hrs (Fig 9). Administration of tamoxifen reduced VEGF-A production ten-fold by Pdx1^{PB}-Cre^{ER}; VEGF^{fl/fl} islets, measured at one week and 1 month after tamoxifen treatment (Fig. 9). The reduction of VEGF-A secretion in Pdx1^{PB}-Cre^{ER}; VEGF^{fl/fl} islets is comparable to that seen in isolated islets from RIP-Cre; VEGF^{fl/fl} mice (Brissova, 2006). VEGF-A expression was unaltered in VEGF^{fl/fl} mice at 7 days and 30 days post tamoxifen (Fig. 9).

Islet Vasculature is altered by reduced VEGF-A expression

Based on prior data from RIP-Cre;VEGF^{fl/fl} studies (Brissova, 2006; Lammert, 2003), we knew that reduced VEGF-A production in islets, during embryogenesis, resulted in decreased vascular density and vessel area. To determine the impact of a reduction in VEGF-A on adult islets, we measured the same vascular parameters. Reduced VEGF-A expression in tamoxifen-treated Pdx1^{PB}-Cre^{ER}; VEGF^{fl/fl} mice led to altered islet vasculature (Fig. 10). Vessel density and area per vessel were similar in Pdx1^{PB}-Cre^{ER}; VEGF^{fl/fl} and VEGF^{fl/fl} islets pre-tamoxifen treatment (Fig.10 B and C).

Vasculature perimeters were unchanged in VEGF^{fl/fl} islets at either one-week or one-month after tamoxifen treatment (Fig.10 B and C). There appeared to be a trend toward decreased density in Pdx1^{PB}-Cre^{ER}; VEGF^{fl/fl} islets, as seen by lectin staining one week after tamoxifen treatment. Vessel density of Pdx1^{PB}-Cre^{ER}; VEGF^{fl/fl} islets was significantly reduced 30 days post tamoxifen treatment (Fig. 10B). The area per vessel of Pdx1^{PB}-Cre^{ER}; VEGF^{fl/fl} islets was significantly reduced at both 7 days and 30 days post tamoxifen treatment (Fig. 10C).

Glucose tolerance in tamoxifen-treated mice

To elucidate the relationship between VEGF-A, islet vasculature, and islet function, we subjected Pdx1^{PB}-Cre^{ER};VEGF^{fl/fl} and VEGF^{fl/fl} mice to intraperitoneal glucose tolerance tests at (A) pre-tamoxifen (B) 7 days after the last tamoxifen injection (C) 30 days after the last tamoxifen injection. Glucose clearance was similar between Pdx1^{PB}Cre^{ER}; VEGF^{fl/fl} and VEGF^{fl/fl} groups pre-tamoxifen treatment (Fig. 11A). The glucose clearance of VEGF^{fl/fl} mice was unaltered by tamoxifen treatment (Fig. 11B). Tamoxifen-treated Pdx1^{PB}-Cre^{ER}; VEGF^{fl/fl} mice had normal fasting blood glucose levels, but there was a slight, but significant delay in glucose clearance in Pdx1^{PB}-Cre^{ER}; VEGF^{fl/fl} mice 7 days post tamoxifen treatment. Interestingly, this delay in glucose clearance was not observed in Pdx1^{PB}-Cre^{ER}; VEGF^{fl/fl} mice 30 days post tamoxifen treatment (Fig. 12). We also monitored random non-fasting blood glucose of Pdx1^{PB}-Cre^{ER}; VEGF^{fl/fl} and VEGF^{fl/fl} mice throughout the course of the experiment and these were normal (data not shown).

Discussion

To determine the efficiency of VEGF-A inactivation in islets of Pdx1^{PB}-Cre^{ER};VEGF^{fl/fl}, we measured VEGF-A protein produced by isolated islets harvested before and subsequent to tamoxifen injection. The level of VEGF-A expression in Pdx1^{PB}-Cre^{ER};VEGF^{fl/fl} islets prior to treatment was similar to that of the VEGF^{fl/fl} islets, indicating that Cre was properly being sequestered in the cytoplasm of this model. We observed a significant reduction in VEGF-A secretion by islets from Pdx1^{PB}-Cre^{ER};VEGF^{fl/fl} islets 7 days and 1 month post injection. In contrast, there was no significant change in VEGF-A secretion from VEGF^{fl/fl} islets at the same time points.

Previous studies suggest that VEGF-A is a major regulator of islet vascularization and islet function (Brissova, 2006; Lammert, 2003). In our study, we demonstrated that inactivation of VEGF-A in adult islets does effect intraislet vasculature. Measurements of the vascular parameters of Pdx1^{PB}-Cre^{ER};VEGF^{fl/fl} islets showed a trend of decreased vessel density at one week and a significant reduction at one month. The area/vessel was reduced at 7 days and at 1 month. This reduction in islet vascular parameters indicates that maintenance of islet vascularization is dependent on VEGF-A production by islet cells and that when VEGF-A levels are reduced, intra-islet vessels regress. How this occurs requires further study into the fate of endothelial cells that line intra-islet vessels and extracellular matrix that comprises the capillary structure. A possible hypothesis is that intra-islet endothelial cells undergo apoptosis and cell death without constant VEGF-A stimulation and that without endothelial cells the basement membrane of capillaries are not maintained. The reduction in islet vessel area at 7 days suggests that the reduced vessel area precedes the loss of vascular structures quantified by measuring vessel density. Previous studies suggest that blood vessel type-specific and tissue-specific characteristics of endothelial cells are under the control of their microenvironment (Lacorre, DA 2004). Additional experiments using 3D reconstruction

of adult islet vasculature and electron microscopy of endothelial cells in our model would determine if the ultrastructure of the endothelial cells is acutely and/or chronically altered when VEGF-A expression is down-regulated.

This change in vascular density in $\text{Pdx1}^{\text{PB}}\text{-Cre}^{\text{ER}};\text{VEGF}^{\text{fl/fl}}$ mice 7 days post injections coincided with a significant delay in glucose clearance. However, a difference in glucose clearance was no longer observed one month after tamoxifen treatment. This initial correlation between islet function and reduced vasculature and vessel area seen at 7 days post tamoxifen treatment, is similar to what has been previously observed in the $\text{RIP-Cre};\text{VEGF}^{\text{fl/fl}}$ studies in our lab (Brissova, 2006), showing that even a 25% reduction in islet vessel density results in impaired islet function. The impairment of glucose clearance at one week could be explained by: 1) the reduction in vessel size causing decreased flow into the VEGF-A mutant islets, this could result in a lower or delayed beta cell response, or 2) alterations in the ultrastructure of the islet endothelial cells could slow the delivery of insulin into the vascular system.

This apparent 'recovery' of islet function seen at 30 days post treatment could be explained by compensatory actions by other angiogenic factors known to be present in endocrine cells. For example, the reduction in islet cell production of VEGF-A, could lead to an upregulation of an angiopoietin or an ephrin, or even another member of the VEGF family. Quantitative and qualitative experiments would help to determine if the levels of other angiogenic factors are altered in response to a reduction of VEGF-A. Or perhaps β -cells compensate for this reduced vessel density by changing their glucose sensitivity or efficiency of insulin secretion. These possibilities could be investigated by examining insulin in isolated islets, by quantifying β -cell gene expression of proteins involved in glucose-stimulated insulin secretion, and by examining β -cell ultrastructure by electron microscopy.

Summary and Conclusions

Pancreatic islets are highly vascularized and receive a much greater blood flow than surrounding pancreatic exocrine tissue. This is likely important in the rapid sensing of the blood glucose and secretion of insulin by the β -cell in response to a glucose ingestion. Earlier data from knockout studies of VEGF-A in islets provide evidence of the importance of this angiogenic factor during development for normal islet function and normal intra-islet vasculature. This thesis focuses on the role of VEGF-A, a major determinant of islet vascularization, in adult islet vasculature and function. This is not only a question of biologic interest, but may be relevant to both the pathogenesis and treatment of diabetes mellitus as discussed in Chapter 1. In type 1 diabetes, islet transplantation is an emerging, but experimental, therapy. While this cell-based therapy has shown promising results, several factors prevent it from being widely adapted as a form of treatment (reviewed in Chapter 1). The work in this thesis is relevant to one of those limitations: revascularization of transplanted islets. In type 2 diabetes, inadequate insulin secretion could result from abnormalities in islet vascularization. Thus, we hope that the studies described in this thesis contribute to a greater understanding of the role of VEGF-A in adult islets and may also have implications for human disease.

To investigate the role of VEGF-A in adult islets, we utilized an inducible Cre/loxP system. We studied two different Cre transgenes that allowed us to temporally and spatially inactivate VEGF-A in pancreatic islets. In Chapter 2, we describe our findings that both RIP-Cre^{ER} and Pdx1^{PB}-Cre^{ER} lines efficiently direct islet cell-specific, Cre-mediated recombination following tamoxifen administration. Surprisingly, we found that RIP-Cre^{ER} mice had small, but significant, amount of Cre-mediated recombination without tamoxifen administration in the R26R reporter line. This was different from a prior report in the literature and prompted further investigation. Interestingly, we found that the Cre-mediated recombination without tamoxifen administration did not occur

using a different reporter line, Z/AP (the reporter line used in the prior report). We found that Cre expression was 4 times greater than RIP-Cre^{ER} compared to Pdx1^{PB}-Cre^{ER} mice. Our comparative studies of the various methods for administering tamoxifen demonstrated that oral or intraperitoneal was not optimal routes of administration - both for reasons of recombination efficiency and impact on the health of the animals. Finally, we found that Cre-mediated recombination was efficiently and safely induced by subcutaneous administration of tamoxifen, with a high percentage of recombination accomplished with 3 doses of 8mg of tamoxifen.

These results with two different Cre lines and two reporter lines indicate that both the promoter driving Cre expression and floxed allele are important variables to consider when using the Cre/LoxP systems. Since the level of Cre^{ER} expression will vary depending of the promoter and every Cre^{ER} transgene will have a different insertion site, investigators must consider this in both the design and interpretation of experiments using a Cre^{ER} transgene. For example, if two promoters have a similar cell-specific expression pattern, but one is highly expressed in that cell type and the other is expressed a lower level, then basal (non-tamoxifen-mediated) recombination may occur in one line but not in the other. Likewise, every floxed allele will be different and the accessibility of the allele to the Cre enzyme may be an important variable. In our studies using the highly active RIP promoter, the R26R allele appeared very sensitive to small amounts of Cre in the nucleus. These findings suggest that if an investigator was using a “sensitive” or an “accessible” floxed allele, there might be some inactivation of the gene of interest without tamoxifen administration. This low level inactivation could affect cell function if the level of expression of the gene of interest was critical and might not be easily detected. We were concerned about such an effect on the floxed VEGF-A allele, especially since prior studies have suggested that islet vascularization is very sensitive

to the level of VEGF-A expression. Thus, we used only Pdx1^{PB}-Cre^{ER};VEGF^{fl/fl} for subsequent studies.

In the studies to investigate the role of VEGF-A in adult islet vascularization, we found that reduced VEGF-A expression in Pdx1^{PB}-Cre^{ER};VEGF^{fl/fl} islets decreased the vascular density and vessel area of islets (Chapter 3). Consequently, this loss of VEGF-A impaired glucose clearance when tested one-week post tamoxifen injection. However, reduced VEGF-A expression had no significant effect on glucose clearance in Pdx1^{PB}-Cre^{ER};VEGF^{fl/fl} mice when tested one-month post tamoxifen injection. In parallel, control experiments, tamoxifen administration did not alter VEGF-A expression, effect glucose tolerance, or reduce vasculature in VEGF^{fl/fl} islets. Additionally, VEGF-A expression in non-treated Pdx1^{PB}-Cre^{ER};VEGF^{fl/fl} islets was in the same range as VEGF^{fl/fl} islets.

Our results indicate that VEGF-A in adult islets also plays an important role in the maintenance of intra-islet vasculature. Intra-islet vasculature appears sensitive to changes levels of VEGF-A post development, suggesting plasticity in the morphology and architecture of islet vasculature. The relationship between VEGF-A expression and islet function is still unclear and further work is needed.

Future Studies

Work on this project is continuing in our laboratory and a number of scientific questions remain unanswered. Potential studies or scientific questions include:

- What are the long-term effects of VEGF-A inactivation in the adult islet? Mice could be treated with tamoxifen and then followed for larger periods of time. Insulin secretion by isolated islets and more sophisticated methods for assessing insulin secretion in vivo (pancreas perfusion or hyperglycemia clamping).
- How would reduced levels of VEGF-A affect islets during transplantation?
- Is there a compensation for the reduced VEGF-A level? The reduction in VEGF-A could lead to an upregulation of an angiopoietin or an ephrin, or even another member of the VEGF family. Quantitative and qualitative experiments would help to determine if the levels of other angiogenic factors are altered in response to a reduction of VEGF-A. Or perhaps there is compensation of β -cells. Can they alter their function and response to glucose? Can they secrete more insulin? One would measure insulin levels and in response to various secretagogues.
- There are also several areas of potential future experiments related to the Cre^{ER} system. For example, a limitation of this work is that the level of Cre protein was not quantified (we assessed relative expression by fluorescence intensity). Immunoblotting of protein extracts for Cre from the two mouse lines is needed to more precisely quantify the relative amount of Cre protein in the two transgenic lines. Likewise, one could also investigate the reasons that the two reporter mice differed in their susceptibility to Cre-mediated recombination. Does this relate to epigenetic modifications, chromatin structure or methylation?

REFERENCES

- Biancone L, Ricordi C: Pancreatic islet transplantation: an update. *Cell Transplant* **11**:309–311, 2002
- Brissova M, Shiota M, Nicholson W, Gannon M, Knobel S, Piston D, Wright C, Powers AC: Reduction in transcription factor pdx-1 impairs normal glucose sensing and insulin secretion by pancreatic islets. *J Biol Chem* **277**: 11225-11232, 2002
- Brissova M, Nicholson WE, Shiota M, Powers AC: Assessment of insulin secretion in the mouse. *Methods Mol Med* **83**: 23-45, 2002
- Brissova M, Shostak A, Shiota M, Wiebe P, Poffenberger G, Kantz J, Chen Z, Jerome WG, Chen J, Baldwin HS, Nicholson W, Bader DM, Jetton T, Gannon M, Powers AC: Pancreatic islet production of vascular endothelial growth factor- α is essential for islet vascularization, revascularization, and function. *Diabetes* **55**: 2974-2985, 2006
- Carlsson PO, Palm F, Mattsson G: Low revascularization of experimentally transplanted human pancreatic islets. *J Clin Endocrinol Metab* **87**: 5418-5423, 2002
- Carlsson PO, Andersson A, Carlsson C, Hellerstrom C, Hoglund E, King A, Kallskog O, Liss P, Mattsson G, Olsson R, Palm F, Sandler S, Tyrberg B, Jansson L: Engraftment and growth of transplanted pancreatic islets. *Ups J Med Sci* **105** :107–123, 2000
- Carlsson PO, Mattsson G: Oxygen tension and blood flow in relation to revascularization in transplanted adult and fetal rat pancreatic islets. *Cell Transplant* **11** :813–820, 2002
- Chang EN, Scudamore CH, Chung SW: Transplantation: focus on kidney, liver and islet cells. *Can J Surg* **47**: 122-129, 2004
- Christofori G, Naik P and Hanahan D: Vascular endothelial growth factor and its receptors, flt-1 and flk-1, are expressed in normal pancreatic islets and throughout islet cell tumorigenesis. *Mol. Endocrinol.* **9**:1760–1770, 1995.
- Costa A, Conget I, Gomis R: Impaired glucose tolerance: is there a case for pharmacologic intervention? *Treat Endocrinol* **1**: 205-210, 2002
- Davalli AM, Ogawa Y, Scaglia L, Wu YJ, Hollister J, Bonner-Weir S, Weir GC: Function, mass, and replication of porcine and rat islets transplanted into diabetic nude mice. *Diabetes* **44** :104–111,1995
- Davalli AM, Scaglia L, Zangen DH, Hollister J, Bonner-Weir S, Weir GC: Vulnerability of islets in the immediate posttransplantation period: dynamic changes in structure and function. *Diabetes* **45** :1161–1167,1996
- Dor Y, Brown J, Martinez OI, Melton DA: Adult pancreatic beta-cells are formed by self-duplication rather than stem-cell differentiation. *Nature* **429**(6987): 41-46, 2004

Drab M, Verkade P, Elger M, Kasper M, Lohn M, Lauterbach B, Menne J, Lindschau C, Mende F, Luft FC: Loss of caveolae, vascular dysfunction, and pulmonary defects in caveolin-1 gene-disrupted mice. *Science* **293**: 2449–2452, 2001.

Dvorak HF, Brown LF, Detmar M, Dvorak AM: Vascular permeability factor/vascular endothelial growth factor, microvascular hyperpermeability, and angiogenesis. *Am J Pathol* **146**: 1029-1039, 1995

Esser, K, Wolburg H, Breier G, Kurzchalia T and W. Risau: Vascular endothelial growth factor induces endothelial fenestrations in vitro. *J. Cell Biol.* **140**: 947–959, 1998.

Fajans SS, Bell GI, Polonsky KS: Molecular mechanisms and clinical pathophysiology of maturity-onset diabetes of the young. *N. Engl. J. Med.* **345** (13): 971-80, 2001

Ferrara N: Vascular Endothelial Growth Factor: Basic science and clinical progress. *Endocrine Rev* **25** (4): 581-611, 2004

Ferrara N, Houck K, Jakeman L, Leung DW: Molecular and biological properties of the vascular endothelial growth family of proteins. *Endocr Rev* **13**:18–32, 1992

Fraga DW, Sabek O, Hathaway DK, and Gaber AO: A comparison of media supplement methods for the extended culture of human islet tissue. *Transplantation* **65**: 1060-1066, 1998

Folkman J: Angiogenesis in cancer, vascular, rheumatoid and other disease. *Nat Med* **1**:27–31, 1995

Folkman J, Klagsbrun M: Angiogenic factors. *Science* **235**:442–447, 1987

Gaber AO, Fraga DW, Callicutt CS, Gerling IC, Sabek OM, Kotb MY. Improved in vivo pancreatic islet function after prolonged in vitro islet culture. *Transplantation* **72**:1730-1736, 2001

Gannon G, Mandriota SJ, Cui L, Baetens D, Pepper MS, Christofori G: Overexpression of vascular endothelial growth factor-A165 enhances tumor angiogenesis but not metastasis during beta-cell carcinogenesis. *Cancer Res* **62**:603–608, 2002

Gu G, Dubauskaite J, Melton D.A: Direct evidence for the pancreatic lineage: NGN3+ cells are islet progenitors and are distinct from duct progenitors. *Development* **129**: 2447–2457, 2002 .

Gerber H.P, Hillan K.J, Ryan A.M, Kowalski J, Keller GA, Rangell L, Wright B.D, Radtke F, Aguet M and N. Ferrara: VEGF is required for growth and survival in neonatal mice. *Development* **126**:1149–1159, 1999.

Gannon M, Gamer LW and C.V. Wright: Regulatory regions driving developmental and tissue-specific expression of the essential pancreatic gene pdx1. *Dev. Biol.* **238**:185–201, 2001

Hendrick JP and Hartl FU: Molecular chaperone functions of heat-shock proteins. *Annual Review of Biochemistry* **62**: 349-384, 1993

- Inoue M, Hager JH, Ferrara N, Gerber HP, Hanahan D: VEGF-A has a critical, nonredundant role in angiogenic switching and pancreatic beta cell carcinogenesis. *Cancer Cell* **1** :193–202, 2002
- Jansson L, Carlsson PO: Graft vascular function after transplantation of pancreatic islets. *Diabetologia* **45** :749–763, 2002
- Jansson L: The regulation of pancreatic islet blood flow. *Diabetes Metab Rev* **10**: 407-416,1994
- Jonsson J, Carlsson I, Edlund T, Edlund H: Insulin- promoter-factor 1 is required for pancreas development in mice. *Nature* **371**: 606-609,1994
- Lakey JRT, Warnock GL, Shapiro AMJ, Korbitt GS, Ao Z, Kneteman NM, Rajotte RV: Intraductal collagenase delivery into the human pancreas using syringe loading or controlled perfusion. *Cell Transplant* **8**:285–292,1999
- Lakey JR, Cavanagh TJ, and Zieger MA: A prospective comparison of discontinuous EuroFicoll and EuroDextran gradients for islet purification. *Cell Transplant* **7**: 479-487, 1998
- Lammert E, Cleaver O, Melton D: Induction of pancreatic differentiation by signals from blood vessels. *Science* **294** :564–567, 2001
- Lee J, Gray A, Yuan J, Luoh SM, Avraham H, Wood WI: Vascular endothelial growth factor-related protein: a ligand and specific activator of the tyrosine kinase receptor Flt4. *Proc Natl Acad Sci USA* **93**:1988–1992, 1996
- Lifson N, Kramlinger KG, Mayrand RR, and Lender EJ: Blood flow to the rabbit pancreas with special reference to the islets of Langerhans. *Gastroenterology* **79**: 466-473, 1980
- Linetsky E, Bottino R, Lehmann R, Alejandro R, Inverardi L, Ricordi C: Improved human islet isolation using a new enzyme blend, liberase. *Diabetes* **46**:1120–1123, 1997
- Mattsson G, Jansson L, Carlsson PO: Decreased vascular density in mouse pancreatic islets after transplantation. *Diabetes* **51**:1362–1366, 2002
- Mathis D, Vence L, Benoist C: β -cell death during progression to diabetes. *Nature(Lond)* **414**: 792-798, 2001
- Menger MD, Jaeger S, Walter P, Feifel G, Hammersen F, Messmer K: Angiogenesis and hemodynamics of microvasculature of transplanted islets of Langerhans. *Diabetes* **38** :199–201,1989
- Ohlsson H, Karlsson K, Edlund T: IPF1, a homeodomain – containing transactivator of the insulin gene. *EMBOJ* **12**: 4251-4259, 1993
- Parr E, Bower K, Lafferty K: Changes in cultured mouse thyroid glands and islets of Langerhans. *Transplantation* **30**:135–141, 1980

Prado CL, Pugh-Bernard AE, Elghazi L, Sosa-Pineda B, Sussel L: Ghrelin cells replace insulin-producing beta cells in two mouse models of pancreas development. *Proc Natl Acad Sci USA* **101(9)** 2924-2929, 2004

Ricordi C, Inverardi L, Kenyon NS, Goss J, Bertuzzi F, and Alejandro R: Requirements for success in clinical islet transplantation. *Transplantation* **79**: 1298-1300, 2005

Ricordi C and Strom TB: Clinical islet transplantation: advances and immunological challenges. *Nat Rev Immunol* **4**: 259-268, 2004

Ricordi C, Lacy PE, Finke EH, Olack BJ, Scharp DW: Automated method for isolation of human pancreatic islets. *Diabetes* **37**:413-420, 1988

Risau W, Flamme I: Vasculogenesis. *Annu Rev Cell Biol* **11**: 73-91, 1995

Risau W: Mechanisms of angiogenesis. *Nature* **386**:671–674, 1997

Rossini AA, Greiner DL, Mordes JP: Induction of immunologic tolerance for transplantation. *Physiol Rev* **79**: 99-141, 1999

Roberts WG and G.E. Palade: Increased microvascular permeability and endothelial fenestration induced by vascular endothelial growth factor. *J. Cell Sci.* **108**: 2369–2379, 1995

Robertson RP: Pancreatic islet transplantation for diabetes: successes, limitations and challenges for the future. *Mol Genet Metab* **74**: 200-205, 2001

Robertson RP: Successful islet transplantation for patients with diabetes: fact or fantasy? *N Engl J Med* **343**: 289-290, 2000

Ryan EA, Lakey JR, Rajotte RV, Korbitt GS, Kin T, Imes S, Rabinovitch A, Elliott JF, Bigam D, Kneteman NM, Warnock GL, Larsen I, Shapiro AM: Clinical outcomes and insulin secretion after islet transplantation with the Edmonton protocol. *Diabetes* **50**:710–719,2001

Sandberg JO, Margulis B, Jansson L, Karlsten R, Korsgren O: Transplantation of fetal porcine pancreas to diabetic or normoglycemic nude mice: evidence of a rapid engraftment process demonstrated by blood flow and heat shock protein 70 measurements. *Transplantation* **59**:1665–1669, 1995

Shapiro AM, Lakey JR, Ryan EA, Korbitt GS, Toth E, Warnock GL, Kneteman NM, Rajotte RV: Islet transplantation in seven patients with type 1 diabetes mellitus using a glucocorticoid-free immunosuppressive regimen. *N Engl J Med* **343** :230 –238,2000

Soriano P: Generalized lacZ expression with the ROSA26 Cre reporter strain. *Nat Genet* **21(1)**: 70-1, 1999

Sutton R, Hammonds P, Hughes D: Isolation of islets from human pancreas using increased incubation temperatures and variable density gradients. *Horm Metab Res* **25**:35-36, 1989

Thai AC and Eisenbarth, GS: Natural history of IDDM. *Diabetes Rev.* **123**, 37–64, 1993

Vajkoczy P, Menger MD, Simpson E, and Messmer K : Angiogenesis and vascularization of murine pancreatic islet isografts. *Transplantation* **60**: 123-127, 1995

Vasir B, Jonas JC, Steil GM, et al.: Gene expression of VEGF and its receptors Flk-1/KDR and Flt-1 in cultured and transplanted rat islets. *Transplantation* **71**:924-935, 2001

Veikkola T and K. Alitalo: VEGFs, receptors and angiogenesis. *Semin. Cancer Biol.* **9** 211–220, 1999.

Wu, K. L., M. Gannon, M. Peshavaria, M. F. Offield, E. Henderson, M. Ray, A. Marks, L. W. Gamer, C. V. Wright, and R. Stein. Hepatocyte nuclear factor 3b is involved in pancreatic b-cell-specific transcription of the *pdx-1* gene. *Mol. Cell. Biol* **17**:6002–6013,1997.