DEVELOPMENT, CHARACTERIZATION, AND ASSESSMENT OF A TISSUE-ENGINEERED PREVASCULARIZED PANCREATIC ISLET ENCAPSULATION DEVICE

by

Alton Michael Hiscox

Copyright © Alton Michael Hiscox 2008

A Dissertation Submitted to the Faculty of the
GRADUATE INTERDISCIPLINARY PROGRAM IN PHYSIOLOGICAL SCIENCES
In Partial Fulfillment of the Requirements
For the Degree of
DOCTOR OF PHILOSOPHY
In the Graduate College
THE UNIVERSITY OF ARIZONA
2008
THE UNIVERSITY OF ARIZONA
GRADUATE COLLEGE

As members of the Dissertation Committee, we certify that we have read the dissertation
prepared by: Alton Michael Hiscox

entitled: Development, Characterization, and Assessment of a Tissue-Engineered
Prevascularized Pancreatic Islet Encapsulation Device

and recommend that it be accepted as fulfilling the dissertation requirement for the

Degree of: Doctor of Philosophy

_________________________________________________ Date: 12-17-07
Stuart Williams, PhD

_________________________________________________ Date: 12-17-07
Sean Limesand, PhD

_________________________________________________ Date: 12-17-07
James Hoying, PhD

_________________________________________________ Date: 12-17-07
Ron Lynch, PhD

Final approval and acceptance of this dissertation is contingent upon the candidate’s
submission of the final copies of the dissertation to the Graduate College.

I hereby certify that I have read this dissertation prepared under my direction and
recommend that it be accepted as fulfilling the dissertation requirement.

_________________________________________________ Date: 12-17-07
Dissertation Director: Stuart Williams, PhD
STATEMENT BY THE AUTHOR

This dissertation has been submitted in partial fulfillment of requirements for an advance degree at the University of Arizona and is deposited in the University Library to be made available to borrowers under rules of the Library.

Brief quotations from this dissertation are allowable without special permission, provided that accurate acknowledgment of source is made. Requests for permission for extended quotation from reproduction of this manuscript may be granted by the copyright holder.

Signed: Alton Hiscox
ACKNOWLEDGMENTS

As with any endeavor of this magnitude, many people were responsible for the research and training that culminated in this dissertation.

I would like to first thank my advisor and mentor, Dr. Stuart Williams. The training I received in Stu’s lab extended well beyond experimental or even scientific training, and included life and professional training. Thank you Stu for your guidance and support.

I would also like to thank the rest of my committee, Dr. Sean Limesand, Dr. Ron Lynch, and Dr. Jay Hoying. Their guidance and expertise were instrumental in my development as a research scientist.

I would also like to thank the people who, with tireless patience, provided me with the technical training needed to complete my research. Most of the skills I possess today are a direct result of the masterful training by Alice Stone. Alice trained me in everything from making solutions, to tissue culture techniques, and experimental design. Alice has been absolutely instrumental in my development as a bench scientist. Vangie Patula’s surgical training taught me to perform surgical techniques that I never dreamed I could ever master. Vangie is, without a doubt, the most talented microsurgeon I have ever known, and I am grateful for the opportunity to have been taught by the best. Last, but certainly not least, Faith Rice taught me everything I know about histology (and more than I wanted to know!). It is certainly obvious that Faith has a child, because she answered my ridiculous questions with the utmost patience and courtesy. Because of Faith, my technical understanding of histology will serve me well in the future.

I would also like to thank the people who provided administrative support. Holly Lopez does not receive enough credit for the services that she provides. Dealing with everything from student stipends to progress reports, Holly was always available to help, or in some cases scold, me as needed. Debbi Howard’s contributions to my training are equally instrumental. Debbi was always on top of the most important thing in a graduate student’s mind: getting paid. I’m not exactly sure how she went about dealing with everyone in the Bursar’s office, but the end result was that she always made sure I was paid on time. Lastly, I would like to thank Charlotte Garcia for answering my endless questions. She made sure I knew where I was supposed to be at any given time, and made sure that my grant submissions ended up where they should have.

The last group of people that I would like to thank are my friends. My friends are second on my list of life priorities (behind my family), and I could not have made it without their constant support. They are too many to name, but they know who they are.
DEDICATION

To the four most important people in my life.

Heather and Hannah for their daily reminder of what’s truly important.

Megan and Jen, without whom I would not be where I am today. I owe you everything.
# TABLE OF CONTENTS

**LIST OF FIGURES** ..................................................................................................................8

**ABSTRACT** ..............................................................................................................................11

**CHAPTER 1 – INTRODUCTION** ............................................................................................13
   Specific aims ......................................................................................................................33

**CHAPTER 2 – AN ISLET-CONTAINING COLLAGEN MATRIX CONSTRUCT AND EXAMINE ISLET RESPONSIVENESS TO GLUCOSE STIMULATION IN COLLAGEN** ............................................................................34
   Introduction .......................................................................................................................34
   Methods ............................................................................................................................36
   Results ...............................................................................................................................43
   Discussion ..........................................................................................................................63

**CHAPTER 3 – A 3-DIMENSIONAL TISSUE-ENGINEERED PREVASCULARIZED CONSTRUCT FOR THE PURPOSE OF SUPPORTING ISLET CELL SURVIVAL** ........................................................................72
   Introduction .......................................................................................................................72
   Methods ............................................................................................................................75
   Results ...............................................................................................................................79
   Discussion ..........................................................................................................................90

**CHAPTER 4 – A PREVASCULARIZED PANCREATIC ENCAPSULATION DEVICE TO ENHANCE ISLET SURVIVAL IN VIVO** ..............................................................................................................95
   Introduction .......................................................................................................................95
   Methods ............................................................................................................................98
   Results ............................................................................................................................102
   Discussion .........................................................................................................................122
TABLE OF CONTENTS - continued

CHAPTER 5 – DISCUSSION AND CONCLUSIONS .....................128
  Summary ...........................................................................128
  Conclusions .....................................................................132
  Scientific Contributions ..............................................134
  Future directions .........................................................137

APPENDICES
  APPENDIX A – Microvessel fragment isolation protocol ..........142
  APPENDIX B – Rat islet isolation protocol ..........................145
  APPENDIX C – Recipes of solutions ...................................147
  APPENDIX D – Permissions ............................................149
  APPENDIX E - Insulin RIA quality assurance data ..................150

REFERENCES ......................................................................154
LIST OF FIGURES

Figure 1-1  Timeline of historical events in diabetes research……18
Figure 1-2  An image of an adult rat pancreatic islet………………22
Figure 1-3  A schematized image of a pancreatic islet illustrating the various cell types within the islet…………………………………………………….23
Figure 1-4  Table of human islet transplantation protocol………..25
Figure 1-5  Schematic representation of the prevascularized pancreatic encapsulation device (PPED)………………31

Figure 2-1  Islet viability data using three independent structural and metabolic viability assays………………………………45
Figure 2-2  An image of the bioreactor setup used to culture isolated islets under flow conditions………………47
Figure 2-3  Radioimmuno assay standard curve used to assess unknown insulin samples………………………………48
Figure 2-4  Radioimmuno assay data for total intracellular insulin content for islets cultured within bioreactors and under static conditions………………49
Figure 2-5  Table showing insulin diffusion and sequestration data for collagen gels placed within solutions of varying insulin concentrations………………………………52
Figure 2-6  Summary statistics for insulin diffusion out of collagen gels…………………………………………………….53
Figure 2-7  Insulin diffusion out of gels polymerized with radioactive insulin……………………………………………………55
Figure 2-8 Summary data for insulin diffusion out of gels polymerized with radioactive insulin for all three washing conditions…………………………..56

Figure 2-9 Glucose stimulated insulin secretion in free islets and isles in collagen gels relative to basal insulin secretion……………………………………59

Figure 2-10 Glucose stimulated insulin secretion in free islets and islets in collagen gels reported as total insulin secretion normalized to intracellular insulin content……………………………………60

Figure 2-11 Glucose stimulated insulin secretion is free islets and islets in collagen gels reported as rates of stimulated insulin secretion per hour per 10 ilsets……………………………………61

Figure 3-1 Microvessel fragment growth and sprouting in vitro……80

Figure 3-2 Microsphere perfusion of cultured microvessel fragments…………………………………………………………..81

Figure 3-3 ELISA standard curve for VEGF secretion……………….83

Figure 3-4 Vascular endothelial growth factor release from microvessel fragments cultured in collagen…………….84

Figure 3-5 Real-time PCR of microvessel fragment construct implants………………………………………………………….86

Figure 3-6 India ink perfused microvessel fragment construct implants………………………………………………………..88

Figure 3-7 FITC-dextran perfused microvessel fragment constructs implants in GFP-mice…………………………………89

Figure 4-1 A schematized image of a one-piece islet-microvessel fragment implant………………………………………………104

Figure 4-2 India ink perfused microvessel fragment implants with and without isolated pancreatic islets………………105
LIST OF FIGURES-continued

Figure 4-3  Quantitative morphometric analysis of implanted microvessel fragment constructs with and without pancreatic islets.................................................................106

Figure 4-4  An H&E image of a 7 day microvessel fragment-islet implant.................................................................107

Figure 4-5  An image of a one-piece microvessel-islet implant With positive insulin staining.................................109

Figure 4-6  Islet viability in vitro in collagen encapsulated islets with and without microvessel fragments.................110

Figure 4-7  A schematized image of the PPED with histological characterization.................................................................112

Figure 4-8  Insulin immunohistochemical stains in PPEDs implanted for 7, 14, and 28 days...........................................113

Figure 4-9  Double immunohistochemical and cytochemical stains of PPEDs implanted for 7, 14, and 28 days........115

Figure 4-10 Double immunohistochemical and cytochemical stains of PPEDs showing an association between non-surviving islets and a negatively stained vasculature..................................................................................116

Figure 4-11 Insulin immunohistochemical stains of isolated pancreatic islets subjected to severe hypoxic insult........119

Figure 4-12 Dextran-perfused PPEDs stained for cell nuclei..........120

Figure 4-13 Comparison of positive control tissue and PPEDs implanted for 28 days using dapi cell nuclear stain and H & E stain.............................................................................121
ABSTRACT

Islet transplantation for the purpose of treating insulin-dependent diabetes is currently limited by several factors, most significantly, islet survival post transplantation. In the following dissertation, a tissue-engineered prevascularized pancreatic encapsulating device (PPED) was designed, developed, and evaluated. Microvessel fragments placed within a 3-dimensional collagen-based matrix produce and secrete vascular endothelial growth factor, and inosculate with the host circulation. Isolated islets placed within collagen gels exhibited four-fold more insulin release in response to glucose stimulation than islets in tissue culture. The insulin released by β-cells in islets encapsulated in collagen exhibited unobstructed diffusion within the collagen gels. Subsequent studies evaluated the ability to create a sandwich comprised of two layers of prevascularized collagen gels around a central collagen gel containing islets. In vitro characterization of the islets within these constructs showed that islets are functional and respond to glucose stimulation. The PPEDs were implanted subcutaneously into SCID mice. Islet survival was assessed after 7, 14, and 28 days. Immunohistochemical analysis was performed on the implants to detect insulin and the presence of intraislet endothelial cells. At all time points, insulin was localized in association with intact and partially dissociated islets. Moreover, cells that exhibited insulin staining were co-localized with intraislet endothelial cells. Lastly, dextran-perfused PPEDs showed host perfusion throughout the implant, including perfusion to structures that are morphologically consistent with
pancreatic islets. These data indicate that the PPED enhances islet survival by supporting islet viability, by maintaining intraislet endothelial cells, and by enhancing reperfusion to the islets.
CHAPTER 1

INTRODUCTION

It is unclear exactly when the term “diabetes” was first applied as a designation for a disorder characterized by a constellation of metabolic symptoms. The first confirmed description of the disorder is found on a 3rd Dynasty Egyptian papyrus by physician Hesy-Ra in 552 B.C.E. The clinical description of the disorder included polyuria, or frequent urination, as the primary manifestation. In the first century C.E. the physician Arateus describes diabetes as “the melting down of flesh and limbs into urine.” Most historical records, however, have the term “diabetes” first appearing in the 11th century, where it was often diagnosed by “water tasters”, who would taste the urine of individuals who were thought to possess the disorder. By this point, it had been well established that individuals suffering from the disorder expelled copious quantities of sweet smelling, and tasting, urine. It was in the 11th century that the term “mellitus”, Latin for “honey”, was added to the term “diabetes”, resulting in the term diabetes mellitus, which has been used to describe the disorder ever since. Although the exact etiology of diabetes remained elusive for the next ten centuries, working theories of the disorder’s origin were adopted by many different cultures, and included a disorder of the kidney, sugar depletion, and a “serious general disorder”. It was not until the end of the 19th century that the root cause of diabetes was identified.
In the late 19th century, two German doctors, Joseph Freiherr von Mering and Oscar Minkowski, began work to identify the etiology of diabetes. They were able to mimic the clinical manifestations of the disorder by removing the pancreata of dogs. These pancreaectomized dogs quickly exhibited high blood sugar, glycosuria, and died within 3 weeks after falling into a ketone-induced coma. Their work was published in 1890, and is credited as the first manuscript to identify diabetes as a pancreatic disorder. Three years later, in 1893, Laguesse identified structures within the pancreas that were thought to mediate the pancreas’s effect on lowering blood sugar. Laguesse named these structures islets of Langerhans, after the German medical student Paul Langerhans who identified the structures in 1869 in his dissertation, but was unable to associate them with a function. Although the islets of Langerhans had been suspected to play a role in sugar metabolism, there was no clinical or experimental evidence available to support this claim. In 1900, a professor of pathology at Johns Hopkins University, named Eugene Lindsay Opie, identified islet degeneration in a biopsy of pancreas obtained from a patient with diabetes. Opie later published his findings, which confirmed the suspicion that the reduced function of the islets of Langerhans was directly related to the occurrence of diabetes mellitus. Although it had been confirmed that the islets of Langerhans are responsible for glucose homeostasis, their exact involvement, and the mechanisms behind their action, would remain elusive. The final piece of the puzzle came several years later, when in 1905, Ernest Henry Starling coined the term “hormone” to identify the chemicals of endocrine glands that are responsible for mediating signals from
gland to target tissue. Subsequently, it was suggested that the islets of Langerhans were endocrine structures that secreted an unidentified hormone that was responsible for blood sugar metabolism. This hormone was later named by Jean de Meyer in 1909, and termed “insuline”\textsuperscript{4}. The term “insulin” would later be used by the pioneers of diabetes research in Toronto, Frederick Grant Banting and Charles Herbert Best\textsuperscript{5}.

Frederick Banting was born in Ontario in 1891. He attended medical school, and later became an orthopaedic surgeon. Banting became interested in isolating insulin and applying the hormone to treat diabetes after preparing for a lecture on the disease in 1920. Banting was aware that the islets of Langerhans were the likely stores of insulin, but had no way of confirming this long held suspicion, nor the means to test this hypothesis to treat the disease. In the spring of 1921 Banting was assigned bench space in the lab of J.J.R. Macleod, the head of the department of physiology at the University of Toronto. Banting began work isolating pancreatic extracts that would contain the hormone insulin. Previous attempts at using pancreas extracts to treat pancreatectomized dogs were modestly successful, but the temporary benefits of using these extracts were accompanied by pain, fever, and abscesses\textsuperscript{6-10}. Banting theorized that whole pancreas extracts were ineffective because of degradation of the hormone insulin from the enzymatic activity from other cells within the pancreatic tissues. Banting developed a protocol for eliminating the cells that secreted the suspected insulin-destroying enzymes, the acinar cells, prior to making the pancreatic extract. In his lab book, Banting wrote: “Diabetus. Ligate pancreatic ducts of
dog. Keep dogs alive till acini degenerate leaving islets. Try to isolate the internal secretion of these to relieve glycosurea." In order to test his theory, Banting required not only bench space, which was provided by Macleod, but also laboratory assistance. Macleod introduced Banting to a young undergraduate lab assistant who was majoring in physiology named Charles Herbert Best. Banting and Best would spend the summer of 1921 testing Banting’s ligation model, and trying to isolate more purified pancreatic extracts. Banting and Best’s first attempt at injecting ligated-pancreas extracts into depancreatized dogs was marginally successful. During the first hour after the initial injection of extract, the dog’s blood sugar dropped from 0.2% to 0.12%. However, the blood sugar failed to drop any lower, despite further extract injections, and the dog died the next morning, probably from surgery-related infection. With the ligated-pancreas extracts’ failures, Banting had the idea to stimulate the pancreas with purified secretin prior to making the extract. The new pancreatic extracts were more successful at lowering blood serum glucose levels, but Banting and Best pressed on, confident that they could develop an even more efficacious extract. It became obvious after some time that the action of trypsin, as well as other harmful enzymes originating from other cells within the pancreatic tissues, could not be completely eliminated with any practical preextraction technique. Banting then recalled that in earlier work by Laguesse, fetal pancreata were morphologically composed primarily of islets of Langerhans, and had relatively fewer acinar cells compared to adult pancreata. Banting and Best then began preparing pancreas extracts from fetal calves. These fetal extracts proved hugely
successful in treating depancreatized dogs. Banting and Best published their work, and were the first researchers to successfully treat diabetes with pancreatic extracts\(^5\).

Although Banting and Best’s fetal calf pancreas extracts were successful in lowering blood serum glucose levels in depancreatized dogs, Macleod began the tedious work of further purifying the extracts to result in pure insulin. Macleod recruited James Bertram Collip to work on purifying the extracts. Collip was a biochemist and had prior experience isolating glandular secretions from tissue extracts\(^12\). In December of 1921, a single dose of Collip’s purified extract was administered to Leonard Thompson, a 14 year old boy with diabetes mellitus\(^13\). Although this represented the first clinical trial in humans of Collip’s purified insulin, the results were not clinically sustainable, and the patient’s blood sugar quickly returned to previous elevated levels. However, treatment of the child resumed in January with one protocol exception: the child would be given regular injections of the extract. The result was a huge success, and regular injections of purified pancreas injections, by this time consisting primarily of insulin, became a routine treatment for diabetes mellitus. Banting and Macleod were awarded the Nobel Prize in 1923; Banting shared his award with his lab assistant Best, and Macleod shared his award with Collip. Figure 1-1 highlights several important milestones in the area of diabetes research and insulin therapy development.
Figure 1-1: A timeline of important milestones in diabetes mellitus research. Please note: distances between events are not to scale.
Since the first clinical use of insulin in 1922, insulin injection therapy has been the predominant clinical approach to maintaining sustainable and physiologically appropriate blood serum glucose levels in patients with diabetes mellitus. However, other therapies have been explored to supply exogenous insulin to diabetic patients. Even before the discovery of insulin, scientists were examining ways to replace the dysfunctional pancreas in diabetic animals. The original pioneer in this field was a Russian born scientist named Oscar Minkowski. Minkowski was the first scientist to associate the clinical symptoms of diabetes with a dysfunction in the pancreas in 1893. Minkowski observed that depancreatized dogs exhibited the metabolic symptoms of diabetes. However, not only did Minkowski properly identify the etiology of diabetes, but he also was the first scientist to reverse the symptoms with transplantation. Minkowski transplanted canine pancreata into depancreatized dogs and noticed a sharp, yet transient, relief of symptoms. Although these experiments failed to achieve sustainable freedom from diabetes symptoms, they represented a fundamentally different clinical and scientific approach to treating diabetes; an approach that would revolutionize the field of diabetes treatment, and an approach that would present significant technical and intellectual challenges.

With the identification of islets of Langerhans in 1893, scientists quickly began work on isolating the hormones, or “extracts”, of the islets for the purpose of treating diabetes mellitus. Although insulin was ultimately purified and mass produced by Eli Lilly et al., the ability to transplant the individual islets remained elusive, as existing technologies and protocols were unable to isolate the islets
from their surrounding tissues. In 1967, Paul Lacy developed a method in which indi-
individual islets could be completely removed from their surrounding acinar tissues. Lacy was able to achieve islet isolation by using an enzyme that dis-
sociated the extracellular matrix of the tissues, composed largely of collagen, with an enzyme called collagenase. With the newly established protocol for islet isolation, Lacy opened the door for a new era of diabetes research, islet transplantation.

The adult human pancreas contains approximately 1-2 million islets, and comprises 1-2% of the mass of the pancreas. The human islet is approximately 100-400 μm in diameter, and contains approximately 2000-3000 cells. Figure 1-2 illustrates an image of an isolated adult rat islet. There are 4 cell types found in the islet: α-cells secrete glucagon, β-cells secrete amylin and insulin, δ-cells secrete somatostatin, and pp cells secrete a pancreatic polypeptide whose function has not been established. Figure 1-3 illustrates a schematic representation of an islet and the cell types within the islet. Although the islets comprise only 1% of the total pancreatic mass, they receive over 15% of its blood flow. The islet vasculature contains capillary networks that are both in series and in parallel with the surrounding exocrine circulation. The size of the islet determines the architecture of the vasculature that serves it, with smaller islets being fed from a single arteriole and the larger islets being supplied by several arterioles. Additionally, the capillaries of the islets exhibit distinct ultrastructures with regard to the endothelium of the exocrine capillaries. For example, the capillaries of the islets are highly fenestrated, containing
approximately 10 times more fenestrae than the exocrine capillaries\textsuperscript{24}. These fenestrae are critical for the proper exchange of chemical messengers and hormones between the blood and the islet cells, and are likely partly maintained by the high amount of vascular endothelial growth factor A (VEGF-A) produced in the cells of the islet\textsuperscript{25,26}. The $\beta$-cells of the islet represent the only physiologic source of insulin, and therefore dysfunctions in pancreatic $\beta$-cells will result in insulin insufficiency, a condition whose only remedy is insulin replacement, either by injection or transplantation.
Figure 1-2: An image of an isolated adult rat pancreatic islet. This islet was isolated by Alton Hiscox using adapted and optimized methods of Lacy, et al.
Figure 1-3: A schematized image of a pancreatic islet illustrating the various cell types within the islet.
Islet transplantation began in earnest in the early 1970s. The first recorded human islet transplantation occurred in 1974 at the University of Minnesota\textsuperscript{27}. Although the trial did not result in clinically significant and sustainable results, it highlighted a number of issues associated with islet transplantation, issues that persist to this day. Since 1974, there have been a number of human trials of islet transplantation, several of which are illustrated in figure 1-4. The protocol for human islet transplantation has varied greatly between research groups, especially with regards to the exogenous islet source. The earlier attempts at islet transplantation used mostly porcine islets\textsuperscript{28-31}, while later attempts adopted human cadaveric islets\textsuperscript{32, 33}. While these studies persist, they are all plagued with several fundamental issues.
Figure 1-4: A brief summary of several human trials of islet transplantation

<table>
<thead>
<tr>
<th>Year</th>
<th>Author</th>
<th>Islet Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>1974</td>
<td>Najarian, et al</td>
<td>porcine</td>
</tr>
<tr>
<td>1995</td>
<td>Groth, et al</td>
<td>porcine</td>
</tr>
<tr>
<td>2000</td>
<td>Shapiro, et al</td>
<td>human</td>
</tr>
<tr>
<td>2005</td>
<td>Wang, et al</td>
<td>porcine</td>
</tr>
<tr>
<td>2006</td>
<td>O’Connell, et al</td>
<td>human</td>
</tr>
<tr>
<td>2007</td>
<td>Elliott, et al</td>
<td>porcine</td>
</tr>
</tbody>
</table>
There are several issues that limit the efficacy of islet transplantation, including the number of islets necessary in order to impart a significant reduction in insulin-dependence, the survival of transplanted islets, and the immunosuppressive therapies used to prevent host rejection of the allografts\textsuperscript{32}. Although it has been estimated that as little as 20\% of the number of endogenous islets are necessary to establish normal blood serum glucose levels\textsuperscript{34,35}, up to 70\% of the transplanted $\beta$-cell mass dies shortly after transplantation\textsuperscript{36-38}. Since this drastic loss of islet implant mass is seen both in immunodeficient and syngeneic animal models, the loss of cell mass is likely caused by hypoxia secondary to lack of adequate perfusion\textsuperscript{39-42}. As a result of this hypoxic insult, the majority of islet mass dies quickly following implantation, with hypoxia-induced apoptosis peaking at 2-3 days following implantation, consequently, the number of islets needed to impart a therapeutic benefit is exorbitant\textsuperscript{43-45}. Hypoxic insult continues for about 2 weeks following implantation, and what remains of the original cell mass begins to stabilize. Additionally, it has been well established that the immunosuppressive medications given to transplant recipients adversely impacts implanted $\beta$-cell survival and function\textsuperscript{46}. Another issue resulting in islet graft failure is mechanical damage associated with packing the islets into large masses for intraportal perfusions. These packing procedures are mechanically damaging to the islets, and lead to cell death\textsuperscript{47}. Lastly, in most human islet transplantation protocols, the isolated islets are injected into the portal vein, where they lodge within the portal circulation of the liver, an organ which metabolizes toxins.
Although the liver is chosen as an effective infusion site because of its high vascular density, several issues are associated with intraportal injections, including portal vein thrombosis, liver stenosis, and destruction of islets as a result of toxic liver metabolites. This close proximity of the transplanted islets to these toxins likely exacerbates islet destruction\textsuperscript{48,49}. These fundamental issues have hindered islet transplantation since its inception, and must be resolved in order for appropriate and effective application of human islet transplantation to achieve normoglycemia.

A number of materials sciences and tissue engineering approaches have been employed to develop an implantable device that maintains islet survival and can be used to reverse hyperglycemia. One of the pioneers in this field is Jim Brauker, who developed a polymer-based bag in which islets could be encapsulated\textsuperscript{50}. The success of Brauker’s materials-approach to resolving the issue of islet survival prompted other researchers to develop a wide array of devices intended to stabilize islet survival and function\textsuperscript{51-54}. In each of these studies, two important milestones indicating successful transplantation were assessed, engraftment and revascularization.

Engraftment is the process in which the transplanted islets adapt to their new environment, and is an essential process for proper islet graft function and survival\textsuperscript{55,56}. Several important steps are included in the engraftment process, but arguably none more important than revascularization and subsequent perfusion to the islets. Studies have shown that islet graft reperfusion occurs between 7 and 14 days of implantation, regardless of the implantation site\textsuperscript{57-60}. Although
revascularization has been shown to occur in implanted islets by means of Doppler perfusion studies and histological analyses, the extent of revascularization has not been adequately characterized. Furthermore, recent studies have suggested that the host-driven revascularization process is insufficient to properly reperfuse the implanted islets to maintain their survival and function\textsuperscript{61,62}. This is a troubling observation considering that the majority of transplanted islets are injected intraportally as a result of the liver’s high vascular density\textsuperscript{63}. With these observations, it is clear that revascularization, and therefore reperfusion, must occur as quickly as possible, and that this process must be stimulated to occur, as the host-driven revascularization process occurs too slowly and is not sufficient to adequately reperfuse the transplanted islets. Although this represents a daunting obstacle, several strategies have been explored by researchers to enhance reperfusion.

With the revascularization process representing the most critical milestone for islet graft success, several researchers have investigated methods to enhance islet graft revascularization. One of the more popular strategies includes the use of various growth factors. Vascular endothelial growth factor (VEGF) is an angiogenic growth factor that plays a role in neovascularization. Although VEGF alone is not sufficient for the formation of a functioning vascular bed, research into the effects of VEGF on islet graft survival have yielded promising results. Investigators have shown that VEGF production by the intraislet endothelial cells can be enhanced if the islets are subjected to hypoxic conditions prior to implantation\textsuperscript{64}. Additionally, researchers have shown that pharmacologic
additions of VEGF \textit{in vitro} can stabilize islet graft survival \textit{in vivo}\textsuperscript{65-67}. Although these studies have produced promising data, there are several issues associated with growth factor-related induction of angiogenesis. First, increasing the amount of VEGF available will not necessarily induce a robust angiogenic response on its own; VEGF receptors (flt and flk-1) must be available to initiate intracellular signaling destined to result in an angiogenic response\textsuperscript{68}. Second, the doses of VEGF given are superphysiologic, and are often 100 times greater than the amount of endogenous VEGF secreted to induce angiogenesis\textsuperscript{69}. Additionally, VEGF is not the only growth factor needed in order to produce and maintain a functioning vascular bed; although VEGF will result in new vessel growth, other growth factors, platelet derived growth factor for example, are needed in order to remodel the newly formed vessels and create a functioning vascular bed with inflow and outflow tracks. With these limitations in mind, an angiogenic and revascularization response that more closely mimics intrinsic physiologic responses must be achieved in order to properly reperfuse implanted islet grafts.

The research presented in this dissertation uses tissue-engineering techniques to enhance revascularization of implanted pancreatic islets. Using a similar method to Lacy’s islet isolation protocol, Roger Wagner has developed a method in which microvessel fragments could be isolated from rat epididymal fat pads\textsuperscript{70}. Additionally, James Hoying has developed a method for encapsulating these microvessel fragments within a 3-dimensional collagen matrix\textsuperscript{71}. It is hypothesized that these microvessel fragments provide the stimulus needed for islet graft reperfusion, and represent the foundation upon which this dissertation
was based. The goal of this study was to develop and test an islet implant construct with accelerated microcirculation formation within and around transplanted islets to support islet cell viability and enhance transplanted islet survival. This study is based on previous work utilizing isolated microvessel fragments in a collagen gel to enhance vascular reperfusion with the host\textsuperscript{71}. Previous work in the lab has shown that microvessel fragments undergo angiogenesis \textit{in vitro} and will insosculate with the host circulation within 7 days of implantation\textsuperscript{72}. Additionally, preliminary data has indicated that microvessel fragments produce and secrete VEGF, a growth factor known to enhance islet viability and maintenance\textsuperscript{65-67}. Therefore, this study investigates whether a prevascularized collagen construct with microvessel fragments will support islet survival. These properties of a 3-dimensional microvessel fragment gel were used to encapsulate isolated islets, resulting in a prevascularized pancreatic encapsulating device (PPED), and the effect of this preformed vasculature on implanted islet survival was examined. Figure 1-5 illustrates the structure of the PPED.
Figure 1-5: A schematic representation of the prevascularized pancreatic encapsulating device (PPED).
According to the United States Diabetes association, diabetes represents the sixth leading cause of death in the US and accounts for a quarter of all Medicare dollars spent. Additionally, more than two-thirds of diabetics die from cardiovascular disease, indicating an intimate association between diabetes and cardiovascular disease. As a result of this association, effective treatments for diabetes would also serve to reduce cardiovascular disease and associated deaths. Although islet transplantation represents a promising therapy for the treatment of insulin-sensitive diabetes, the lack of prompt and adequate perfusion to islets following implantation remains a significant limitation. Moreover, hypoxia-related islet death severely limits the long-term viability of transplanted islets. A current approach to limiting the amount of time the islets remain hypoxic subsequent to transplantation is to enhance the rate of reperfusion to the islet implants, thus mitigating the effects of hypoxia on transplanted islets. This research project was designed to address the problem of islet hypoxia, caused by a lack of rapid perfusion to transplanted islets. Specifically, this research project builds upon the lab’s expertise in creating tissue-engineered constructs containing a preformed vasculature to increase perfusion to the implanted islets.

The long-term goal is to create an islet construct that, when transplanted, can remain viable indefinitely, and thereby can be used to permanently treat insulin-sensitive diabetes. The objective for this study was to create an islet construct which has been encapsulated within a preformed vasculature to enhance perfusion to the islets. The central hypothesis driving this research is that the transplanted islets’ failure to function is the result of inadequate perfusion to the
implants, and that by accelerating reperfusion, thus limiting the amount of time the islets remain hypoxic, islet stability and viability can be greatly enhanced.

Specific Aims

Specific Aim 1: *Develop an islet-containing collagen matrix construct and examine islet responsiveness to glucose stimulation in collagen.*

*Working Hypothesis:* Collagen-encapsulated islets will be structurally and metabolically viable, and will respond to glucose stimulation.

Specific Aim 2: *Evaluate a 3-dimensional tissue-engineered prevascularized construct for the purpose of supporting islet cell survival.*

*Working Hypothesis:* Prevascularized collagen gels will provide the revascularization stimulus needed for islet cell survival, and will inosculate with the host vasculature in vivo.

Specific Aim 3: *Assess the ability of a prevascularized pancreatic encapsulation device to maintain islet survival in vivo.*

*Working Hypothesis:* A prevascularized pancreatic encapsulation device will maintain islet survival longer than a non-prevascularized implant.
CHAPTER 2

AN ISLET-CONTAINING COLLAGEN MATRIX CONSTRUCT AND EXAMINE ISLET RESPONSIVENESS TO GLUCOSE STIMULATION IN COLLAGEN.

Introduction

The development of the first islet isolation protocols in 1967 quickly highlighted the problems associated with removing pancreatic islets from their native microenvironment. There are many issues resulting in the drastic decline in viability of pancreatic islets in vitro. First, as islets are highly vascularized structures, due to their large metabolic demands, removing islets from their surrounding tissues severs their vascular supply, and the islets become ischemic and subsequently hypoxic. One result of this hypoxic event is initiation of programmed cell death. Although islets in culture can receive nutrients and oxygen from their surrounding media, cells within the center of islets are susceptible to diffusion barriers. This diffusion barrier typically results in cell death in the center of islets, as the cells around the periphery of the islet are closest to the nutrient rich media. Second, extracellular contacts between the islets and the surrounding extracellular matrix proteins are disrupted during the isolation procedure. Consequently, cells that lose contact with their extracellular matrix can undergo anoikis, or programmed cell death initiated by removing inhibiting apoptotic signals through integrin binding. Third, the isolation
procedure itself is mechanically and enzymatically destructive. The procedure involved enzymes which disrupt cell-cell interactions, and also uses procedures, such as centrifugations and filtering steps, which impart shearing forces on the islets. These three issues combine to result in an isolation procedure yielding damaged or dying islets. Improving islet yields and the quality of isolated islets is an emerging field that includes protocol modifications including buffer recipe changes, enzyme type and concentration changes, pancreas perfusion modifications, and varying the islet purification steps\textsuperscript{74}. Additionally, as culturing the islets is becoming an inevitability, several researchers are investigating ways in which varying culturing methods and techniques can be used to maintain islet viability and survivability \textit{in vitro}. These methods include culturing the islets with various growth medias and in different biochambers\textsuperscript{77-79}. In order for an efficacious islet transplantation protocol to exist, islet isolation and culturing techniques must be refined to result in the most structurally sound and viable islets possible.

It has been well established that the extracellular matrix of native pancreatic islets plays a crucial role in islet structure and function\textsuperscript{75, 80-82}. Cell-matrix interactions are critical for proper intracellular signaling events that lead to cell proliferation, differentiation, migration, and suppression of apoptosis\textsuperscript{83-89}. Cells that are disrupted from their extracellular matrix connections can undergo apoptosis in a process called anoikis\textsuperscript{75, 76, 90}. Additionally, research has also suggested that various metalloproteinases (MMP-2) can degrade the islet extracellular matrix, resulting in $\beta$-cell dysfunction and consequent
hyperglycemia\textsuperscript{91}. The extracellular basement membrane of the native islet contains several important proteins, including collagens I, III, IV, and V\textsuperscript{92, 93}. Therefore, in order for transplanted islet grafts to maintain structural and functional viability, the islets must be encapsulated within a matrix that not only provides structural support, but also maintains extracellular contacts that are critical for normal islet cell function. Specific aim #1 was designed to address the issue of islet-extracellular matrix interactions. Specifically, this aim investigated encapsulating islets within a rat tail type I collagen matrix, and the effects the collagen matrix would have on islet function and response.

**Specific Aim 1:** *Develop an islet-containing collagen matrix construct and examine islet responsiveness to glucose stimulation in collagen.*

*Working Hypothesis: Collagen-encapsulated islets will be structurally and metabolically viable, and will respond to glucose stimulation.*

**Methods**

*Islet isolations:* For islet isolations, male Sprague-Dawley rats were used (350-500 grams). All islet isolations were performed using sterile technique, and in compliance with IACUC approved protocols. Islet isolations were performed using adapted methods of Lacy as modified by Limesand, et al, and are briefly described\textsuperscript{16, 94}. After administration
of 0.6cc of Nembutal® (30 mg/kg) intra-abdominally using a 1cc syringe and 18 gauge needle, the animal’s ventral aspect was shaved with an electric shaver. The shaved area was sprayed with an antiseptic wash, and the abdominal cavity was opened with dissecting scissors from the genitals to the diaphragm. The liver was inverted using sterile cotton swabs, and the common bile duct was located using a surgical microscope with a 16x objective (Zeiss). A 4-0 silk suture was placed under the bile duct distal to the last portal branch, and an incision was made proximal to the suture. The common bile duct was cannulated with PE50 tubing attached to a syringe hub. The syringe was filled with liberase® solution at a concentration of 0.3 mg/ml (Roche, Indiana). The pancreas was then clamped across the duodenum using a hemostat, and 12 ml of the digestion solution was perfused into the pancreas. The pancreas was removed with dissecting scissors, and placed in a 50 ml tube containing 5 ml of the digestion solution. The tube was placed in a water bath at 37°C for 20-30 minutes. The digested pancreas was then washed with 30 ml of cold quenching buffer (Hanks Balanced Salt Solution with 10% fetal bovine serum [FBS]), and centrifuged in a Beckman TJ-6 centrifuge (Beckman Coulter, Fullerton, CA) at 1.0 RCF (relative centrifugal force in g’s) for 5 minutes to separate free islets from exocrine tissue. Once separated, the islets were purified on a Ficoll® (polysucrose) gradient by resuspending the islet pellet in 8 ml of 25% polysucrose, and layering on 5 ml each of 23%, 20% and 11% polysucrose. The tube was centrifuged at 1.4 RCF for 20 minutes, and the 20% layer containing the islets was removed. The islets
were washed with cold quenching buffer, and placed in equilibration buffer (Kreb’s Ringer buffered solution, 0.1% BSA, 2.0 mM glucose).

Cell viability assay: Upon isolation, islets were either picked immediately using a p20 pipetman and dissecting scope with a 4x objective, or incubated for 90 minutes at 37°C in islet equilibration buffer. To pick the islets, a p20 pipetman with tip was used to aspirate the islet and the surrounding 10 µl of media under a dissecting scope with a 4x objective. Ten islets were picked and placed into individual wells of a 48 well plate, and three independent viability assays were performed. In each assay, the experiment was performed on 5 groups (n=5) of 10 islets each. In the first assay, dithizone (0.25 mg/ml) was added to the wells, and incubated for 5 minutes. Red viable islets were then counted, and then divided by the total number of islets to give the percent viable islets. In the second assay, dimethylthiazol tetrazolium (MTT, 5 mg/ml) was added to the wells, and incubated for 30 minutes. Dark purple viable islets were then counted, and then the number was divided by the total number of islets to give the percent viable islets. In the last assay, Live/Dead solution (5 µl stock calcein, 20 µl stock ethidium homodimer, 10 ml PBS) was placed into the well, and incubated for one hour. The number of live cells (green) was then counted, and divided by the number of total cells (green and red) to get a ratio of live to total cells.

Bioreactor Setup: Islets were isolated and encapsulated within collagen gels (3 mg/ml) at a concentration of 100 islets/200 µl of collagen. To encapsulate the
islets, cold non-polymerized rat tail type-1 collagen was mixed with 1x DMEM (5 mM glucose) on ice. The islets were then spun down at 1.0g for 5 minutes, and the islet pellet was suspended in the non-polymerized collagen mix. The collagen was pipetted into individual wells of a 48 well plate (200 µl), and the collagen was polymerized at 37°C for 30 minutes. The islet containing collagen gels were then placed within the flow chamber of the bioreactor setup (T.G.I., Hawaii), and control gels were placed within 50 ml tubes. The flow chambers were connected in series with a peristaltic pump (Watson Marlow, Cornwall, UK) and media reservoir using gas-permeable tubing, and flow was introduced at a rate of 9 ml/minute. After 7 and 14 days of culturing at 37°C with 5% CO₂, the gels were removed from the biochamber and control tubes, and were homogenized in 1 ml of an acid ethanol solution (50% 1N HCl and 50% 70% ethanol) with a tissue sonicator to release intracellular insulin. The homogenized gels were assessed for the presence of insulin using an RIA kit (Linco®, cat # RI-13K). Intraassay validation was confirmed using internal quality control standards. Since only two bioreactors were available, each treatment group (bioreactor vs. static conditions) was repeated twice with two samples per group (a total of n=4 for each treatment group).

Insulin diffusion and sequestration into collagen gels: 200 µl collagen gels were polymerized in a 48 well plate at a concentration of 3 mg/ml (collagen diluted in 1X DMEM containing 5mM glucose as previously described. After polymerization, gels were placed in 1 ml of three different insulin solutions (1.6
ng/ml, 8 ng/ml, and 76 ng/ml) for one hour at room temperature. The gels were then placed in a PBS wash for one hour at room temperature. The gels were washed a total of three times, and then homogenized to release any bound insulin using acid ethanol and a tissue sonicator. All five supernatants (original incubation, three washes, and gel homogenate) were assayed for the presence of insulin with an RIA kit (Linco®, cat # RI-13K). Each treatment group (1.6 ng, 8.0 ng, and 76 ng) was repeated 5 times (n=5). The assay was validated with QC controls, and standard curves across several assays confirmed interassay validity.

**Insulin diffusion out of collagen gels:** Collagen gels were polymerized in a 48 well plate containing I^{125}-labeled insulin. The gels were then removed from the well, and washed with three different washing protocols: 1 hour at room temperature with 1X PBS, overnight at 4°C with 1X PBS, and room temperature for one hour in a 10x wash solution volume (PBS). The wash solutions, the gels, and the liquid remaining in the wells were all assayed for radioactivity using a Gamma® 4400 counter (Beckman Coulter, Fullerton, CA). Each treatment group was performed with 5 different gels (n=5).

**Glucose stimulated insulin secretion:** Islets were placed in 2.0 mM glucose, 20.0 mM glucose, or 20.0 mM glucose on ice for one hour in a 1.5 ml tube to extract insulin from the islets. The supernatants were saved and frozen at -20°C. After thawing, radioimmuno assays (Linco® Research, assay # RI-13K, see appendix E) were performed to quantify the amount of insulin produced by the implants.
The RIA kit uses radioactively labeled insulin to compete with the insulin within the samples for antibody binding. Lastly, cell lysates were taken by sonicating the islets in acid ethanol and assaying for total insulin content to normalize the data. Two statistical comparisons were conducted. First, the percent of basal for each treatment group (free and collagen islets) were examined by taking the normalized insulin secretion data and comparing the relative secretion to the basal secretion within that treatment group, with basal secretion representing 100%. Second, the absolute normalized insulin secretion in each treatment group was examined by plotting the absolute normalized insulin secretion for each treatment group to indicate amounts, rather than percent basal, secretion. Due to the minimal numbers of islets available, the two treatment groups were examined using two different islet picks, and each treatment within each pick was conducted 3 times (n=3). To correct for inter-isolation variances in responsiveness, basal responses to glucose stimulation was conducted on a single third pick to confirm that the two picks could be appropriately compared. The result of this third pick was identical to the comparison between the first two picks, confirming that the two treatment groups could indeed be compared.

*Islet construct implantation:* For implantations, female severe combined immunodeficient (SCID) mice were used (CB-17/ICR background bred at the Arizona Cancer Center). The mice weighed approximately 22-25 grams. All implantations were performed using sterile technique, and followed IACUC approved protocols.
Female SCID mice were anesthetized with 0.3 ml of 2.5% avertin solution using a 1cc syringe and 22 gauge needle. The dorsal aspect of the left and right haunches were prepared for surgery. An incision was made and blunt dissection with a hemostat was performed to make a subcutaneous pocket. The implant was then removed from the well plate using a sterile spatula, and the implant was placed in the pocket. The incision was closed with sterile skin staples.

*Islet construct explantation:* All explantations were performed using sterile technique, and in compliance with IACUC approved protocols.

Mice were anesthetized with 0.3 ml of 2.5% avertin solution using a 1cc syringe and 22 gauge needle, the skin staples were then removed, and an incision made inferior to the implant site. The skin was peeled back. Using fine scissors and forceps, the implant was teased away from the skin and the surrounding tissues; a small amount of tissue surrounding the implant was also removed for histological comparison.

*Insulin staining:* Paraffin sections of implants were washed in xylene for 20 minutes, and rehydrated in ethanol solutions (100% to 50%). The slides were washed in DCF-PBS, and blocked for one hour at room temperature (rabbit serum, 0.1% BSA, 0.1% evaporated milk). The slides were washed in DCF-PBS for ten minutes, and guinea pig anti-swine primary insulin antibody (Dako, Germany) was applied (1:100 dilution). The slides were placed in a 37°C
incubator for 90 minutes. The slides were then washed with DCF-PBS for 15 minutes, and rabbit anti-guinea pig secondary-HP (Invitrogen, Carlsbad, CA) was applied (1:200 dilution). The slides were placed in a 37°C incubator for 90 minutes, and then washed in DCF-PBS for 15 minutes. The slides were mounted with Permoun® (Invitrogen, Carlsbad, CA), and stored in a light-controlled case. 8 implants were assessed for the presence of insulin (n=8).

Results

*Islet isolation optimization and quality validation*

A total of 38 islet isolation preparations were performed on 130 animals before the optimum viability was achieved (>90%). Three independent viability assays were performed on the isolated islets under two different conditions and the results are provided in figure 2-1. Islets were examined immediately following isolation, or islets were examined following 90 minutes of equilibration at 37°C in equilibration buffer. The first assay performed was a dithizone assay, which examines the presence or absence of intracellular insulin granules. The second assay was a dimethylthiazol tetrazolium assay (MTT), which examines cellular metabolism. The last assay was a calcein/ethidium homodimer live/dead assay, which assesses cellular structure and metabolism. The results of all three viability assays indicate that, using our isolation technique, islets are structurally and metabolically viable. Specifically, using the MTT and live/dead assays, cells
that were equilibrated for 90 minutes have higher viability than islets not
equilibrated. Although 38 total preparations were performed, the viability data
presented represent the last 3 islet isolation preparations, and 5 samples of islets in
each of the last three preparations were examined with the three viability assays
(n=5).
Figure 2-1: Cell viability data for three independent viability assays, DTZ, MTT, and Live/Dead, under two conditions, no equilibration and 90 minutes equilibration. Each treatment group was performed on 5 samples (n=5). Statistical significance was examined using a two-sided student’s t-test, *p < 0.05. Figure was taken from Hiscox, et al, Journal of Tissue Engineering, 2007 (in press)

<table>
<thead>
<tr>
<th></th>
<th>DTZ †</th>
<th>MTT ‡</th>
<th>Live/Dead §</th>
</tr>
</thead>
<tbody>
<tr>
<td>No Equilibration</td>
<td>86.3 (5.4)</td>
<td>78.3 (6.2)</td>
<td>79.4 (1.1)</td>
</tr>
<tr>
<td>90 Minute Equilibration</td>
<td>87.1 (5.3)</td>
<td>95.6 (2.7)</td>
<td>90.6 (1.8)</td>
</tr>
</tbody>
</table>

† - Islets were either assayed immediately after isolation, or allowed to equilibrate for 90 minutes at 37°C
‡ - Dithizone assay, chelates zinc and results in reddish-brown viable cells
§ - [4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide assay, results in purple crystal formation in metabolically active cells
€ - Calcein/ethidium homodimer assay, results in fluorescent green structurally intact and viable cells or fluorescent red structurally damages cells
Islets placed within bioreactors under flow conditions maintain intracellular insulin longer than islets cultured under static conditions

To examine the effect of culturing islets under flow conditions on intracellular insulin preservation, islets were encapsulated within collagen gels and cultured for 7 and 14 days under two conditions: islet gels were either placed within bioreactors and subjected to constant media flow, or islets were placed in static culturing dishes. In both treatment groups, islets were provided the same volume of culture media (see appendix C). Figure 2-2 illustrates an image of the bioreactor setup, which includes flow chambers (A), a peristaltic pump (B), and media reservoirs (C). After 7 and 14 days of culturing, islet gels were homogenized, and islets were lysed to free intracellular insulin. Additionally, islet cell extracts at day 0 were taken and used as a positive control against which the treatment group sample would be assessed; the amount of insulin in each treatment group was standardized to the amount of starting insulin. The insulin concentrations in each treatment group were assayed using an insulin RIA kit from Linco®. The standard curve for the RIA is shown in figure 2-3, and the equation generated from the best fit plot was used to assess insulin concentrations of the unknown samples. Figure 2-4 shows the RIA data for the amount of intracellular insulin in each treatment group relative the amount of starting intracellular insulin, and shows that islets placed in bioreactors under flow conditions showed statistically significantly more intracellular insulin than islets placed under static culturing conditions.
Figure 2-2: An image of the bioreactor setup used to culture islets under flow conditions. A-biochambers into which islets were placed, B-peristaltic pump, C-media reservoir. The entire setup was placed inside a 37° C incubator with 5% CO₂. The biochambers (A) were provided as a generous gift from Tissue Genesis, Inc.
Figure 2-3: RIA standard curve. The equation from the best fit plot was used to examine insulin concentrations in unknown samples. Each standard sample was assayed in duplicate, and the averages at each concentration are represented above. Assay quality assurance and quality control data are provided by Linco® and can be found in appendix E.
Figure 2-4: Intracellular insulin concentrations in islets placed within biochambers (BR) and islets placed within static culturing conditions (ST). Both treatment groups were normalized to the amount of starting intracellular insulin, and their values are represented as a percentage of starting insulin. Each treatment group contained 4 samples (n=4) Statistical significance was examined using a two-sided student’s t-test *p < .01.
Collagen gels do not bind or sequester free insulin

Collagen gels were placed in three different insulin solutions, low (1.6 ng/mL), medium (8 ng/mL), and high (76 ng/mL) concentrations of human insulin. After an incubation of one hour, the gels were placed in three subsequent washes of PBS for an hour each. Finally, the gel was homogenized to release any bound insulin, and all five solutions were assayed for the presence of insulin. The data presented in figure 2-5 indicates that for all insulin concentrations examined, the PBS washes showed only trace amounts of insulin, and that the diffusion out of the gels is relatively constant. Additionally, the gel homogenates also showed only trace amounts of insulin. No evidence of saturation within the gels is seen, as the highest amount of insulin remaining within the gel was 0.52 ng, which is less than the amount of insulin that entered the gel in each of the treatment groups. Additionally, figure 2-6 illustrates summary statistics comparing the amount of insulin that remained in each gel to the total amount that entered the gel. These data show that the amount of insulin that remained in the gel after entering was 24%, 14%, and 7% for low, medium, and high starting concentrations respectively. Additionally, it is estimated that these gels had approximately 20% dead space, so the predicted values of the amount of insulin that would enter the gel in each treatment group is 0.256, 1.28, and 12.16 ng of insulin for the low, medium, and high insulin amounts respectively. However, the data indicate that 0.5, 1.82, and 7.42 ng of insulin in each treatment group actually entered the gel; in the first two treatment groups, more insulin than was expected entered the gels. Lastly, from the gel volume and gel dead space calculations
(200 µl gels, 80% void volume = 160 µl), the amount of insulin that is predicted to remain inside the gel after the three washes is <0.01, .01, and 0.04 ng for the low, medium, and high concentrations of insulin respectively (theoretically, 86% of all insulin inside the gel should have diffused out with each wash given the gel and wash volumes: 160 µl void volume/1000 + 160 µl wash volume = 14%, 100%-14% = 86%). Combined, these data indicate that insulin diffusion throughout the collagen gel occurs unobstructed and freely, however, a larger than expected amount of insulin remains within the gels, suggesting possible non-covalent interactions with the collagen.
Figure 2-5: Insulin diffusion and sequestration data showing the amount of insulin in the original incubation solution, the amount of insulin in each PBS wash, and the amount of insulin remaining within the gel after three washes. Values are presented as means with the standard error. Each treatment group was examined using 5 different samples (n=5).


<table>
<thead>
<tr>
<th>Incubation Solution†</th>
<th>First Wash ng (SEM)</th>
<th>Second Wash ng (SEM)</th>
<th>Third Wash ng (SEM)</th>
<th>Gel Homogenate ng (SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low [Insulin]</td>
<td>1.6 (0.05)</td>
<td>.14 (0.003)</td>
<td>.12 (0.002)</td>
<td>.12 (0.001)</td>
</tr>
<tr>
<td>Medium [Insulin]</td>
<td>8.0 (0.64)</td>
<td>.56 (0.01)</td>
<td>.52 (0.002)</td>
<td>.26 (0.003)</td>
</tr>
<tr>
<td>High [Insulin]</td>
<td>76.0 (2.0)</td>
<td>3.0 (0.08)</td>
<td>2.6 (0.02)</td>
<td>.52 (0.002)</td>
</tr>
</tbody>
</table>

† - Collagen gels were incubated in three different concentrations of insulin for one hour at 23°C
‡ - After the original incubation, the collagen gels were placed in DCF-PBS for one hour in three different washes at 23°C
× - After the three washes, the collagen gels were homogenized to assess any bound insulin
Figure 2-6: Insulin diffusion and sequestration summary data showing the amount of insulin remaining inside the gel after three washes and the amount of insulin that washed out. These data represent the location of the insulin that actually entered the gel after the original incubation (n=5).
Insulin diffuses out of collagen gels with minimal diffusion barrier

In the second series of studies, collagen gels were polymerized containing I\(^{125}\) labeled insulin (5 \(\mu\)Ci/mL). After polymerization, the gels were placed into three different washing conditions: one hour wash with equal volume of wash solution to gel (1 ml:1 ml), overnight wash with equal volume of wash solution to gel (1 ml:1 ml), and one hour wash with 10-fold more wash volume than gel (1 ml:10 ml), and the amount of insulin diffusion out of the gel was examined by measuring the radioactivity using a gamma counter. Under all three washing conditions, radioactively labeled insulin could be found in 4 places: insulin could have remained in the gel, insulin could have diffused out of the gel into the wash, insulin could have remained within the well plate and not incorporated into the gel, or insulin could have bound to transfer pipets or other dishes and is therefore inconsequential for these experiments. Figure 2-7 illustrates the amount of insulin in all 4 places for the all washing conditions. For all three experiments, detectable amounts of insulin either remained behind in the well plate or was lost, ostensibly by adhering to transfer pipets or culturing dishes. Figure 2-8 illustrates the data for the insulin that was incorporated into the collagen gels for all three washing conditions, and indicates that greater than two-thirds of all insulin polymerized within the collagen gel diffused out of the gel.
Figure 2-7: The amount of radioactively labeled insulin found in the gel, the wash, the well, and unknown for all three washing conditions. (n=5)
Figure 2-8: Data showing the amount of insulin diffusion out of the collagen gel relative to the amount of insulin that was polymerized into the collagen gels in all three washing conditions. Each washing condition was performed on 5 different samples (n=5).

Islets placed within collagen gels respond more robustly to glucose stimulation than islets not encapsulated within collagen gels

Isolated islets were hand picked and either placed into 1.5 ml centrifuge tubes, or polymerized into collagen gels. The free islets and islets in collagen gels were incubated in three different solutions, 2.0 mM glucose, 20.0 mM glucose, and 20.0 mM glucose on ice, representing basal secretion, stimulated secretion, and inhibited secretion of insulin respectively. The supernatants were saved, and the islets were lysed to obtain total intracellular insulin. After normalization to the total intracellular insulin content, the percent of basal secretion for the free islets and the islets in collagen gels for the three treatment groups was analyzed. Figure 2-9 illustrates the data for glucose stimulated insulin secretion (GSIS) for free islets, as well as islets placed within collagen. Both groups were stimulated to secrete insulin, and also insulin secretion was successfully inhibited, showing that the insulin secretion was glucose responsive. The free islets exhibited a statistically significant glucose stimulated insulin release of 336% (± 41) of basal secretion, while the islets placed in collagen showed an insulin secretion of 176% (± 76) of basal secretion. In addition to comparing the percentage of basal insulin secretion in stimulated islets, the total insulin release in each treatment group was examined to assess differences in absolute insulin secretion between treatment groups. In this experiment, the absolute values of insulin secretion normalized to intracellular insulin content were assessed. Figure 2-10 illustrates that islets placed in collagen secreted approximately four times more insulin than free islets in each treatment group, and all treatment groups showed statistically
significant differences. From these data, although the percentage increase over basal secretion is more for free islets than islets in collagen (336% (± 41) vs. 176% (± 76) respectively), islets in collagen secreted more insulin over basal when stimulated than free islets (7.7 ng/hour/10 islets vs 3.8 ng/hour/10 islets), and therefore responded more robustly to glucose stimulation. Figure 2-11 illustrates the rates of insulin secretion per hour per 10 islets in free islets and islets encapsulated within a collagen matrix. Lastly, it should be noted that the gels were not washed prior to lysing the collagen-encapsulated cells. Therefore, the amount of intracellular insulin that was observed was more for collagen-encapsulated islets as this amount represents the amount of intracellular insulin content plus the amount of secreted insulin that may have been sequestered within the collagen gels. This would effectively confound these data by normalizing the secreted amounts to a larger amount, which would result in a smaller observed amount of secreted insulin; if the secreted data are normalized to artificially elevated intracellular content data, then the resulting secreted data would appear smaller than actual amounts.
Figure 2-9: Glucose stimulated insulin secretion in islets encapsulated within collagen and islets not encapsulated within collagen (free). All insulin concentration values were normalized to total intracellular insulin concentrations and reported relative to the amount of insulin secreted under basal conditions (non-stimulated). Asterisk represents a statistically significant increase in the 20 mM treatment group over the 2.0 mM treatment group for free islets. Each incubation within both treatment groups contained 3 different samples from two different islet preparations (n=3). Statistical significance was assessed using a 2-sided student’s t-test. *p < 0.001 Hiscox, et al, Journal of Tissue Engineering, 2007 (in press).
Figure 2-10: The absolute values of insulin concentrations in GSIS experiments in encapsulated vs. free islets. All values were normalized to total intracellular insulin concentrations. Asterisks represent statistically significant increases in each treatment group over their corresponding treatment groups for the “free” islets. Statistical significance was examined using a 2-sided student’s t-test. *p < 0.05. Hiscox, et al, Journal of Tissue Engineering, 2007 (in press).
Figure 2-11: The rate of insulin secretion per hour per 10 islets in free islets and islets encapsulated within a collagen matrix.
Collagen gels containing islets alone do not support islet survival in vivo

Collagen gels containing islets only were implanted subcutaneously into SCID mice. The implants were removed after 7 days and examined for surviving islets, as indicated by an insulin immunostain. At 7 days of implantation, no insulin containing cells were found within the implanted collagen gels, indicating that none of the islets had survived.
Discussion

Upon isolation, pancreatic islets are inherently unstable. The isolation process is mechanically and chemically destructive, and not only severs all vascular connections, but also disrupts the linkage between the extracellular matrix proteins around the islet cells and the exocrine cells of the islets, a linkage that confers protection from programmed cell death. The isolation procedure is a multi step process that involves a number of surgical and tissue culture techniques. In the process of refining and optimizing the isolation procedures, many variables were considered. These variables included changes in enzyme concentration and amounts, changes in digestion time and agitation, changes in filtering and purification steps, and changes in centrifugation steps and other potentially mechanically disruptive procedures. Ultimately, an islet isolation protocol was achieved that resulted in metabolically and structurally viable cells. The main technical obstacle in specific aim #1 was the development and optimization of an islet isolation protocol. Although the original isolation protocol was developed by Lacy et al in 1967, the islets isolated in this specific aim involved significant alterations to Lacy’s original protocol. Although earlier isolation protocols resulted in greater numbers of islets, preliminary viability assays indicated that as many as 50% of the islets were not viable (data not shown), thus prompting investigations into refining and optimization of the isolation procedure. Figure 2-1 illustrates metabolic and structural viability data for islets isolated with the revised protocol. The results of these trials indicated that islets were more viable than islets isolated in previous protocols. Three
separate, independent viability assays were chosen for this specific aim because, while each assay only reveals a small piece of information relative to overall islet health, combined the three assays provide a more detailed description of islet health. Specifically, the DTZ assay provides information on intracellular insulin content, the MTT assay provides information on intracellular mitochondrial metabolism, and the Live/Dead assay provides information on both cell structure and cytoplasmic metabolism. Additionally, these data also indicate that allowing the islets to equilibrate for 90 minutes prior to picking and assessment increased the resulting viability. Several factors may account for this increase in viability. First, allowing the islets to incubate for 90 minutes assures that any damaged islets will not survive this 90 minute period, and so will not be picked for further assessment; islets that may have been chosen for viability examination 90 minutes earlier have fallen apart, and will no longer be used. Also, the isolation procedure uses enzymes and salt solutions that are harmful to the islets. Allowing time for the cells to equilibrate and rebalance their intracellular environments makes the cells more receptive to the viability reagents. Essentially, non-equilibrated cells may not necessarily be non-viable, they just may not be receptive to the viability reagents because their intracellular environments have not reestablished a state of homeostasis.

Although culturing of islets prior to transplantation is not ideal, because of the large number of islets necessary in order to impart a therapeutic benefit, culturing of islets for a brief period of time is indicated in many cases. As such, culturing methods that maintain islets survival and intracellular insulin granules
will improve the overall efficacy of islet transplantation protocols. Data for intracellular insulin content from islets placed within bioreactors indicates that islets placed under flow conditions maintain their intracellular insulin stores longer than islets cultured under static conditions. Although these data do not suggest a mechanism to explain the increase in islet survival, several ideas exist which could address the difference. First, media taken from the bioreactors was statistically significantly less acidic than media taken from the static culturing dishes (data not shown). A possible explanation for this difference is that islets placed under static culturing conditions acidify the media as they undergo programmed cell death. Additionally, the media within the bioreactors is constantly exchanged with fresh media from a media reservoir, so changes in media pH are buffered and protected. Another possible theory to explain why islets within bioreactors maintain intracellular insulin longer is that islets placed under flow conditions are subject to slight shearing forces, forces that have known proliferative effects in other cell types, including bone.95

Another critical question that was addressed in this specific aim was whether or not insulin diffuses through a collagen gel, and if the collagen matrix would bind to and sequester released insulin. Clearly, an insulin producing implant that inhibited insulin diffusion would not be effective, therefore, insulin diffusion out of collagen had to be demonstrated. Given the volume of the collagen gel (200 µL) and the predicted void volume (80%), we can divide the overall void volume of the gel by the amount of solution into which the collagen gels are placed (160 µL/1000 + 160µL = 14%). The resulting number predicts
that, given free diffusion and complete equilibration, 14% of the insulin should remain within the collagen gel, and 86% of insulin should diffuse out of the collagen gel in each incubation. In experiments where collagen gels were placed in an insulin-containing solution, and then washed, the data shows that, when corrected for collagen gel void volume, insulin is able to enter the gel at amounts that are slightly larger than predicted, illustrating non-obstructed diffusion of insulin into the gel. However, the insulin that diffuses into the gel diffuses out with slightly slower diffusion kinetics than those predicted given the gel and wash volumes. However, the overall amount of insulin that diffused out of the collagen gel was actually greater than is predicted, indicating that these collagen gels diffuse insulin at slower, more steady rates, and given time, will diffuse insulin completely. These two observations have two possible explanations, but they are not mutually exclusive. First, the observation that insulin enters the collagen gel at predicted amounts could suggest that collagen gels are not resistant to insulin diffusion. The second observation indicates that a small percentage of insulin may remain within the gel, either sequestered or mechanically trapped, which may have implications for the overall amount of insulin secreted by islets bound within collagen gels. Certainly insulin that is within the collagen matrix diffuses out, as evidenced by the two diffusion experiments. Additionally, collagen does not seem to bind insulin specifically, as the diffusion kinetics indicated that the collagen-insulin binding relationship is non-specific and non-saturable. The implication from these experiments is that a collagen matrix represents a good scaffold for the secretion of insulin.
These diffusion experiments have implications for the eventual function of these implants. A critical function of this implant, ultimately, will be its ability to secrete insulin, and for that secreted insulin to enter the vasculature of the host. There are at least two ways for secreted insulin to enter the vasculature. First, the insulin can enter the vasculature by direct islet-host inosculation, whereby the insulin is secreted and directly taken up by the intraislet vasculature. Second, the insulin can be diffused into the interstitium, and taken up by the surrounding vasculature. This mode of insulin uptake can only occur if insulin indeed can diffuse into the surrounding interstitial spaces. A requirement confirmed with these experiments.

The last set of experiments was designed to assess the effect of a collagen matrix on insulin sensitivity. Typically, islets will respond to elevations in glucose concentrations by secreting insulin. This event is initiated by glucose receptors, which mediates intracellular signaling cascades ultimately resulting in elevations in intracellular calcium, inducing insulin granules to fuse with the cell membrane and release insulin\textsuperscript{96}. Additionally, islets will secrete basal levels of insulin without the presence of insulin secretagogues. Generally, glucose stimulation results in an increase in insulin secretion over basal levels of 5-300%. This wide range of values is attributable to variations in islet size, inter-species differences, and variances in the islet isolation procedure, however, it is generally accepted that the larger the response to glucose, the more functional and viable the islets. Figure 2-11 illustrates data from glucose stimulated insulin secretion experiments (GSIS) in which free islets and islets encapsulated within a collagen
matrix were examined for glucose responsiveness. The data show that although free islets increased their secretion over basal levels more than collagen encapsulated islets, islets within collagen gels responded to glucose stimulation to a level that is consistent within the literature to be considered highly responsive. More significantly, however, is the observation that islets placed within a collagen-based matrix release 4 times more insulin than free islets at all treatment groups examined, suggesting that rat-tail type-I collagen is a secretagogue or potentiator for stimulated insulin release. Although a smaller percentage increase over basal secretion was seen in islets in collagen compared to free islets (176% vs. 336% respectively), the absolute increase in insulin secreted from islets in collagen is much larger than the amount of insulin secreted from free islets. Free islets secreted 3.8 ng/hour more insulin when stimulated while islets in collagen secreted 7.7 ng/hour more insulin when stimulated.

There are several ways to normalize insulin secretion in GSIS experiments. First, insulin can be normalized to islet number. However, this normalization is unreliable as the sizes of islets varies considerably, and larger islets have, presumably, more intracellular insulin. Second, insulin content can be normalized by DNA content. This method is more reliable than islet number, but is hampered by non-islet tissues. If acinar or non-islet tissues contaminate the islet prep, the DNA within these tissues will contribute to overall DNA count, and provide an inaccurate determination of islet DNA amount. The last method of insulin normalization is to use the intracellular insulin content. This method is the most reliable and accurate way of normalizing insulin content, and was the
method used in these GSIS experiments. Although intracellular insulin content is more accurate than other methods, the presence of a collagen matrix potentially confounds the results, particularly when considering the diffusion experiments which suggest that some insulin may be trapped within the gels. Therefore, in the collagen encapsulated islets, the total intracellular content is actually the intracellular insulin plus the amount of insulin that may be trapped within the gel. The functional result of this problem is that the intracellular content may appear to be elevated, which provides smaller than expected normalized secretion values. However, since the secretion values observed in collagen encapsulated islets was greater than those of free islets, this confounder only increases the amount of insulin secretion, and does not effect, functionally, the interpretation of the data. The amount of insulin that was secreted from the islets within collagen gels may actually be higher than was detected, therefore it can be estimated that the insulin secreted by islets within collagen gels may actually be significantly higher than free islets.

It has been suggested in the literature that enhancing outside-in signaling via various extracellular matrix proteins can stabilize isolated islet structure and function\textsuperscript{80}. These data not only corroborate this hypothesis, but also applies this understanding to create an implant that releases more insulin than previous implant constructs. Lastly, our study has implications for the total number of islets necessary to impart a therapeutic benefit on insulin-sensitive diabetics. Given previous estimates of the number of islets needed to reverse hyperglycemia, collagen-based implants can impart the same therapeutic efficacy
with fewer numbers of islets. Consequently, the risk of developing post-transplant hypoglycemia may exist when using collagen-based implants without considering the effect of the collagen on insulin secretion. However, hypoglycemia is protected against by several physiologic mechanisms, including glucagon and somatostatin secretion, as well as autonomic regulation.

Additionally, to eliminate the possibility of developing hypoglycemia secondary to implantation, studies in which the minimal number of islets necessary in order to reverse hyperglycemia using collagen-based implants must be performed.

Specifically, implants containing a range of different islet numbers could be implanted, and dose-response curves could be develop to assess the effectiveness, pharmacokinetics, and toxicity of the islet implants.

Lastly, collagen gels containing freshly isolated islets were implanted for 7 days, and then examined for surviving islets. Despite preliminary characterization of the implant, no surviving islets were seen in the 7 day implants. It was then hypothesized that the islets had not survived because they were not perfused adequately, and therefore an implant that increases perfusion to the islets would need to be developed.

The data resulting from experiments conducted in specific aim #1 indicate that islets isolated using the protocol outlined in the methods, and expanded upon in appendix B, are metabolically and structurally viable. Additionally, the data show that culturing these islets, for a short period of time, in a bioreactor under flow conditions increases the preservation of their intracellular insulin content longer than islets cultured under static conditions. These data also suggest that
insulin diffusion out of a collagen gel occurs rapidly, and with minimal diffusion barrier, and that collagen does not bind specifically and sequester insulin. Lastly, these data show that islets encapsulated within a collagen gel are responsive to glucose stimulation, and secrete approximately 4 times more insulin that islets that are not encapsulated within a collagen gel. Combined, these data indicate the feasibility of using a collagen-based 3-dimensional scaffold to support islet structure and function.
CHAPTER 3

A 3-DIMENSIONAL TISSUE-ENGINEERED PREVASCULARIZED CONSTRUCT FOR THE PURPOSE OF SUPPORTING ISLET CELL SURVIVAL

Introduction

One current approach to enhancing the function of transplanted islet implants is to induce an angiogenic and neovascularization response to and within the islets\textsuperscript{66, 97}. Islets are vascularized structures located within the tissues of the pancreas. The cells that comprise the islet include α, β, δ, and γ cells, with the β-cells representing the sole source of physiologic insulin\textsuperscript{98}. The islet contains an intrinsic microvascular network that is supplied by several arterioles. Consequently, because the islet contains its own intrinsic microvascular network, interactions between cultured microvessel fragments and the islet’s microvasculature may help stabilize the vasculature, and consequently enhance perfusion to the islets. As a result of these observations, the use of cultured microvessel fragments to provide a supportive role for transplanted islets will be examined. Any positive effect that microvessel fragments may have on transplanted islet function, vasculature, and perfusion would represent a novel means by which islet viability can be enhanced. This research aim examined the characteristics of microvessel fragments encapsulated within a 3-dimensional collagen matrix in an attempt to enhance transplanted islet survival, presumably
by promoting neovascularization and the maintenance of the islet microcirculation, conditions that many researchers suggest is necessary for long-term islet graft survival\textsuperscript{99, 100}.

Blood vessel angiogenesis and maturation are physiologic events that, given a complete understanding of mechanisms and consequences, could potentially be used for therapeutic purposes\textsuperscript{101, 102}. The process of angiogenesis, which is defined as new vessel growth from existing vascular structures, is initiated and partially maintained by vascular endothelial growth factor (VEGF)\textsuperscript{103, 104}. Although other growth factors are required in order for vascular remodeling, maturation, and specialization to produce fully functioning vascular beds, the use of VEGF to promote an angiogenic response is a therapy that offers the potential to treat ischemic tissues and mitigate hypoxic insult\textsuperscript{105, 106}.

It has been reported that angiogenesis can be induced by a variety of different growth factors and pharmacological treatments\textsuperscript{67, 107}. Additionally, transgenic expression of vascular endothelial growth factor (VEGF) within β-cells, under control of the insulin promoter, has been shown to enhance islet insulin-production\textsuperscript{66}. This enhanced islet function was also shown to be associated with greater perfusion within the implants, suggesting a direct relationship between perfusion and islet function. However, these studies are limited in their application by not supporting crucial vascular events subsequent to angiogenesis that are required for a physiologically relevant reperfusion event. For example, angiogenesis without proper vascular remodeling will lead to new vessel formation, but not necessarily a functioning vascular bed\textsuperscript{108}. In order for
reperfusion to be increased, a more physiologically relevant model of angiogenesis, neovascularization, and vascular remodeling must be applied. This approach therefore utilizes microvessel fragments to stabilize the intraislet microvasculature, and also to support a vascular bed capable of inosculating with the host’s vasculature. Prior work in the lab has shown that microvessel fragments, when cultured in collagen, can sprout, elongate, and undergo angiogenesis. When co-cultured with isolated islets, these microvessel fragments represent a novel means of stabilizing the intraislet vasculature, promoting inosculatedation with the host vessels, and consequently enhancing implanted islet function.

Specific Aim 2: Evaluate a 3-dimensional tissue-engineered prevascularized construct for the purpose of supporting islet cell survival. Working Hypothesis: Prevascularized collagen gels will provide the revascularization stimulus needed for islet cell survival, and will inosculate with the host vasculature in vivo.
Methods

Microvessel fragment isolation: Microvessel fragments were isolated from epididymal rat fat using the methods described in Hoying et al, and are briefly described. For microvascular fragment isolations male Sprague-Dawley rats were used (350-500 grams). All microvascular fragment isolations were performed using sterile technique, and in compliance with IACUC approved protocols.

After administration of 0.6 CC Nembutal® (30 mg/kg) with a 1 cc syringe and 18 gauge needle intra-abdominally, an incision was made on the ventral surface to expose the peritoneal cavity near the gonads, and the distal two-thirds of the epididymal fat pad was excised. Once the epididymal fat had been removed, the rat was euthanized with an overdose of Nembutal®. The epididymal fat was minced with fine scissors, and digested in a collagenase solution (20 mg collagenase/40 ml PBS with BSA). The digestion mixture was washed in PBS/0.1% BSA, and centrifuged. The fragment pellet was resuspended in PBS/0.1% BSA, and the mixture strained in a 500 μm nylon mesh to remove fibrous debris from the microvascular fragments. The fragments were then captured in a 30 μm nylon filter. Once isolated, the microvascular fragments were placed in 200 μl of type-I collagen gel, placed in the well of a 48 well plate, and incubated for seven days in an incubator at 37° C with 5% CO₂ in culture media (1X DMEM/10% FBS).
In vitro microsphere perfusion of microvessel fragments: The following experiment was conducted by Carlos Chang in Dr. Jay Hoying’s lab as part of his dissertation research. Microvessel fragments were isolated and encapsulated within collagen gels as previously described. The collagen gel was then placed in the channel of a PDMS mold polydimethylsiloxane) (60 µm x 60µm). After 7 days of culturing at 37° C in an incubator, 10 µm fluorescent microspheres were perfused through the channel using a peristaltic pump.

VEGF extraction from cultured microvessel fragments: Microvessel fragments were isolated and cultured as previously described. After 4 days of culturing, the media was obtained and frozen, and fresh media was added to the gels. The media was obtained again after the 7th day of culturing and frozen. The days 1-4 and 4-7 media were assessed for the presence of VEGF. Each treatment group (day 0 control, days 1-4, and days 4-7) had 4 gels each from the same isolation (n=4).

VEGF quantification in conditioned media: Conditioned media was taken as previously described. VEGF was quantified using a rat VEGF-A ELISA kit (Cell Sciences, Canton, MA), and intraassay validation was confirmed with internal quality control samples (n=4).

Microvessel construct implantation: For implantations, female severe combined immunodeficient (SCID) mice were used (CB-17/ICR background bred at the
Arizona Cancer Center. The mice weighed approximately 22-25 grams. All implantations were performed using sterile technique, and followed IACUC approved protocols.

Female SCID mice were anesthetized with 0.3 ml of 2.5% avertin solution using a 1cc syringe and 22 gauge needle. The dorsal aspect of the left and right haunches were prepared for surgery. An approximately 5 mm incision was made and blunt dissection with a hemostat was performed to make an approximately 1 cm subcutaneous pocket. The implant was then removed from the 48 well plate using a sterile spatula, and the implant was placed in the pocket. The incision was closed with sterile skin staples.

Assessment of perfusion in implanted microvessel fragment gels: Microvessel fragment gels were created and implanted as previously described. The following experiment was conducted by Helen Chen in the lab of Dr. James and is unpublished data. After 21 days of implantation, approximately 0.75 cc of TRITC-dextran (Millipore, Billerica, MA) was injected into the tail vein (25 mg/ml) and allowed to circulate for ten minutes. The implant was removed and immediately fixed in 4% paraformaldehyde.

For other implants, India ink was used to visualize perfusion. The left ventricle was cannulated with PE50 tubing, and the right atrium was incised. Dialyzed India ink (Invitrogen, Carlsbad, CA) was forced into the left ventricular cannula using a syringe pump at a constant pressure of 100 mmHg. The implants were visualized, and images were taken prior to implant removal and fixation.
**Microvessel construct explantation:** All explantations were performed using sterile technique, and in compliance with IACUC approved protocols.

Mice were anesthetized with 0.3 ml of 2.5% avertin solution using a 1cc syringe and 22 gauge needle, the skin staples were then removed, and an incision made inferior to the implant site. The skin was peeled back. Using fine scissors and forceps, the implant was teased away from the skin and the surrounding tissues; a small amount of tissue surrounding the implant was also removed for histological comparison.

**Real-time PCR on explanted microvessel fragment constructs:** The following experiment was performed in collaboration with Dr. Kevin Greer in the lab of Dr. Jay Hoying as part of his doctoral dissertation research, and Dr. Chad Steining during his post-doctoral fellowship in the lab of Dr. Jay Hoying, and represents unpublished data. Microvessel fragment constructs were created and implanted by Kevin Greer. After 7, 14 and 28 days of implantation, the implants were removed. An RNA extraction was performed using a Qiagen® RNA extraction kit by Kevin Greer (Qiagen, Valencia, CA). Real-time PCR was run on the extracts by Chad Steining, and the data were normalized to day 0 implants. Each treatment group had 3 samples (n=3).
**Results**

*Microvascular fragments grow in vitro and are patent*

Upon isolation, fat-derived microvascular fragments were cultured in rat tail type I collagen (3 mg/ml) at a concentration of 20,000 microvascular fragments per ml of collagen. The overall size of a 200 µl collagen gel in a 48 well plate (diameter 1.11 cm) is a cylinder with a diameter of 1.11 cm and height of 0.32 cm. The fragments were cultured in DMEM containing 10% fetal bovine serum, and placed in a 37° C incubator with 5% CO₂. Images of the microvascular fragments were taken at days 1, 3, and 7, and are illustrated in figure 3-1. These images illustrate new vascular sprouts originating from existing microvascular fragments, indicating that an angiogenic response has occurred.

Figure 3-2 illustrates images of cultured microvascular fragments containing fluorescent microspheres. Fragments were cultured in 60 µm x 60 µm channels for 7 days, then perfused with 10 µm fluorescent microspheres. As these microspheres were placed in media which flowed through the PDMS mold lumen, the only way for microspheres to enter the microvascular fragments is if the fragments had a patent lumen, therefore these images provide direct evidence that cultured microvascular fragments are patent and able to support perfusion.
Figure 3-1: Images of isolated microvessel fragments at days 1, 3, and 7 showing evidence of angiogenesis and vascular sprouting. The images were taken with an inverted microscope. These images represent microvessel fragments that were isolated by Helen Chen and imaged by Helen Chen and Faith Rice and are unpublished data. These images are provided by and used with permission from Dr. Jay Hoying and Dr. Stuart Williams.
Figure 3-2: Fluorescent microsphere perfusion in cultured microvessel fragments. These images show green 10 µm fluorescent microspheres within red GS-1 labeled cultured vessels. The images were taken with a confocal microscope. This experiment was performed by Carlos Chang in the lab of Dr. Jay Hoying during his doctoral dissertation research project, and represents unpublished data (manuscript in preparation). These images are provided by and used with permission from Carlos Chang and Dr. Jay Hoying.
Microvessel fragments were cultured in a 3-dimensional collagen matrix as previously described. The microvessel fragment cultures were supplied media containing 10% FBS. After the 4th day of culturing, the media was removed from the fragment gels and placed in a -20°C freezer, and fresh media was applied. After the 7th day of culturing, the media was again removed from the fragment gels and preserved. Additionally, fresh media at day 0 was also preserved; this media was never in contact with microvessel fragment gels, and represents a baseline measurement of VEGF levels. The three medias, day 0, days 1-4, and days 4-7, were analyzed using a VEGF ELISA kit, and VEGF levels in each media was quantified. A standard curve was developed using VEGF standards supplied in the ELISA kit, and is provided in figure 3-3. After the standard curve was produced, the equation of the line was used to quantify the VEGF concentrations in each of the three medias. Figure 3-4 illustrates the amount of VEGF secreted by microvessel fragments at days 1-4 and 4-7. Day 0 media was VEGF-free, and therefore represented an appropriate negative control for the VEGF assay. From days 1-4, the microvessel fragments produced 30.1 pg/ml of VEGF while microvessel fragments at days 4-7 produced 135.1 pg/ml of VEGF, a statistically significant difference over days 1-4 media.
Figure 3-3: ELISA standard curve for VEGF quantification. The equation of the line was used to quantify VEGF levels in unknown samples. The standards were plated in duplicate, and the averages at each concentration are represented by the points on the standard curve.
Figure 3-4: VEGF secretion in microvessel fragment cultures at days 0, days 1-4, and days 4-7. Media was taken from 4 separate gels of the same isolation (n=4). Statistical significance was assessed using a 2-sided student’s t-test P < 0.05
VEGF transcript is upregulated in implanted microvessel fragment gels

Microvessel fragments were isolated and placed within 3-dimensional collagen gels as previously described. Fragment gels were implanted subcutaneously in female severe combined immunodeficient mice (SCID). Implants were removed at days 7, 14, and 28, and processed with Qiagen RNA isolation kits. The samples were amplified with real time PCR, using dynactin as a “house keeping gene”, and normalized to day 7 figures. Figure 3-5 illustrates the RT-PCR data, and shows that VEGF mRNA is upregulated at days 14 and 28 relative to VEGF mRNA levels at day 7. Specifically, VEGF mRNA expression is 4.02 times greater than the levels at day 7, and 3.7 times greater than the levels at day 7. Although the difference between days 14 and 28 were not statistically significant, both days 14 and 28 VEGF mRNA levels are statistically significantly elevated over day 7 levels.
Figure 3-5: Real time PCR data showing VEGF mRNA levels in microvessel fragment gels implanted for 7, 14, and 28 days. Dr. Kevin Greer was responsible for implantation and RNA extraction and Dr. Chad Steining was responsible for performing the RT-PCR. The data was normalized to day 7 mRNA levels, and dynactin was used as a house keeping gene. Day 14 and 28 implants showed statistically significantly more VEGF transcript over day 7 controls. Three implants at each time point were assessed (n=3). p < 0.001.
Implanted microvessel fragment gels inosculate with the host vasculature

Microvessel fragment gels were constructed and implanted subcutaneously. Prior to removal, the mouse was perfused with a marker to indicate areas of perfusion. Figure 3-6 shows images of host-derived India ink perfused microvessel fragment gels after 21 days of implantation. Prior to removal of the implant, India ink was perfused through the left ventricle under constant pressure from a syringe pump (100 mmHg). Figure 3-6 shows images of the implant containing India ink perfused vessels, showing evidence of implant-host inosculation, and implant perfusion.

Microvessel fragments have also been isolated from green fluorescent protein-expressing mice. These microvessel fragments express GFP under control of the tie-2 promoter, and the endothelial cells of these fragments fluoresce green. Microvessel fragment gels with tie-2-GFP vessels have been implanted, and prior to removal, the animal was perfused with rhodamine-labeled dextran to indicate areas of implant-host inosculation and perfusion. Figure 3-7 shows images of GFP-labeled implant-derived microvessel containing host-perfused rhodamine dextrans. As the dextrans were host-derived, the only way in which GFP vessels could contain the dextrans is by direct inosculation with the host circulation; therefore, figure 3-7 shows direct evidence that microvessel fragment gels inosculate with the host circulation by 21 days of implantation.
Figure 3-6: India ink perfused microvessel fragment implants. These images show India ink perfused vessels within microvessel fragment implants, showing evidence of implant-host inosculation. The implants are indicated by white circles. The images were taken with a Nikkon camera attached to a dissecting microscope with a 4x objective. These experiments were performed in collaboration with Dr. Gabriel Gruionu.
Figure 3-7: GFP-labeled microvessel perfused with host-derived rhodamine-dextran. These images show red host-derived dextran inside implant-derived green GFP-vessels, showing direct evidence of implant-host inosculation. Image A represents 7 day implants and image B represents 21 day implants. These images were obtained by Helen Chen in Dr. Jay Hoying’s lab (data unpublished), and were provided by and used with permission from Dr. Stuart Williams and Dr. Jay Hoying.
Discussion

The purpose of this specific aim was to examine the characteristics of a tissue-engineered microvessel fragment containing collagen gel that would be supportive of implanted pancreatic islets. As perfusion has been identified as the most crucial event necessary in order to sustain implanted islet graft survival, these microvessel fragment gels were assessed for their ability to initiate and sustain critical vascular events, including angiogenesis, neovascularization, inosculutation, and consequent reperfusion.

Figure 3-1 shows that cultured microvessel fragments, when encapsulated within a 3-dimensional collagen matrix and placed within a 37°C incubator with 5% CO₂ and supplied with 10% fetal bovine serum, are capable of supporting angiogenic events which ultimately result in vascular sprouting and new vessel formation. While the exact events that lead to this angiogenic response are not fully understood, an angiogenic response is elicited nonetheless, an angiogenic response which has implications for not only the growth and maintenance of the microvessel fragments, but also of any vascularized tissues requiring perfusion.

Vascular endothelial growth factor is an angiogenic growth factor known to support new vessel growth, as well as maintenance of other cells and tissues, including pancreatic islets \(^{109,110}\). With evidence showing that microvessels grow in culture and undergo angiogenesis, VEGF production in cultured microvessel was examined. Figure 3-4 shows VEGF secretion in cultured microvessel fragments at day 0, days 1-4, and days 4-7. VEGF secretion is present at days 1-
4, and is statistically significantly greater at days 4-7. It is important to note that VEGF was completely absent in the feeding media (day 0), and therefore any quantified VEGF is directly attributable to VEGF secretion from the cultured microvessel fragments. Additionally, all of the media was taken at day 4, representing VEGF secretion by microvessels at days 1-4, and completely replaced with fresh media so that the second time point, days 4-7 represented VEGF secretion only for those days, and is not cumulative from days 1-7. In other words, since all of the media was taken at day 4, the larger quantity of VEGF secretion at days 4-7 is directly attributable to secretion for those days, not including the first four days of culturing. While it is not known if the elevated VEGF levels are due to increased production and secretion per cell, or due to the larger vascular mass due to angiogenic events leading to new vessel growth, the microvessel fragments from days 4-7 represent a source of growth factors that are physiologically mediated and controlled, a situation that is absent when pharmacologic VEGF is added. It is worth noting that the amount of VEGF that is typically added to islet grafts is an order of magnitude larger than the amount of VEGF secreted by these microvessel fragments. Although this may indicate that the VEGF secreted by microvessel fragments is inadequate to support islet survival, it is likely that the amount of VEGF added pharmacologically by many research groups is in gross excess, and contributes to ultimate vascular collapse due to an uncontrolled angiogenic responses leading to a very dense vascular bed that cannot be appropriately remodeled; because these microvessel are secreting VEGF at levels that are at a more physiologically relevant level, the resulting
vascular beds are more appropriately remodeled, which requires growth factors that are consequentially produced by the endothelial cells of the microvessels\textsuperscript{111}. Although an angiogenic response has been shown in figure 3-1, and supported by figure 3-4, these images do not show important morphological characteristics of the vessels, namely, these images do not distinguish between vascular cords or patent vessels.

Figure 3-2 illustrates images of cultured vessels containing perfused fluorescent microspheres. As these microspheres were perfused \textit{in vitro}, these images show direct evidence that cultured microvessels are indeed patent and able to support lumenal flow; the only way that microspheres could enter the cultured microvessel is if the vessels are patent and able to support lumenal flow. The combined evidence showing that cultured microvessel can support angiogenic events leading to vascular sprouting and new vessel formation, and evidence showing that these vessels are patent, leads to the question of inosculation and perfusion \textit{in vivo}.

Since the ultimate goal of using these microvessels is to support islet survival by enhancing reperfusion, the question of inosculation and perfusion must be answered. First, real time PCR was performed on these implants at various time points to show the pattern of VEGF expression. Figure 3-5 shows the real time data for microvessel fragment implants at 7, 14, and 28 days. The data shows that VEGF is statistically significantly upregulated at 14 and 28 post implantation. These data suggest that implanted microvessels produce factors that are known to stimulate an angiogenic response, an event which is necessary, but
not sufficient, in order for inosculation and consequent perfusion to occur. Figure 3-6 illustrates images of India ink perfused microvessel fragment gels at 21 days of implantation. These images show that host-derived India ink is perfusing the subcutaneous implant. It should be noted, however, that these images do not show direct evidence of inosculation with the implant. Although vessels within the implant are perfused with India ink, it is possible that the perfusion occurred as a result of host vessels penetrating the implant without inosculating with the vessels within the gel. Although this distinction is inconsequential from the perspective of perfusing the gel (i.e. perfusion without inosculation is still sufficient to support islet cell survival), inosculation with the host would result in faster reperfusion of the construct than host vessel penetration. It is important to note that negative control implants, that is, implants that contain no microvessel fragments, do not become perfused at any time points assessed. That is, India ink perfusion to empty collagen gels has never been shown. To examine more directly host-implant inosculation, GFP-labeled microvessel fragments were implanted into non-GFP mice, and rhodamine-conjugated dextrans were perfused into the host mouse prior to implant removal at day 21. Figure 3-7 shows images of the green vessels within the implant containing host-derived dextran. Like the microsphere perfusion experiment in vitro, the only way for the implant-derived vessels to contain host-derived dextran is to receive perfusion from the host. This experiment shows direct evidence that microvessel fragment gels can inosculate with the host by 21 days of implantation.
The ultimate goal of this aim was to determine the characteristics of these microvessel fragment gels that would support islet graft survival. The resulting data shows that microvessel fragments undergo and stimulate angiogenesis, that the angiogenic event is correlated with VEGF production and secretion by the microvessel fragments, and that when implanted the microvessel fragments inosculate with the host by 21 days of implantation. Combined, these data indicate that microvessel fragments possess critical characteristics that would support islet graft survival by enhancing an angiogenic event resulting in islet graft reperfusion.
CHAPTER 4

A PREVASCULARIZED PANCREATIC ENCAPSULATION DEVICE TO MAINTAIN ISLET SURVIVAL IN VIVO.

Introduction

Isolated and transplanted islets are highly susceptible to ischemic insult associated with severing vascular connections, and therefore their survival and clinical efficacy are highly diminished within the first 48 hours of transplantation. As a result, in current islet transplantation protocols the islets are transplanted in regions of dense vascularization to enhance the rate of reperfusion to the islet grafts. These areas include the portal circulation, the omental pouch, and the renal capsule. Although these sites represent areas of high vascular density, many issues are associated with transplanting islets in these sites, including thrombosis, irreversible metabolic damage to the liver, and drug metabolite-associated islet toxicity. Consequently, other non-invasive implantation sites are currently being investigated to mitigate the effects of islet transplantation into high risk areas.

Islet transplantations for the purpose of reversing insulin-sensitive diabetes have been undertaken since the early 1970s. Although progress has been made in the areas of islet isolations and encapsulation techniques, a drastic shortage in islets available for transplantations remains a concern. Consequently, many
techniques have been explored and developed to help stabilize islet survival. Included in these attempts are exploring various encapsulation techniques, and the interactions between islets and their extracellular matrix that may help stabilize and maintain islet survival. One example includes encapsulation within a silicone scaffold, which has been shown to enhance angiogenesis and connective tissue formation\textsuperscript{121}. In these silicone-based scaffolds, collagen fibril alignment and further connective tissue maturation was seen by 4 weeks of implantation\textsuperscript{122}. While biomaterial research in this field certainly has advantages, including the possibility to confer immunoprotection, biomaterials alone are unlikely to produce an islet graft which accurately mimics the native microenvironment of the islet; tissue engineering approaches which utilize \textit{ex vivo} tissues to stimulate various physiologic responses represent a more feasible and complete solution to enhancing transplanted cell survival.

In an attempt to minimize the invasiveness of islet implantation procedures, researchers have investigated the possibility of islet transplantation into low risk and highly accessible locations. In one example, islet transplantation in the groin, with modification of the vasculature in that region, has shown that islet reperfusion and connective tissue growth occurs at a rate that is amenable to islet survival\textsuperscript{121,123}. Immunohistochemical analysis from these implants has revealed positive insulin and glucagon staining after 3 weeks of implantation, indicating that $\alpha$ and $\beta$ cells within these implants have survived\textsuperscript{124}. Additionally, islets have been implanted subcutaneously in various prevascularized scaffolds
with successful results$^{125-127}$. These studies indicate that subcutaneous islet implant sites are a feasible option to high risk implantation sites.

It has been previously demonstrated that intraislet endothelial cells contribute to the revascularization and reperfusion process in isolated and transplanted islets, and therefore loss of the intraislet vasculature has been associated with islet death$^{99}$. Consequently, as a result of diffusion distances and the size of islets, cells within the middle of islets undergo hypoxic insult before the cells of the periphery, the result is central islet cell necrosis$^{128,129}$. Therefore, any subcutaneous implant must be able to maintain the intraislet vasculature in order to prevent central core necrosis.

The goal of specific aim #3 was to develop and assess an islet implant construct with accelerated microcirculation formation within and around transplanted islets to support islet cell viability and enhance islet transplantation efficiency. This aim is based on previous work utilizing isolated microvessel fragments in a collagen gel to enhance vascular reperfusion with the host. Previous work has shown that microvessel fragments undergo angiogenesis in vitro and will insosculate with the host circulation within 7 days of implantation. Additionally, data has indicated that microvessel fragments produce and secrete VEGF, a growth factor known to enhance islet viability and maintenance$^{109,110}$. Therefore, in this study we investigate whether a prevascularized collagen construct with microvessel fragments will support islet survival. These properties of a 3-dimensional microvessel fragment gel were used to encapsulate isolated
islets, resulting in a prevascularized pancreatic encapsulating device (PPED), and the effect of this preformed vasculature on implanted islet survival was examined.

**Specific Aim 3:** *Assess the ability of a prevascularized pancreatic encapsulation device to maintain islet survival in vivo.*

*Working Hypothesis:* A prevascularized pancreatic encapsulation device will maintain islet survival longer than a non-prevascularized implant.

**Methods**

*One-piece vessel fragment-islet gel construction:* Microvessel fragments were isolated from rat epididymal fat as previously described (chapter 3). Concurrently, pancreatic islets were isolated as previously described. At the conclusion of both isolation protocols, the two tissues were combined and placed within a single collagen gel (200 µl). The gels contained approximately 3,500 microvessel fragments and 200 islets. The collagen gels were then immediately implanted into subcutaneous pouches of SCID mice as previously described; the microvessel fragments were not cultured.

*Vessel quantification:* One-piece microvessel fragment-islet gels were implanted for 21 days. Prior to explantation, the animal was perfused with dialyzed India ink as previously described. The implants were imaged after the skin was removed. Three images each from two of the implants were loaded into
morphometric software for analysis. The software examines the number of vessels that intersect a single artificial line extending down the center of the image from the top to the bottom, and quantifies overall vessel number provided adequate and representative images are loaded into the software. The implants were then removed and fixed in 4% paraformaldehyde for 4 hours. The implants were processed, embedded in paraffin blocks, and sectioned. A total of 2 implants were assessed for vessel numbers (n=2), and therefore statistical assessment was not possible.

*In vitro islet viability with and without microvessel fragments:* Islets were isolated, and encapsulated within a collagen gel both with and without microvessel fragments. The gels were placed in an incubator, and DTZ was added to three gels at three different time points (3 hours post isolation, 15 hours post isolation, and 27 hours post isolation) within each treatment group (with and without fragments) to assess the number of viable islets (n=3).

*PPED construction:* One-hundred fifty µl of type-I collagen gel containing freshly isolated islets 100-300) was placed on top of a seven day collagen gel containing microvessel fragments (approximately 3,500) as previously described. A second seven-day microvessel fragment gel was then placed on top, and the three layers allowed to fuse together, as the middle islet collagen gel polymerizes, for 45 minutes.
Fixation and processing: The PPEDs and associated tissues were placed in 4% paraformaldehyde for 4 hours. The implants were then placed in a 0.5% paraformaldehyde solution overnight. The fixed implants were then placed in a Tissue-tek® processor (Tissue-tek, Reddington, CA). Fixed and processed implants were then embedded in paraffin, and 6 µm sections were obtained for histological analysis.

Insulin staining: Paraffin sections of implants were washed in xylene for 20 minutes, and rehydrated in ethanol solutions (100% to 50%). The slides were washed in DCF-PBS, and blocked for one hour at room temperature (rabbit serum, 0.1% BSA, 0.1% evaporated milk). The slides were washed in DCF-PBS for ten minutes, and guinea pig anti-swine primary insulin antibody (Dako, Germany) was applied (1:100 dilution). The slides were placed in a 37°C incubator for 90 minutes. The slides were then washed with DCF-PBS for 15 minutes, and rabbit anti-guinea pig secondary-FITC (Invitrogen, Carlsbad, CA) was applied (1:200 dilution). The slides were placed in a 37°C incubator for 90 minutes, and then washed in DCF-PBS for 15 minutes. The slides were mounted with Mowiol® (Invitrogen, Carlsbad, CA), and stored in a light-controlled case. At each timepoint, 3 implants were assessed along side 3 controls (n=3 for both PPED and controls).

Insulin/GS-1 lectin double stain: A double cytochemical stain was performed using the same procedure described above, with the exception that D-PBS was
used rather than DCF-PBS, and the blocking solution did not contain milk. Additionally, the GS-1 lectin (Invitrogen, Carlsbad, CA) was applied after the insulin secondary had been washed off (1:40 dilution), and placed in a 37°C incubator for 90 minutes. The slides were then washed in D-PBS for 15 minutes, and mounted with Mowiol®. At each timepoint, 3 implants were assessed along side 3 controls (n=3 for both PPED and controls).

*Islet hypoxic insult:* Islets were isolated and placed within collagen gels as previously described. Upon encapsulation, the islets were tightly wrapped in several layers of plastic wrap, and incubated at -20°C overnight. The gels were then fixed, processed, embedded, and sectioned. This protocol insures that islet cells die from both hypoxic insult (apoptosis) and freeze-shock (necrosis).
Results

*Islets enhance angiogenesis and vascular density*

It has been previously demonstrated that microvessel fragment gels are pro-angiogenic, inosculate with the host vasculature *in vivo*, and are patent and support perfusion. Although other cell types have been investigated for their pro-angiogenic benefits, the effect of isolated pancreatic islets on microvessel fragment growth was unknown. In this experiment, pancreatic islets and microvessel fragments were coencapsulated within a 3-dimensional collagen matrix. A schematized image of these implants is provided in figure 4-1. These constructs were then implanted subcutaneously into SCID mice, and were removed after 28 days. Prior to removal, the animal was perfused with India ink to visualize areas of patency and perfusion. Figure 4-2 illustrates an images of India ink perfused constructs both with and without pancreatic islets. These images suggest that constructs containing pancreatic islets had a higher vascular density than constructs not containing islets, however, implants containing only islets and no microvessel fragments, did not demonstrate India Ink perfusion. Therefore, these data indicate the microvessel fragments within collagen gels are needed in order to enhance perfusion to the implant, and the presence of isolated islets potentiates this response. To quantify this difference, images were analyzed using morphometric software. Figure 4-3 illustrates the summary data from this quantitative morphometric analysis, and indicates that pancreatic islets have a
positive effect on angiogenesis and vascular density. To examine islet structure in the gels, implants were removed and stained with hematoxylin and eosin (H&E). Figure 4-4 illustrates the resulting micrograph, and indicates that islets within these implants are structurally and morphologically normal.
Figure 4-1: A schematized diagram of the one-piece islet-microvessel fragment implant. The implants contained 20,000 microvessel fragments per ml of collagen and 100-300 islets in a total volume of 200 µl of collagen.
Figure 4-2: India ink perfused microvessel fragment subcutaneous implants with and without pancreatic islets. Images of 2 different implants with and without pancreatic islets (n=2). These images were taken with a Nikkon camera attached to a dissecting microscope with a 4x objective. Note: pancreatic islets are not visible in these images.
Figure 4-3: A quantitative morphometric analysis of implanted microvessel fragment constructs with and without pancreatic islets. Three images from 2 different implants within each treatment group were loaded into morphometric software that examined vessel density by counting the number of vessels that crossed a single artificial line extending from the top to the bottom of the image through the center. Statistical assessment was not possible because of the sample size (n=2).
Figure 4-4: An H&E stain of a microvessel fragment-islet implant removed after 7 days, showing morphologically normal islets. Size bar is 100 µm.
One-Piece implant assessment and PPED rationale

One-piece microvessel fragment-islet implants were examined for their ability to maintain islet cell survival. Insulin stains were performed on one-piece implants as well as collagen gels containing only islets (no microvessel fragments, n=8 for each). Although the one-piece gel showed enhanced vascularization, the collagen-only gel showed no evidence of implant-host inosculation or perfusion. Additionally, with the exception of a single one-piece vessel-islet implant, all other attempts at staining for insulin were negative. Figure 4-5 illustrates the only, irreproducible, positive insulin stain in a 21 day one-piece implant. Although the insulin stain was not reproducible in sections of these constructs, the image shows India ink perfused vessels penetrating the implant, suggesting that perfusion throughout the implant can occur. Additionally, islets were cultured with and without day 0 microvessel fragments and encapsulated within collagen gels. Figure 4-6 illustrates DTZ viability data in cultured islets both with and without microvessel fragments, and indicates that the day 0 microvessel fragments alone are not sufficient in order to stabilize islet viability and enhance islet survival. Therefore, it was hypothesized that the day 0 vasculature was too immature to support islet survival, and by the time the implants were perfused, the islet had already died. Therefore, an implant had to be developed which used more mature microvessels that are able to increase the rate of reperfusion to the implant. As a result of the inability to reproduce positive insulin staining, the one-piece design was revised, and the PPED was developed which allowed for the microvessel fragments to culture for 7 days prior to implantation.
Figure 4-5: Positive insulin stain in a sectioned one-piece microvessel fragment-islet subcutaneous implant. The brown spot (black arrow) indicates insulin localization using an HRP-conjugated antibody for insulin. This image was taken with an inverted microscope with a 10x objective. This image also shows black India ink perfused vessels within the implant.
Figure 4-6: *in vitro* islet viability in islets encapsulated with collagen gels with and without microvessel fragments. Viable islets were assessed using a DTZ assay on collagen gels at three different time points, and with two different treatment groups: islets were either encapsulated in collagen gel alone, or encapsulated with freshly isolated microvessel fragments. No comparisons were statistically significant using a student’s t-test. n=3.
Prevascularized pancreatic encapsulating devices (PPEDs) were able to maintain islet survival at 28 days post-implantation

A two-piece islet-microvessel fragment implant was developed, and a schematized image of the device, along with histological characterization, is provided in figure 4-7. PPEDs were implanted for 7, 14, and 28 days, fixed, stained for insulin, and then evaluated morphologically. Attempts at implanting islets in collagen gel alone without a preformed vasculature did not maintain islet survival and no insulin positive cells or microvasculature were detected at any time point (n=3 at day 3 and 7), suggesting that the prevascularized gels within which the islets were encapsulated are required for islet survival. Figure 4-8 shows immunohistochemical stains of a control pancreas tissue and implants at the three time points. At all three time points, detectable amounts of insulin were present (87% of all PPEDs examined at all time points). Additionally, the staining showed that insulin production is present in fully intact, as well as partially dissociated islets. These data indicate that islets placed within a prevascularized collagen-based structure maintain their ability to produce insulin longer than islets implanted without a preformed vasculature, and that collagen-based implants are able to maintain their structure at 28 days of implantation.
Figure 4-7: A schematized image of the PPED with histological characterization. The green staining represents insulin immunostaining and the red staining indicates GS-1 lectin endothelial cells. This image was created by merging two images from two different 28 day PPEDs (a GS-1 image of the two outer prevascularized layers and a double insulin-GS-1 stain of the inner islet layer). The image was taken using a fluorescent scope and a Sony camera with a 10x objective. Hiscox, et al, Journal of Tissue Engineering, 2007 (in press).
Figure 4-8: Immunohistochemical insulin stains of PPEDs after 7, 14, and 28 days of subcutaneous implantation. The top row of images was taken using a 10x objective, and the bottom row of images was taken using a 20x objective. At each time point, 3 different implants were examined for insulin presence (n=3). Size bars = 100 µm.
Surviving islets are associated with a positively stained intraislet vasculature

Double immunohistochemical stains were performed on the removed implants. Specifically, the implants were stained for insulin and endothelial cells using FITC-conjugated and rhodamine-conjugated antibodies respectively. Figure 4-9 shows immunofluorescent stains, illustrating a correlation between islet viability and the presence of intraislet endothelial cells. These data indicate that a preformed vasculature is necessary in order to maintain insulin production within islets placed in a collagen gel, and that a positive association between insulin content and the presence of a vasculature exists. Additionally, a insulin/endothelial double stains have been performed which indicate that islets which do not contain surviving cells are not associated with evidence of a vasculature. Figure 4-10 illustrates negative insulin staining in association with negative endothelial cell staining, supporting the hypothesis that a surviving vasculature is necessary in order for islet cell survival in vivo.
Figure 4-9: double immunochemical and cytochemical stains of PPEDs implanted for 7, 14, and 28 days. The green staining indicates insulin immunostain and the red staining indicates GS-1 lectin endothelial cells. The images were taken with a fluorescent microscope using a 10x objective. At each time point, insulin and endothelial cell presence was assessed in three different implants (n=3). Size bar = 100 μm. Hiscox, et al, Journal of Tissue Engineering, 2007 (in press).
Figure 4-10: Insulin/endothelial cell stains in 14 day PPEDs showing an association between non-surviving islets and a negatively stained vasculature. The red endothelial cell stain is a GS-1 lectin conjugated to TRITC and the green insulin stain is an insulin immunostain conjugated to FITC. These images represent the negative control for the islets in figure 4-8. Size bars=100 µm.
Islets within PPEDs are perfused

Although host-implant inosculation has been previously demonstrated in specific aim #2, the ability of this reperfusion to supply implanted islets has yet to be demonstrated. To assess islet perfusion, PPEDs were implanted for 28 days into SCID mice. Prior to implant removal, the mouse was perfused with .750 cc of FITC-conjugated dextran for ten minutes (MW: 2,000,000) to visualize areas of patency and perfusion near islet structures. The implants were then removed, fixed, and sectioned for histochemical analysis. Additionally, for comparative purposes, isolated islets were subjected to in vitro hypoxic freeze-shock insult for 24 hours at -20°C, and then immediately fixed and processed for histochemical analysis. Figure 4-11 illustrates images of insulin-stained pancreatic islets that were subjected to hypoxic freeze-shock insult. These images show islets devoid of internal insulin staining, indicating that hypoxia-induced apoptosis, or freeze-shock induced necrosis, occurred from the inside of the islet, the area of the islet furthest away from nutrient and gas exchange. Dextran-perfused PPEDs were also visualized. Figure 4-12 illustrates images of dextran-perfused implants. Panel A shows a high magnification image of a dextran perfused vessel (green=dextran, blue=dapi nuclear stain, red=GS-1 endothelial cell stain). Panel B shows an image of a perfused structure consistent in size and morphology to pancreatic islets (green=dextran, blue=dapi nuclear stain). To aid in the determination of the identity of the perfused structure in figure 4-12, dapi nuclear stains and H & E stains of positive control pancreas tissues were performed and compared to the PPED stains. Figure 4-13 shows comparisons of dapi and H & E
stains in positive control, and indicates similarities in size, morphology, and staining patterns to sample PPEDs. Combined, these data provide suggestive evidence that islets implanted within PPEDs are perfused.
Figure 4-11: Insulin immuno stains in isolated pancreatic islets subjected to severe hypoxic freeze-shock insult. The green staining indicates insulin immunostain, and shows no evidence of intraislet insulin content.
Figure 4-12: Images of Dextran-perfused PPEDs that were stained for cell nuclei (blue). The green staining indicates host-derived fluorescent dextran, the blue staining represents DAPI nuclear staining. A: a dextran-perfused vessel within the PPED. B: a dextran-perfused cellular structure within the PPED. These images were taken with a fluorescent microscope with a 20x objective. Size bars = 100 µm. Arrow indicates islet.
Figure 4-13: Images comparing DAPI nuclear stains and H & E stains of positive control pancreas tissues and PPED sections. Size bars = 100 µm. Arrows indicate islets. Note: the image on the bottom left of this figure is the same image as panel B of figure 4-12.
Discussion

The purpose of this specific aim was to assess the ability of a prevascularized pancreatic encapsulation to maintain islet cell survival as indicated by the persistence of intracellular insulin granules. Islets placed in collagen had previously demonstrated enhanced glucose responsiveness \textit{in vitro}, and prevascularized collagen gels have been shown to be proangiogenic and enhance inosculation and subsequent reperfusion \textit{in vivo}. Therefore, these data represent the rationale for encapsulating islets within a prevascularized collagen gel.

It had been previously demonstrated in specific aim #2 that microvessel fragments secrete known angiogenic growth factors that could benefit coencapsulated cells. Additionally, the intraislet vasculature represents important vascular elements that have been investigated for their ability to support revascularization\textsuperscript{99}. Therefore, the ability of isolated pancreatic islets to contribute to the revascularization process was investigated. Microvessel fragments and pancreatic islets were coencapsulated within a collagen gel, and implanted for 28 days. The data indicated that microvessel implants containing islets were better perfused and had a denser vascular network than implants not containing pancreatic islets. It should be mentioned that India ink perfusion shows the presence and location of intact, patent vascular beds, and cannot reveal the presence of vascular beds whose arterioles are not patent or are physiologically closed or shunted. Therefore, while India ink perfused vessels are
patent, non India ink perfused vessels are not necessarily occluded or non functioning, they may simply be shunted. These data support the hypothesis that implants containing islets and microvessel fragments inosculate and are better reperfused once implanted than implant constructs containing either microvessel fragments or pancreatic islets; the addition of pancreatic islets to microvessel fragments potentiates angiogenic and reperfusion events. These data are corroborated by the primary literature whereby other cell types have been shown to have angiogenic benefits, including parathyroid tissue\textsuperscript{130,131}. Additionally, as VEGF is produced and secreted from endothelial cells, maintaining the intraislet vasculature is critical for the observed enhanced vascular response. Previous work has shown that the intraislet endothelial cells contribute to the revascularization process\textsuperscript{99}. This observation can be explained by two mechanisms. First, preservation of the intraislet vasculature is critical for inosculation of the host circulation to the islet circulation; if the intraislet vasculature is destroyed, there would be no vasculature to support reperfusion within the islet. Second, the intraislet vasculature represents a rich source of growth factors, growth factors that are mutually beneficial to the islets as well as the microvessel fragments. Consequently, maintenance of the intraislet endothelial cells is important and has direct consequences on angiogenesis, revascularization, inosculation, and reperfusion.

Figure 4-7 illustrates that insulin positive cells are seen associated with an intraislet vasculature. As a result of previously described benefits of preserving the intraislet vasculature, it is perhaps not surprising that islets whose vasculature
remains intact and present also maintain their insulin granules. Additionally, islets whose vasculature was not detected did not maintain their insulin granules, indicating a direct correlation between the presence of an intact intraislet vasculature and islet survival in vivo. Consequently, if the maintenance of an intraislet vasculature enhances angiogenesis, and is required for proper inosculcation and revascularization, reperfusion of these implanted islets can be inferred. Several lines of evidence presented within specific aim #3 support this hypothesis. It should be noted, however, that the morphology of the preserved intraislet vasculature, or that of the coencapsulated microvessel fragments, has not been examined in this research project. As previously described, the intraislet vasculature of the islets is highly fenestrated. These fenestrae are critical for the proper transmission of chemical signals to and from the islets, as well as appropriate insulin diffusion into the vasculature, however, the degree of fenestrations within the vasculature of encapsulated islets has not been assessed, and would represent future experiments assessing the functionality of these devices.

In order for islet survival to persist, the cells of the islet must be perfused, either through direct inosculcation with the intraislet vasculature, or indirectly by perfusing the implant within approximately 100 µm of the islets. Islet reperfusion requires the preservation of the intraislet vasculature, or formation of a new vasculature, a criterion shown in figure 4-10, where a positive association between an intact intraislet vasculature and maintenance of intracellular insulin was seen. Additionally, the presence of the intraislet vasculature has been shown
to enhance the vascular density of the implants in figure 4-3. Consequently, this neovascularization was shown to inosculate with the host and subsequent perfusion was demonstrated in figure 4-7. Figure 4-11 demonstrates the direct consequence of non-perfused islets, that is, islets lacking adequate perfusion have necrotic cores as the internal islet cells are subjected to the most severe hypoxic insult. The end result being that the islets necrose and/or apoptose from the inside-out, producing rings of positive insulin staining. These ring-like insulin stains have never been demonstrated in the PPED, suggesting that each positively stained islet is adequately perfused. The last line of evidence supporting the hypothesis that islets implanted within PPEDs are reperfused is shown in the dextran-perfused PPED experiment.

Prior to removal, the host was perfused with FITC-conjugated dextran. The implants were then stained for cell nuclei. Dextran perfusion was demonstrated throughout the PPED, and also seen around and within structures morphologically resembling pancreatic islets. The resemblance between the structures shown to be perfused within PPEDs and islets from a positive control pancreas section was indicated by dapi cell nuclear stains and H & E stains and was shown in figure 4-13. Although absolute confirmation that the perfused structures within PPEDs was not attained through positive insulin staining, the morphological resemblance to known islets, achieved through two separate stains, as well as the location of the structures within the PPEDs represent suggestive evidence that these perfused structures are pancreatic islets.
Several experimental and methodological issues arise which could explain the absence of positive insulin staining in these perfused PPEDs. In all previous attempts at insulin staining within PPEDs, positive results were attained. Consequently, the possibility that the encapsulated islets did not survive is unlikely. However, these perfused PPEDs were frozen in OCT and sectioned in a cryostat, where all previous PPEDs were paraffin embedded and sectioned in a microtome. Although the differences in processing and handling frozen sections vs. paraffin sections are ostensibly trivial, differences are present and could possibly result in anomalous staining results. It should be noted that positive controls (whole mouse pancreas) stained positive for insulin using the same staining procedure, indicating that the staining procedure works for this application. The most likely explanation for these negative insulin staining results is the processing and fixation of frozen sections. A patent filed in the United States Patent Office discusses the issues associated with the processing of collagen-based samples within OCT frozen blocks (patent approved August 9, 2004, #5336616). The authors of the patent application detail explicit processing and drying steps needed for collagen-based OCT blocks, steps that are not necessary for paraffin-embedded blocks. As collagen-based tissues are especially susceptible to perturbations in processing and fixation steps, optimization of the pre-staining processing protocol for collagen-based OCT frozen samples would need to occur.

Despite the absence of immunocytochemical localization of insulin, evidence is presented which indicates that islets within PPEDs have survived at
28 days post-implantation and are possibly perfused. Islets and microvessel fragments are known to produce an angiogenic response resulting in the formation of a dense and patent vascular bed. The resulting vascular bed has been shown to inosculate with the host and support host perfusion. Islets within PPEDs were shown to maintain their intracellular insulin granules, and islets with intracellular insulin granules were positively associated with the preservation of an intact intraislet vasculature. The presence of an intraislet vasculature has been associated with the production of known angiogenic growth factors, and has also been shown to be required for islet reperfusion. Lastly, host-derived dextran has been shown throughout PPEDs, including to and within structures which resemble pancreatic islets. Combined, these data indicate that PPEDs are able to support islet survival and suggest that islets within PPEDs are perfused.
CHAPTER 5

DISCUSSION AND CONCLUSIONS

Summary

The overall purpose of this research project is to develop, examine, and characterize a tissue-engineered islet implant constructed using a preformed vasculature. This research project can be divided into three separate, yet experimentally linked, research aims. The first aim was designed to assess the effects of encapsulating isolated islets within a collagen type I matrix. The second aim was designed to examine the characteristics of a 3-dimensional collagen gel containing microvessel fragments isolated from rat epididymal tissue. The last specific aim was to characterized the effect of combining a preformed vasculature and a 3-dimensional islet containing collagen-based gel on islet graft survival.

Specific Aim 1: Develop an islet-containing collagen matrix construct and examine islet responsiveness to glucose stimulation in collagen.

Working Hypothesis: Collagen-encapsulated islets will be structurally and metabolically viable, and will respond to glucose stimulation.

This first aim was designed to examine the effects of encapsulating isolated pancreatic islets within collagen type I on islet function. As a first step,
islet isolation protocols were tested for their ability to produce structural and metabolically viable islets. After several iterations of isolation procedures, the final optimized isolation protocol yielded islets which were examined with three separate viability assays, and were shown to be structurally and metabolically viable cells. Additionally, this experiment indicated that 90 minutes of post-isolation equilibration is required to yield maximally viable and responsive islets. Also, insulin diffusion characteristics through collagen gels was examined to assure that insulin diffusion throughout collagen gels is not inhibited, and that the collagen gels do not bind to or sequester free insulin. Data from these experiments shows that insulin is not irreversibly bound to the collagen matrix, that very little insulin actually diffuses into the collagen gel, and that the insulin that is present within collagen gels diffuses out quickly and with little evidence of diffusion barriers. All three of these diffusion characteristics are well within preferred parameters for an insulin producing and excreting implant. Lastly, the effects of a collagen matrix on islet responsiveness were examined. Results from these experiments showed that islets encapsulated within a collagen matrix responded more vigorously to glucose stimulation, and secreted 4 times more insulin than islets not encapsulated within collagen gels. Combined, these studies show that encapsulated islets are healthy and viable, insulin diffuses out of collagen gels with acceptable kinetics, and that encapsulated islets respond more robustly to glucose stimulation than free islets.
Specific Aim 2: *Evaluate a 3-dimensional tissue-engineered prevascularized construct for the purpose of supporting islet cell survival.*

*Working Hypothesis: Prevascularized collagen gels will provide the stimulus needed for islet cell survival, and will inosculate with the host vasculature in vivo.*

In this second specific aim, a 3-dimensional collagen gel containing microvessel fragments was examined for characteristics that would provide benefits to islet implants. These microvessel fragments were shown to grow and produce new vascular sprouts in culture, as well as produce and excrete vascular endothelial growth factor, a growth factor known to initiate angiogenic events and stabilize islet grafts. Additionally, these microvessel fragments were shown to be patent in culture, and are able to support flow. Upon implantation into subcutaneous dorsal pouches in SCID mice, these microvessel fragment gels were shown to upregulate VEGF transcript production at days 14 and 28 relative to day 7. Lastly, these fragment implants were shown to inosculate with the host circulation, and support host-derived perfusion. Combined, these experiments show that microvessel fragment gels produce known islet-stabilizing growth factors, and enhance reperfusion upon implantation, two critical characteristics that are amenable to islet graft survival.
Specific Aim 3: *Assess the ability of a prevascularized pancreatic encapsulation device to maintain islet survival in vivo.*

*Working Hypothesis: A prevascularized pancreatic encapsulation device will maintain islet survival longer than a non-prevascularized implant.*

In this third and final specific aim, the effects of a preformed vasculature on the survival of collagen-encapsulated islets post implantation was examined. First, collagen-encapsulated islets were shown to have proangiogenic benefits on microvessel fragments. Data from this experiment shows that vascular density and consequent perfusion is enhanced in microvessel fragment gels when islets are present. As a result, insulin containing islets were examined at 7, 14, and 28 days, and data from these experiments show that islet survival persists at all three time points. This data supports the use of PPEDs to enhance islet survival considering that islets encapsulated in collagen without a preformed vasculature do not survive at any of the time points examined. Histological examination shows that insulin containing islets are seen associated with a surviving intraislet vasculature. An observation that is corroborated by the primary literature in which it is known that an intraislet vasculature is required for production of islet-stabilizing growth factors and reperfusion. Lastly, dextran perfused PPEDs show perfused structures that resemble pancreatic islets in size, shape, and morphology as confirmed with a nuclear dapi stain and H & E stain. Additionally, non-perfused islets produce distinct ring-like insulin stains as necrotic cores are
present, a staining pattern that has never been demonstrated in islets encapsulated within PPEDs. Combined, these data indicate that a PPED is able to maintain islet survival longer than non-vascularized implants, and also provide suggestive evidence that islets encapsulated within PPEDs are perfused.

Conclusions

Combined, it is possible to use the collective data from all research aims to hypothesize a possible series of events and mechanisms that lead to the final experimental observation, islet survival. Upon removal from the animal, the epididymal fat pad becomes hypoxic. As the microvessel fragment isolation procedure proceeds, the hypoxic insult continues. As the fragments are isolated and encapsulated within a 3-dimensional collagen matrix, the hypoxic insult results in VEGF production from the endothelial cells of the microvessels. The microvessels, now cultured at 37° C with 5% carbon dioxide and fed with DMEM containing 10% serum, can begin to stabilize and recover from the isolation procedure. As the microvessels are cultured, vascular sprouting ensues, which increases the number of cultured microvessels within the gel, and VEGF production increases. This increase in VEGF secretion not only initiates an angiogenic response benefiting the microvessels, but it also saturates the gel with growth factors that will later benefit the islets.

After seven days of microvessel culturing, rat pancreata are perfused with liberase enzyme solution. As the islet isolation procedure ensues, the islets and
associated vasculature is subject to mechanical, enzymatic, chemical, and hypoxic insult. These strains lead to the demise of many islets, however, some islets survive the procedure, and are placed in a Petri dish, in equilibration buffer, for 90 minutes. During this 90 minute equilibration phase, heavily damaged islets disintegrate, sloughing off cells, and die. The remaining islets, however, reestablish intracellular homeostasis. As the intraislet vasculature is hypoxic, the endothelial cells of the vasculature begin to secrete growth factors, including VEGF. As the islets are placed in collagen gel, the collagen matrix provides a binding scaffold for extracellular integrins, a binding event that halts programmed cell death. This integrin binding not only stabilizes anti-apoptotic signaling pathways, but also provides structural scaffolding which allows the islets to space evenly throughout the gel so that islets do not pack together, a phenomenon known to be destructive to isolated islets. Additionally, integrin binding is linked to intracellular signaling cascades that result in an increase in intracellular calcium levels. This increase in calcium levels induces insulin granule docking with the cell membrane, and subsequent insulin release from the cell. Additionally, this leads to an increase in insulin transcription and production.

Once the islet gel is encapsulated by the seven-day-old microvessel fragments, the gels are implanted subcutaneously into SCID mice.

With the fusion of the two gels, the amount of VEGF within both gels is able to quickly initiate an angiogenic response within the microvessels and presumably within the intraislet vasculature. Additionally, there is a wound-associated healing response that occurs at the site of implantation. An event that
supplies a number of growth factors, including VEGF, that initiate and maintain and angiogenic and revascularization response. In the first 48 hours of implantation, although a robust angiogenic and neovascularization response has occurred, most of the implanted islets have died due to overwhelming hypoxia, however, the islets that have survived are now stabilized by the revascularization response. Between 3 and 7 days of implantation, as the angiogenic response begins to slow down, the vascular remodeling phase begins, sustained by growth factors derived from circulating platelets and cytokines secreted from macrophages. After seven days of implantation, the vessels within the PPEDs have inosculated with the host circulation, and can support perfusion. The islets that have survived to this point are now perfused, and with the support of the collagen scaffold, can stabilize and achieve homeostatic balance.

Scientific Contributions

The most altruistic goal of any scientific endeavor is to expand upon the existing body of scientific knowledge. Indeed, the one of the most important indicators of scientific validity is the ability of new scientific material to enrich current scientific ideas. As such, the body of work represented by this dissertation adds to the existing scientific literature by contributing new scientific concepts and ideas, as well as corroborating previously held posits.
Since the initial introduction of islet isolation protocols in 1967, it was quickly established that the isolation procedure is intrinsically destructive to the islets. Although strategies to enhance islet survival were considered, little work into the areas of refining and optimizing isolation procedures for the purpose of enhancing islet viability was pursued; therefore, the effect of modifying isolation procedures on resulting islet viability remained relatively poorly understood. Indeed, current islet isolation modifications are being investigated by several groups, however, these endeavors are being pursued for the purpose of increasing islet yield rather than enhancing islet viability\textsuperscript{132-134}. As such, one of the research goals of this dissertation was to optimize the isolation procedure for the purpose of increasing islet viability at the expense of greater islet yields. To this end, the resulting islet isolation procedure, although resulting in fewer numbers of islets, resulted in significantly more metabolically and structurally viable islets. This result contributes to the literature because it suggests that manipulations in the islet isolation procedure can enhance islet viability. Furthermore, this experiment suggests specific ways in which changes in the procedure can be employed to increase cell viability. Combined with existing literature on improving islet yields, this work can be used to develop an islet isolation protocol that optimizes yield without sacrificing viability.

Additionally, encapsulating isolated islets within a 3-dimensional collagen matrix produced novel results which corroborate existing hypotheses discussing the importance of extracellular matrix proteins on islet stability and function. With the observation that islets within a collagen matrix secrete 4 times more
insulin than islets not encapsulated within collagen, the theory of anoikis associated with islets removed from their native pancreatic tissues represents a plausible explanation for the experimental observations. Prior to this experiment, several scientists had hypothesized that one of the reasons isolated islets are unstable and lose function is that disruption of the cell-matrix contacts removes anti-apoptotic signals, and islet cells subsequently die. This experiment supports this hypothesis by showing, directly, that islets within collagen gels are more responsive to stimuli.

Lastly, the use of a tissue-engineered prevascularized collagen gel to stimulate reperfusion represents significant methodological and intellectual scientific contributions. The need to reperfused transplanted cells and tissues in order to attain functionality and preservation of the graft has been well established; implanted tissues that lack perfusion cannot function or survive. To that end, researchers have investigated a number of ways to enhance reperfusion, including inducing angiogenesis using various growth factors and agonists of new vessel growth and development, as well has implanting grafts within highly vascularized regions, such as the renal capsule or hepatic circulation. These approaches, however, contain intrinsic flaws that limit their effectiveness. However, using a prevascularized collagen matrix in which to encapsulate isolated cells represents a fundamental change in strategies, and mitigates some of the issues associated with previous reperfusion strategies; rather than stimulate an angiogenic response, the necessary blood vessels are transplanted such that all of the required components for successful reperfusion are contained within a single
implant device, eliminating the dependence on angiogenic responses to produce an environment in which reperfusion can occur. Not only do these prevascularized devices supply the necessary vessels, but the vessels within the device provide important growth factors that enhance the reperfusion processes, as well as provide established vessels with which inosculation with the host can occur. With these prevascularized devices, all of the necessary components of reperfusion are supplied, and therefore reperfusion can occur to the encapsulated cells regardless of the site of implantation.

Future Directions

The ultimate goal of most life sciences scientific researchers is to understand a biologic or physiologic process, and use that understanding to benefit humankind, either through gains in scientific knowledge or more directly, through clinical translation. The research presented in this dissertation has clear and obvious clinical applications. However, a number of preliminary steps, all of which lying outside the scope of this research project, must be accomplished prior to clinical application.

The research presented in this dissertation used rat derived microvessel fragments, and rat derived pancreatic islets implanted into immunocompromised mice. Before the process of upscaling can ensue, several preliminary studies must be completed, the first of which is completion of efficacy studies using the current
implant structure and composition. PPEDs must be implanted into a diabetic animal model to assure that reversal of hyperglycemia occurs. These hyperglycemic models can either be streptozotocin-induced hyperglycemia, non-obese diabetic genetic animals, or Zucker diabetic rats. Upon confirmation of the PPEDs clinical efficacy in small animals, the process of upscaling can continue.

Although rat derived isolated islets were examined in this dissertation, ultimate clinical translation to human recipients requires a more abundant source of islets. Although islets encapsulated within collagen gels secrete 4 times more insulin than non-collagen encapsulated islets, human recipients receive about 10,000 islets per kilogram of body weight. Using rat islets and the current isolation protocol, this would mean that islets would have to be isolated from approximately 11,000 rats in order to provide the estimated 800,000 islets required to reverse hyperglycemia in humans. To this end, other sources of islets must be identified to meet this demand. The most likely source would be porcine islets, which have been used in previous and ongoing human clinical trials of islet transplantations. However, porcine islets and rat islets may vary in many respects, including isolation procedure, collagen gel density, resistance to hypoxia, and glucose responsiveness. Therefore, porcine islets must be used in experiments that were presented in this dissertation, including viability experiments, in vivo survival experiments, and glucose stimulated insulin secretion experiments. Upon confirmation that porcine islets behave as expected when encapsulated within PPEDs, experiments on porcine islets within diabetic models must be undertaken to examine the effect of hyperglycemia on porcine-
containing PPEDs, as well as to estimate the number of porcine islets required to reverse hyperglycemia.

With the successful completion of preliminary studies investigating the use of porcine islets, the process of upscaling can begin. Human recipients of porcine islets require approximately 1600 times more islets than mouse models in order to attain clinical relevance. As a result, there are three aspects of the proposed research which will need to be upscaled: the microvessels, the islets, and the collagen scaffold.

In order to attain 1600 times more islets, the porcine islet isolation procedure would have to be optimized in order to balance maximal yield with maximal viability, two aspects of islet isolation which are negatively correlated. With the successful refinement of the porcine islet isolation procedure, the number of islets derived from pigs that are needed for human clinical trials can certainly be attained.

Another aspect of upscaling involves the construction of the collagen scaffold. Since the collagen matrix is a critical aspect of the presented research, large reinforced collagen gels will need to be attained. Experiments in which collagen gels are increased in size by a factor of 1600 must be done in order to examine their structural integrity. Inevitably, reinforcing the large collagen gels will be indicated, so reinforcing strategies will need to be investigated. These strategies include increasing the density of the collagen gels, and the effects that this will have on the encapsulated microvessels and islets, as well as the use of biostable polymers and materials, such as nylon.
Additionally, different transplantation sites could be investigated to resolve the most clinically beneficial area for transplantation. Alternative sites of transplantation include the mesenteric pouch, various intraabdominal locations, and possibly the renal capsule.

In order to attain 1600 times more microvessels than existing PPEDs, a larger source of vascularized fat must be used from which microvessels can be isolated. Current studies are investigating the possibility of using human derived fat, obtained from liposuction fat, to isolate microvessel fragments. This fat source is abundant in the human population, and the possibility of using the recipients own fat from which to isolate the microvessel fragments brings up the possibility that these autograft microvessel gels can confer a level of immunoprotection, the last consideration for clinical translation.

Current strategies for human islet transplantation trials involve the use of immunosuppressive, antirejection medications in order to prevent a host-derive immune attack on the grafts. Although these medications are effective at protecting the islet grafts, they have many negative side effects, including infection and the development of insulin resistance. To mitigate the issue of host rejection of islet grafts, immune isolation strategies must be investigated. The most probable strategies for immunoisoliating the implanted islets involve the use of biostable polymers, such as polytetraflouroethylene (PTFE), or nonreactive gels, such as calcium alginate. These immunoisolating strategies will have to prevent immune attack on transplanted cells while allowing for gas and nutrient exchange to occur. This obstacle represents a series of daunting research studies,
research studies which will likely occupy the lifelong career objectives of scores of scientists.

As can be seen, even with the successful completion of the research aims represented by this dissertation, a large amount of work is required in order to clinically translate this research. Like many aspects of science, the successful answering of a single question brings about the asking of a dozen more questions.
APPENDIX

APPENDIX A-RAT FAT DERIVED MICROVESSEL FRAGMENT ISOLATION PROCEDURE

Micro-fragment isolation from Rat  Shaleen Botting  02/14/2005

You will need:

Solutions:
About 2ml of 4 x DMEM (filter sterilized) Not pH’d
About 150ml - 0.1% BSA in DCF-PBS (filter sterilized)
10ml per rat (2mg/ml) Type I collagenase [Worthington Biochem Lot# M1C4749]
in (0.1% BSA-DCF-PBS)(filter sterilized)
Rat-tail collagen Type I [BD Biosci Lot# 001184 5.06mg/ml]
1mM NaOH (filter sterilized)

A. Surgery:

Instruments needed
a. 4x4 gauze sterile
b. Lg forceps, Lg scissors, med tweezers, med scissors
c. 30ml PBS-BSA in a sterile 50ml conical tube

1. Animal is anesthetized initially with Isoflurane, then with
   1ml IP injection of 1 mg/ml Nembutal sodium soln.
2. Prep abdomen with Novlasan.
3. Clamp skin with forceps, start incision cutting towards the
   flanks and upwards to expose scrotum.
4. Place a sterile 4x4 gauze pad under the exposed area
5. Make two small incisions on each sack exposing the
   epididymal fat pads.
6. Gently pull the fat out of the body, laying on the sterile 4x4.
7. Gently pull the fat away from the vein, cutting with a small
   pair of scissors if needed. Place the epididymal fat into the
   sterile tube of 0.1%BSA.
8. Note the final volume of liquid plus fat in the 50ml tube,
   this will give you an estimate of the amt of fat isolated.
B. Vessel microfragments isolation is performed in tissue culture hood under sterile conditions.

You will need:
- Shaking waterbath set to 37°C
- 25ml flask with small stirbar and screw top (autoclaved)
- 500μm screen cut to 75x75mm square (autoclaved)
- 30μm screen cut to 75mm round (autoclaved)
- Screening Frame (autoclaved)
- small tweezers (autoclaved)
- petri dishes (Sterile, but not cell culture treated)
- 15ml conical centrifuge tubes, Sterile
- 50ml conical centrifuge tubes, Sterile

Preparation and digestion of fat
1. Divided fat pads into 3 groups, placing one part in a petri dish at a time
   a. Quickly cut up each group with curved scissors until about 1-2mm size.
   b. This should take around 10mins total; fat should be minced well enough to not clog the tip of a 10ml pipette used in the next step.
2. Add approximately an one volume of 2mg/ml collagenase (Worthington lot# M1C4749 ) to the fat in the petri dish
3. Using a 10ml pipette transfer to the 25ml flask measuring the total volume when transferred to the small flask.
4. Add additional collagenase to the flask until you have added 1.5times the volume of fat.
5. Mixed up and down a few times with pipette before digestion.
6. Place in shaking water bath 37°C for 6-9mins.
   a. After 6mins remove from bath and look for extent of digestion. There should be ‘some’ small undigested pieces, but not a lot. Return to bath rechecking every minute. The digestion time should not exceed 9mins.
7. Mixed up and down a few times with 10ml pipette before dividing into two 15ml tubes, wash out the flask with 5ml of sterile PBS.
8. Centrifuged at 400rcf for 4 mins to separate fat from fragments
9. Gently decant off the top layer of fat and liquid, leaving the pelleted fragments behind.
10. Wash 2 times with ample PBS+BSA, pellet at 400rcf for 4mins. During the addition of PBS+BSA, titrate the pellet a few times to break it up.
11. After last spin, removed media with pipette (gently) and added 10ml of PBS to each 15ml tube.

Size exclusion isolation of fragments
1. Presoak the 30um filter in a sterile petri dishes with 15ml PBS-BSA to cover the filter for a few mins.
2. Using sterile tweezers, place the 500um filter on top of an open petri dish, and the 30um filter on top of the screening frame on a second petri dish.

3. Slowly pass the total volume thru the large filter in outward concentric circles, wash the tube out with 5ml PBS+BSA then pass thru large filter in the same manner to wash.

4. The elutant captured in the petri dish of the 500um filter is then carefully passed thru the 30um filter in outward concentric circles. This should be done quickly.

5. Quickly rinse the small filter with 5ml of PBS+BSA.

6. With sterile tweezers transfer the 30um filter to clean petri dish containing 15ml of PBS+BSA.

7. Using a 10ml pipette, rinse the filter very well by flushing the surface vigorously to remove all of the fragments.

8. Collect the total volume from washing off the small filter in a 50ml conical tube, note the total volume of liquid.

   a. To count fragments, gently mix the 50ml tube and remove 2 samples of 20ul with a 200ul pipette tip and place on a glass slide.

      i. Note: Clip off about 20% of the 200ul tip in advance with sterile scissors

      ii. Count # fragments were per 20ul drop under 10x. The total fragments can be calculated by [<#counted>*total volume in ul / 20].

9. Centrifuge for 4mins at about 400rcf. Gently remove all liquid except about 50ul !!.

Culturing isolated fragments in Rat Tail collagen matrix

1. Collagen is mixed in a sterile 15ml tube on ice with 4xDMEM with a cold pipette at the ratio {3 collagen: 1 DMEM}

   i. Determine the amount of collagen matrix needed for 15,000 fragments per ml, and make up an extra ml.

   ii. Adjust Ph by addition of 1mM NaOH, until color of 1xDMEM

2. Mix the microfragments gently by flicking the tube. Completely resuspend the fragments in the appropriate amount of collagen, mixing well with a cold pipette. Be careful not to introduce air bubbles when mixing.

3. Immediately pipette the mixture into a 48 well plate, you should put about 250ul per well.

4. Place 48well plate into incubator for at least 20-30 mins at 37C until collagen is solidified.

5. Add an equal volume of media (10% FBS/DMEM Media) to top of the collagen matrix.

6. Incubation continued in basement at 37C.

7. Change Media after 4days.

8. Sprout growth should be observable by day 5.
APPENDIX B-RAT PANCREATIC ISLET ISOLATION PROCEDURE

Surgical procedure:

1. Anesthetize the rat with Phenobarbital (.1 cc/100 grams)
2. Shave the ventral area of the rat from neck to lower genitalia
3. Make an incision from upper genital region to thoracic diaphragm
4. Remove distal sternum
5. Open the thoracic cavity through the diaphragm
6. Cut the superior vena cava, bleeding the animal into the thoracic cavity
7. Flip the liver up, securing with 4 x 4 gauze and hemostats
8. Expose the bile duct, excising connective and fat tissues
9. Clamp the proximal duodenum with a hemostat
10. Insert a ligature under the bile duct at the distal most region, below the second bifurcation
11. Using spring loaded microscissors, nick the common bile duct
12. Insert a stretched PE 50 tube into the bile duct, and secure with ligature
13. Pulse inject 15 ml of cold liberase solution
14. Remove the distended pancreas, beginning with the distal splenic region
   Place pancreas in 50 ml tube containing 5-7 ml of cold liberase solution

Isolation procedure:

1. Place the pancreas in a shaking water bath set at 37° C
2. Digest for 22-27 minutes, checking tube every 5 minutes
3. Remove the tube from the water bath, and add 30 ml of cold quenching buffer
4. Mix tube by inverting 5 or 6 times, and place tube in ice bath for ten minutes
5. Aspirate the top 30 ml of solution, making sure not to disturb the pellet
6. Add 30 ml of cold quenching buffer, and centrifuge for 4 minutes at 1000g
7. Aspirate the solution down to the pellet
8. Resuspend the pellet with 7 ml of 25% polysucrose solution
9. Add 23% polysucrose solution slowly, one drop at a time
10. Add 20% polysucrose solution slowly, one drop at a time
11. Add 11% polysucrose solution slowly, one drop at a time
12. Centrifuge for 20 minutes at 1400g
13. Remove the 20% layer with a 10 ml pipet, taking half of the 11% and half of the 23% layers
14. Add room temperature quenching buffer, and mix in a 50 ml tube by inverting 5 or 6 times
15. Centrifuge for 4 minutes at 1000g

Culturing/encapsulation
1. Place islets in a 30 mm Petri dish in 5 ml of equilibration buffer
2. Place Petri dish in 37° C incubator for 90 minutes
3. Using a p20 pipet, pick islets under a dissecting scope
4. Place islets in nonpolymerized collagen gel (3 mg/ml) or culture in CMRL
### APPENDIX C - MEDIA AND SOLUTION RECIPES

<table>
<thead>
<tr>
<th>Product Name</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Type I collagen (3 mg/ml)</strong></td>
<td>- Type I collagen&lt;br&gt;- 1x DMEM&lt;br&gt;- 1N NaOH</td>
</tr>
<tr>
<td><strong>Fragment media</strong></td>
<td>- 1x DMEM&lt;br&gt;- 10% FBS</td>
</tr>
<tr>
<td><strong>Collagenase</strong></td>
<td>- 80 mg collagenase&lt;br&gt;- 40 ml DCF-PBS/0.1% BSA</td>
</tr>
<tr>
<td><strong>Liberase (0.3 mg/ml)</strong></td>
<td>- 224 µl Liberase&lt;br&gt;- 24 ml Hanks Balanced Solution</td>
</tr>
<tr>
<td><strong>Quenching Buffer</strong></td>
<td>- 1000 ml Hanks BSS&lt;br&gt;- 110 ml FBS</td>
</tr>
<tr>
<td><strong>25% Polysucrose</strong></td>
<td>- 108 g polysucrose&lt;br&gt;- 324 ml Hanks BSS&lt;br&gt;- 8.1 ml HEPES&lt;br&gt;- 33 mg DNase</td>
</tr>
<tr>
<td><strong>Dithizone stock (2.5 mg/ml)</strong></td>
<td>- 200 mg DTZ&lt;br&gt;- 80 mL DMSO</td>
</tr>
<tr>
<td><strong>Working Dithizone (0.250 mg/ml)</strong></td>
<td>- 80 ml stock dithizone&lt;br&gt;- 0720 ml Hanks BSS</td>
</tr>
<tr>
<td><strong>Shipping media</strong></td>
<td>- 500 ml CMRL&lt;br&gt;- 55 mL FBS&lt;br&gt;- 12.5 ml 1M HEPES&lt;br&gt;- 2.5 ml Pen/Strep&lt;br&gt;- 25 mg DNase&lt;br&gt;- 50 mg trypsin inhibitor</td>
</tr>
<tr>
<td><strong>Krebs Ringer Buffered Solution</strong></td>
<td>- 3.36 g NaCl&lt;br&gt;- 0.185 g KCl&lt;br&gt;- 0.185 g CaCl₂&lt;br&gt;- 0.1 g MgCl₂&lt;br&gt;- 1.01 g NaHCO₃&lt;br&gt;- 1.3 g HEPES&lt;br&gt;- 500 ml MilliQ water</td>
</tr>
<tr>
<td><strong>Islet equilibration buffer</strong></td>
<td>- 50 ml KRB&lt;br&gt;- .5 g BSA&lt;br&gt;- 18 mg D-glucose</td>
</tr>
<tr>
<td><strong>2.0 mM glucose</strong></td>
<td>- 50 mL KRP&lt;br&gt;- 18 mg D-glucose</td>
</tr>
<tr>
<td><strong>Islet lysing buffer</strong></td>
<td>- 25 ml 70% ethanol&lt;br&gt;- 25 ml 1N HCl</td>
</tr>
<tr>
<td><strong>20.0 mM glucose</strong></td>
<td>- 50 ml KRB&lt;br&gt;- 194 mg D-glucose</td>
</tr>
<tr>
<td><strong>100% stock Avertin</strong></td>
<td>- 10 g tribromoethanol&lt;br&gt;- 10 ml tert-amyl alcohol</td>
</tr>
<tr>
<td><strong>2.5% working Avertin</strong></td>
<td>- 40 ml warm DCF-PBS&lt;br&gt;- 1 ml 100% stock Avertin</td>
</tr>
<tr>
<td>Ingredient</td>
<td>Amount</td>
</tr>
<tr>
<td>--------------</td>
<td>----------</td>
</tr>
<tr>
<td>D-PBS</td>
<td></td>
</tr>
<tr>
<td>-0.2 g KCl</td>
<td></td>
</tr>
<tr>
<td>-0.2 g KH₂PO₄</td>
<td></td>
</tr>
<tr>
<td>-8.0 g NaCl</td>
<td></td>
</tr>
<tr>
<td>-1.15 g Na₂HPO₄</td>
<td></td>
</tr>
<tr>
<td>-0.1 g MgCl₂</td>
<td></td>
</tr>
<tr>
<td>-0.1 g CaCl₂</td>
<td></td>
</tr>
<tr>
<td>-1000 ml water</td>
<td></td>
</tr>
</tbody>
</table>
APPENDIX D-PERMISSIONS

Non-copyrighted materials:

Figure 3-2 was used with permission from Dr. Jay Hoying and Carlos Chang.

Figures 3-1 and 3-7 were used with permission from Dr. Stuart Williams.

Copyrighted materials:

Figure 1-3 was used with written permission from Lippincott Williams and Wilkins. The image was originally published in the Textbook of Medical Physiology [9th edition], page 972. [permission pending]
APPENDIX E-INSULIN RIA QUALITY ASSURANCE DATA

The following validation data are provided by Linco Research®.

RI-13K-Rev. 04/10/06 LINCO Research

XII. ASSAY CHARACTERISTICS

A. Sensitivity
The lowest level of Rat Insulin that can be detected by this assay is 0.1ng/ml when using 100μl sample size.

B. Performance
The following parameters of assay performance are expressed as Mean ± Standard Deviation.

ED80 = 0.2 ± 0.02 ng/ml
ED50 = 0.8 ± 0.04 ng/ml
ED20 = 3.0 ± 0.36 ng/ml

C. Specificity
The specificity (also known as selectivity) of an analytical test is its ability to selectively measure the analyte in the presence of other like components in the sample matrix.

Rat Insulin I 100%
Rat Insulin II 100%
Human Insulin 100%
Human Proinsulin 69%
Porcine Insulin 100%
Sheep Insulin 100%
Hamster Insulin 100%
Mouse Insulin 100%
Rat C-Peptide ND
Glucagon ND
Somatostatin ND
Pancreatic Polypeptide ND
IGF-I ND
Human IGF-I ND
Human IGF-II ND
ND-not detectable

D. Precision
Within and Between Assay Variation

Sample Mean Within Between
No. ng/ml % CV % CV

1 0.5 2.2 8.9
2 0.8 1.4 9.1
Within and between assay variation was performed on five Rat Serum samples containing varying concentrations of Rat Insulin. Data (mean and % CV) shown are from five duplicate determinations of each serum sample in five separate assays.

Varying concentrations of Rat Insulin were added to five Rat Serum samples and the Insulin content was determined by RIA. Mean of the observed levels from five duplicate determinations in five separate assays are shown. Percent recovery was calculated on the observed vs. expected.

F. Linearity
Effect of Serum Dilution

Sample Volume Observed Expected % of
No. Sampled ng/ml ng/ml Expected
Aliquots of pooled Rat Serum containing varying concentrations of Insulin were analyzed in the volumes indicated. Dilution factors of 1.0, 1.33, 2.0, and 4.0 representing 100 µl, 75 µl, 50 µl and 25 µl, respectively, were applied in calculating observed concentrations. Mean Insulin levels and percent of expected for five separate assays are shown.

RI-13K-Rev. 04/10/06
LINCO Research

XII. ASSAY CHARACTERISTICS (continued)

G. Example of Assay Results
This data is presented as an example only and should not be used in lieu of a standard curve prepared with each assay.

<table>
<thead>
<tr>
<th>Tube #</th>
<th>ID</th>
<th>CPM Ave</th>
<th>CPM Ave</th>
<th>Net CPM</th>
<th>% B/Bo</th>
<th>ng/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Totals</td>
<td>22694</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>''</td>
<td>23052</td>
<td>22873</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>NSB</td>
<td>748</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>''</td>
<td>728</td>
<td>738</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Bo</td>
<td>12080</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>''</td>
<td>12036</td>
<td>12058</td>
<td>11320</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Standards</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>0.1 ng/ml</td>
<td>10709</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>11309</td>
<td>11009</td>
<td>10271</td>
<td>90.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>0.2 ng/ml</td>
<td>9781</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>10001</td>
<td>9891</td>
<td>9153</td>
<td>80.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>0.5 ng/ml</td>
<td>7169</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>7309</td>
<td>7239</td>
<td>6501</td>
<td>57.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>1.0 ng/ml</td>
<td>5099</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>5043</td>
<td>5071</td>
<td>4333</td>
<td>38.3</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
15 2.0 ng/ml 3376
16 3492 3434 2696 23.8
17 5.0 ng/ml 2004
18 2154 2079 1341 11.9
19 10.0 ng/ml 1499
20 1497 1498 760 6.7
Controls/Unknown
21 QC 1 7633
22 7629 7631 6893 60.9 0.44
23 QC 2 3903
24 3857 3880 3142 27.8 1.69
25-n Unknown

XIII. QUALITY CONTROLS

Good Laboratory Practice (GLP) requires that Quality Control (QC) specimens be run with each standard curve to check the assay performance. Two levels of controls are provided for this purpose. These and any other control materials should be assayed repeatedly to establish mean values and acceptable ranges. Each individual laboratory is responsible for defining their system for quality control decisions and is also responsible for making this system a written part of their laboratory manual. The ranges for Quality Control 1 and 2 are provided on the card insert or can be located at the Linco Research website www.lincoresearch.com.

Recommended batch analysis decision using two controls (Westgard Rules)4:

1. When both controls are within ±2 SD. Decision: Approve batch and release analyte results.

2. When one control is outside ±2 SD and the second control is within ±2 SD. Decision: Hold results, check with supervisor. If no obvious source of error is identified by the below mentioned check of systems, the supervisor may decide to release the results.

Technician check of systems:
1. Check for calculation errors
2. Repeat standards and controls
3. Check reagent solutions
4. Check instrument
References


70. Wagner, R.C. and Matthews, M.A. The isolation and culture of capillary endothelium from epididymal fat. Microvascular research 10, 286, 1975.


