TISSUE ENGINEERING A PANCREATIC SUBSTITUTE BASED ON RECOMBINANT INTESTINAL ENDOCRINE CELLS

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TISSUE ENGINEERING A PANCREATIC SUBSTITUTE BASED ON RECOMBINANT INTESTINAL ENDOCRINE CELLS

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LIST OF ABBREVIATIONS

B10	Histidine to Aspartic Acid Substitution at Position 10 of the B Chain of Insulin
C57Bl6/J	C57 Black 6 Jackson Labs Strain of Mouse
CMV	Cytomegalovirus
CRE	cAMP Responsive Element
DMEM	Dulbeco's Modified Eagle Medium
DNA	Deoxyribonucleic Acid
EGFP	Enhanced Green Fluorescent Protein
FRIC	Fetal Rat Intestinal Cells
FBS	Fetal Bovine Serum
GIP	Gastric Inhibitory Polypeptide
GK	Glucokinase
GLP-1	Glucagon-like Peptide-1
GLUT2	Glucose Transporter 2
HBSS	Hank's Balanced Salt Solution
IBMX	3-isobutyl-1-methylxanthine
IDD	Insulin-Dependent Diabetes
IgG	Immunoglobulin G
MH	Meat Hydrolysate
mPGp	Mouse Proglucagon Promoter
NOD	Non-Obese Diabetic
PBS	Phosphate Buffered Saline
PC1/3	Prohormone Convertase 1/3

PC2	Prohormone Convertase 2
PCR	Polymerase Chain Reaction
RIA	Radioimmunoassay
R_{neo}	Neomycin Resistance
SIS	Small Intestinal Submucosa
STZ	Streptozotocin
SV40	Simian Virus 40
TEPS	Tissue Engineered Pancreatic Substitute(s)
TRITC	Tetramethylrhodamine Isothiocyanate

SUMMARY

Cell-based treatments for insulin-dependent diabetes (IDD) may provide more physiologic regulation of blood glucose levels than daily insulin injections, thereby reducing the occurrence of secondary complication associated with IDD. An autologous cell source is especially attractive for regulatory and ethical reasons and for circumventing the need for immunosuppression, which is currently standard for islet transplantation. Our approach focuses on using adult non-β-cells engineered for physiologic insulin secretion. Specifically, we utilize enteroendocrine L-cells, which naturally exhibit regulated secretion of GLP-1 in response to physiologic stimuli, and upon genetic engineering, co-secrete insulin in a regulated manner. The overall goal of this project is to develop a tissue engineered pancreatic substitute based on a recombinant enteroendocrine cell line and test the efficacy of the pancreatic substitute by implantation into diabetic mice. The specific aims of this thesis were to (1) to modify murine L-cells for regulated insulin secretion and evaluate the insulin secretion properties of the recombinant cells; (2) to incorporate insulin-secreting L-cells into an implantable construct containing small intestinal submucosa (SIS) and to evaluate insulin secretion from the construct in vitro; and (3) to test the efficacy of the tissue engineered pancreatic substitute in vivo by implanting it intraperitoneally in mice made diabetic by streptozotocin. Thus, this proposal takes a tissue engineered pancreatic substitute for IDD from in vitro development to in vivo testing.

CHAPTER 1

INTRODUCTION

Insulin-dependent diabetes is a chronic disease which increases the morbidity and reduces the life-expectancy of the nearly five million people affected by this disease in the United States alone, based on the most recent Figures provided by the CDC (National Diabetes Fact Sheet, CDC 2007). Though insulin injections and infusions can manage the disease, exogenous insulin is not sufficient to prevent the onset of very serious secondary complications including heart and kidney disease, peripheral nerve damage, and blindness. Cell-based therapies, such as allogeneic islet transplantation, which is currently clinically implemented at a small scale, provide better physiological blood glucose regulation less invasively than traditional insulin therapy, thereby potentially improving lifestyle and reducing the occurrence of secondary complications. Though the improvement of lifestyle gained by becoming insulin-independent must be balanced with the side-effects of the immuno-suppressive regiment used to prevent rejection of the allograft, and long-term graft survival has not been widely achieved in order to assess whether there is indeed a reduction in the occurrence of secondary complications (Bertuzzi, Marzorati et al. 2006). Another drawback of allogeneic islet transplantation is that it may not be applied to a large population because of severe limitations in human donor tissue.

Other cell-based treatments of IDD include the use of encapsulated β-cell lines or xenogeneic islets; differentiation of adult or embryonic stem cells toward a β-cell ph

enotype; and genetic engineering of non-β-cells toward the development of β-cell surrogates. As with allogeneic islet transplantation, the use of cell lines and xenogeneic islets also carry the burden of recipient immunosuppresion in order to prevent their rejection. The generation of insulin-secreting cells from embryonic stem cells is

hampered by the fact that pancreatic cells appear late during embryonic development. Thus, generation of insulin-secreting cells from embryonic stem cells would involve the sequential activation and deactivation of a considerable number of genes controlling first, the generation of endoderm lineage cells, the subsequent generation of the precursors of pancreatic cells, and finally differentiation to insulin-secreting cells (Docherty, Bernardo et al. 2007). Adult stem cells, particularly those coming from the pancreas, seem to be easier to fully differentiate but are more difficult to proliferate (Soria, Bedoya et al. 2005). Furthermore, the transplantation of the newly derived β-cells would be subject to destruction by the autoimmunity that caused the onset of diabetes in the first place (Monti, Scirpoli et al. 2008) and allograft rejection for the case of β-cells derived from embryonic stem cells.

The use of non-β-cells from the patients themselves relaxes the requirements of availability and immune acceptance, but is hindered by the need to reconstitute the complex regulatory system of insulin production and secretion inherent to the β-cell. Several potential cell sources have been investigated in attempts to producing β-cell surrogates, including endocrine pituitary cells (Hughes, Quaade et al. 1993; Motoyoshi, Shirotani et al. 1998), muscle cells (Yin and Tang 2001), skin cells (Lei, Ogunade et al. 2007), and liver cells (Huang, Thule et al. 1995; Thule, Liu et al. 2000; Thule and Liu 2000; Olson, Paveglio et al. 2003). Though great strides have been made in this area, each of these potentially autologous cell sources has its drawbacks.

Recently, enteroendocrine cells (specialized endocrine cells of the gastrointestinal tract) have emerged on the scene as a promising cell source for engineering surrogate β-cells. The unique connection between their naturally secreted incretins and insulin secretion makes the engineering of enteroendocrine cells for regulated insulin secretion an appealing approach in terms of the dynamic release of insulin, as well as the compatibility of incretins and insulin in glycemic normalization. A novel study by Anthony Cheung and colleagues in 2000 demonstrated that insulin produced and

secreted by genetically modified intestinal K cells of transgenic mice prevented those animals from becoming diabetic when injected with streptozotocin (Cheung, Dayanandan et al. 2000). Similarly, transgenic mice which produced human insulin in gastric G cells, displayed a meal-regulated increase in the level of transgenic insulin and a corresponding decrease in blood glucose levels (Lu, Sternini et al. 2005). These are important proof-of-concept studies which showed that enteroendocrine cell-produced insulin can provide regulation of blood glucose levels. Much work remains, however, in translating the results seen in transgenic mice to adult models of IDD.

The most direct application suggested by these transgenic mice for treating IDD in an adult animal would be to genetically engineer the appropriate enteroendocrine cells in their natural environment, that is, in vivo gene therapy. Besides significant difficulties inherent to delivering and stably expressing recombinant insulin in L-cells in vivo, a major challenge of in vivo gene therapy is in carefully titrating the treatment, so as not to overdose the patient with the transgene, as this might result in sustained over-production of insulin, an error that could be fatal. Performing the necessary genetic modifications in a closely controlled manner outside of the body would greatly enhance safety. A TEPS would not only provide the safety and quality control of in vitro gene therapy, but could additionally allow for retrieval of the construct and cells, if necessary, and for the non-invasive monitoring of the implant by, e.g., nuclear magnetic resonance techniques (Constantinidis and Sambanis 1998; Stabler, Long et al. 2005; Stabler, Long et al. 2005)

Previously, our lab engineered the human L-cell line, NCI-H716 to release insulin in response to nutrient administration (Tang and Sambanis 2003) and showed preferential transduction by adeno-associated virus for L-cells over enterocytes in a co-culture model (Tang and Sambanis 2004). These studies laid the foundation for developing a method for gene delivery to L-cells in mixed cultures such as primary intestinal isolates. But due to the complex anatomy of the intestinal epithelium and low percentage of L-cells, establishing primary cultures of enteroendocrine cells is difficult and these cultures do

not survive in vitro for extended periods. Initially, we tried several protocols in attempts to isolate primary intestinal epithelial cells found in structures called crypts (APPENDIX A) but were unsuccessful in maintaining cultures with significant levels of viable cells. As these primary cultures made for poor test beds because of their severely limited shelf-life, the focus of these studies was again placed on L-cell lines. GLUTag cells are a reasonable choice as a model system and would represent a closer allograft model for mice than the previously investigated human NCI-H716 cells. Thus, using GLUTag cells was a more direct avenue toward advancing L-cell mediated insulin therapy to a tissue-engineered treatment for adult mouse models of IDD.

The research in CHAPTER 3 was aimed at developing and characterizing a recombinant L-cell line for regulated insulin secretion. This was approached by stable transfection of the murine GLUTag L-cell line with a vector encoding human insulin and neomycin resistance under the control of constitutive promoters. The derived clonal cell line, GLUTag-INS, was then evaluated in vitro to determine if secretion of recombinant insulin and endogenous glucagon-like peptide-1 were regulated in the same manner by nutrient and putative secretagogues and to determine the time-course of insulin secretion.

The work in CHAPTER 4 was to develop an implantable three-dimensional construct which retained the important insulin secretory properties of the cells and to study its behavior in vitro. Hydrogels are frequently used in metabolic tissue engineering and have proven to be sufficient barriers against allograft rejection in mice. As such, tissue engineered devices, in which GLUTag-INS cells were suspended in the natural agarose hydrogel evaluated against counterparts that incorporated either pre-aggregated GLUTag-INS spheroids or small intestinal submucosa. Induced insulin secretion tests were performed on these types of constructs to determine which configuration was appropriate for maintaining this important characteristic of recombinant L-cell function.

This project was further advanced in CHAPTER 5 when the tissue engineered pancreatic substitute based on recombinant L-cells and small intestinal submucosa

developed in CHAPTER 4 was implanted intraperitoneally in diabetic mice and the performance of the construct was evaluated by examining the retrieved constructs and the physiological effects on the mice.

Each of the aforementioned chapters contains detailed motivation, research methods, results, and discussion. General information on IDD and current and potential cell-based treatments are reviewed in CHAPTER 2. Conclusions and potential future directions are discussed in CHAPTER 6. Preliminary studies in understanding how best to transfer genetic material to L-cell lines can be found in APPENDIX B and a peripheral study looking to achieve L-cell specific gene expression through use of a cloned proglucagon promoter is discussed in APPENDIX C.

In summary, this thesis is devoted to increasing the knowledge of developing recombinant β-cell surrogates for the treatment of IDD. Enteroendocrine cells could potentially be of autologous origin (derived from the patient themselves), thus, potentially alleviating the immune acceptance challenges with allo- and xenogeneic cells. The ideal human therapy envisioned would entail surgical retrieval of intestinal stem cells (which give rise to enteroendocrine cells), genetically engineering these cells ex vivo, incorporating them into a tissue engineered device which may be monitored and retrieved if necessary, and re-implanting them into the patient. This thesis evaluated the potential of a tissue engineered pancreatic substitute using a recombinant L-cell line. Because these cells were not autologous in origin, a more direct path toward studying insulin-secreting L-cells as part of a tissue engineered pancreatic substitute was possible. An alternatively envisioned human therapy may rely on an engineered allogeneic cell source such as the one evaluated in this thesis given the inherent difficulties in working with primary L-cells or if a suitable number of intestinal stem cells could not be isolated and engineered by a tolerable size of resected intestine. Alternatively, it may be that primary intestinal stem cells can be isolated and manipulated ex vivo, but may function better upon re-implantation if implanted by themselves. Though this approach may not

facilitate their easy retrieval if needed, it would be preferred to other tissue engineering approaches if they exhibited inferior cell function.

CHAPTER 2

BACKGROUND

2.1 Diabetes

Diabetes is a significant health problem affecting an estimated 23.6 million people in the United States alone. Among adults diagnosed with diabetes 27% report either taking insulin alone or in combination with oral medications (National Diabetes Fact Sheet, CDC 2007). Those taking insulin may have either type 1 (juvenile) or type 2 (adult onset) diabetes. Type 1 diabetes results from the complete loss of insulin producing cell mass (β-cells of pancreatic islets) due to autoimmune attack, whereas when type 2 diabetes is diagnosed, the body is producing enough insulin but it is unable to use it. This is a phenomenon called insulin resistance and overtime the body's production of insulin also becomes impaired. As insulin is required for maintaining blood glucose concentrations within a physiological range, exogenous insulin is necessary for all type 1 diabetics and many type 2 diabetics. As such, insulin-dependent diabetes (IDD) is defined as those patients requiring the use of exogenous insulin to control blood glucose levels and the number of insulin-dependent diabetics in the U.S. is nearly 5 million people based on the most recent Figures provided by the CDC (National Diabetes Fact Sheet, CDC 2007).

2.2 Insulin Therapy

The tight physiological range of glucose in normal human subjects is maintained by the careful balance of the secretion of insulin and glucagon, hormones with antagonistic effects; and these rates of secretion are precisely regulated. β-cells are responsible for maintaining a basal level of insulin during times of fast (including each night during sleep) and responding rapidly to the glycemic load following each meal.

The post-prandial response of β-cells is biphasic in nature with an initial rapid release phase which lasts for 5 -10 minutes, followed by a second phase in which the insulin secretion rate slowly increases over a period of several hours. Thus in order for diabetic patients to mimic the insulin secretion profile by using exogenous insulin, multiple injections may be required. In addition, several types of insulin are available which have varying characteristics of activity (onset, peak, and duration). For example, a single injection of long-acting insulin, used to maintain the basal insulin level, has an onset time of one hour, no peak, and a duration of 20-26 hours, may be taken once daily (NIH 2008). Depending on the patient's requirements, this may be supplemented with several injections of rapid-acting or short-acting insulin before a meal to mimic the post-prandial response. Insulin, a protein hormone, is predominately delivered by subcutaneous injection or infusion by syringe or insulin pump, though Bentley Pharmaceutical's intranasal spray, Nasulin^{TM} is in phase II clinical trials and other delivery routes for insulin including an insulin pill, buccal sprays, and a transdermal patch are under investigation (NIH 2008).

Although diabetes is considered a chronic disease, treatable with daily injections of insulin and careful monitoring of one's diet, even the most vigilant insulin therapy cannot reproduce the precise metabolic control present in the non-diseased state. The poor temporal match between glucose load and insulin action leads to a number of complications including increased risk of heart disease, kidney failure, blindness, amputation due to peripheral nerve damage, diabetic ketoacidosis, or hyperosmolar coma (National Diabetes Fact Sheet, CDC 2005). A 10-year, multi-center study conducted by the Diabetes Control and Complications Trial Group (1993), suggest that aggressive monitoring and insulin therapy can postpone the onset of these secondary complications, but that exogenous insulin therapy alone cannot prevent them. Cell-based therapies, which provide continuous regulation of blood glucose, would provide more physiological

control thereby potentially alleviating many of the diabetes-related health problems and revolutionizing diabetes care.

2.3 Current and Future Cell-Based Therapies

Several directions are being considered for cell-based therapies of IDD including implantation of allogeneic or xenogeneic islets; differentiation of adult or embryonic stem or progenitor cells into insulin-secreting cells; genetic engineering of autologous non-β-cells; implantation of encapsulated insulinoma cell lines; and in vivo gene therapy. The first three approaches will be discussed in this chapter, and the variety of potential cell therapies is depicted in Figure 2.1.

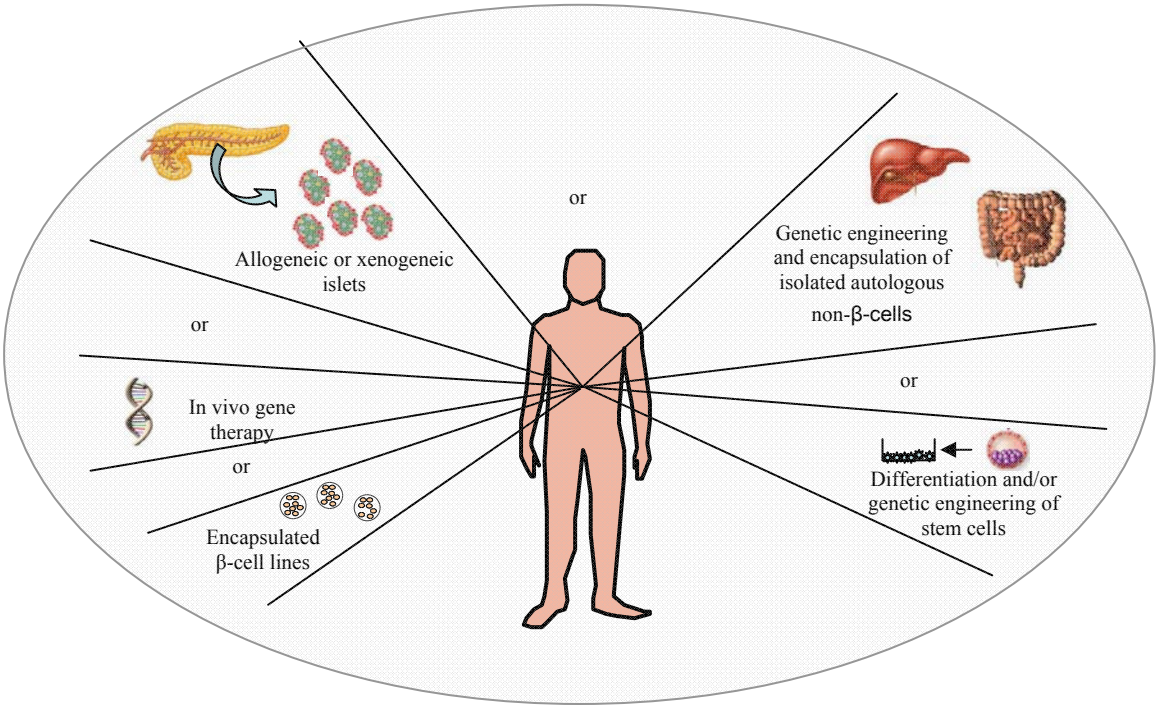


Figure 2.1 The spectrum of cell-based therapies for the treatment of insulin-dependent diabetes.

2.3.1 Islet Transplantation

2.3.1.1 Allogeneic Islet Transplantation

Islet transplantation involves the isolation of islets from a donor pancreas and the subsequent re-implantation of the purified islets into the liver of the diabetic recipient via the portal vein as shown in Figure 2.2. This procedure is far less invasive than the surgery required for the implantation of the whole organ, yet still requires the use of life-long immunosuppressive drugs to prevent rejection of the donor tissue. Sometimes, the side-effects of the drugs used to prevent rejection are deemed worse than the inconvenience of exogenous insulin therapy and the threat of secondary complications.

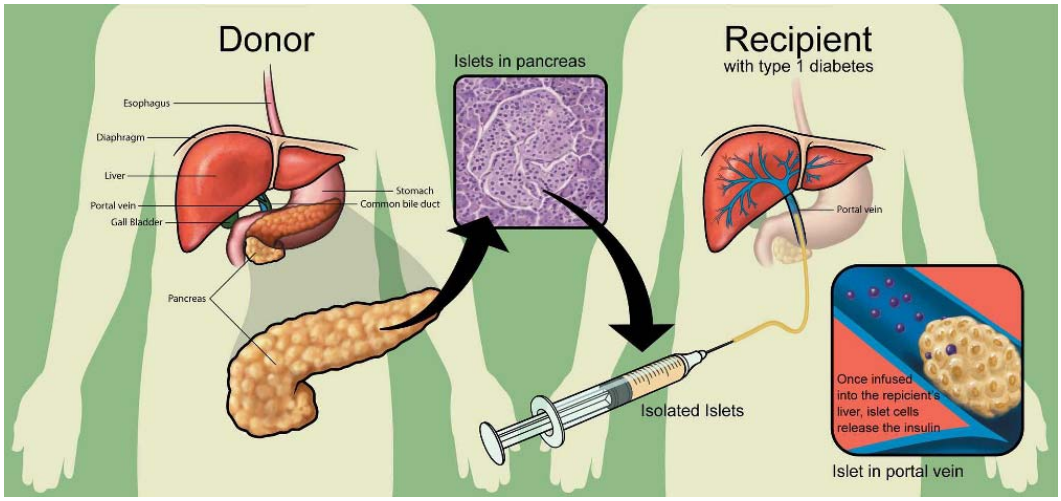


Figure 2.2 Overall process of islet transplantation. Islets are purified from the donor pancreas and later, infused into the portal vein of the diabetic recipient so that they become lodged in the microvasculature of the liver. Illustration by Giovanni Maki (Naftanel and Harlan 2004).

The islet transplantation protocol developed by physicians at the University of Edmonton (Shapiro, Lakey et al. 2000; Logberg, Sgan et al. 2003) dramatically improved the survivability of allogeneic islet grafts, with 80% of patients being insulin independent one year post-transplantation (Bertuzzi, Marzorati et al. 2006). A five year follow-up however, revealed that although 80% of patients still had detectable C-peptide, only 10%

remained insulin independent, indicating that there was a progressive decline in graft function (Bertuzzi, Marzorati et al. 2006). Reasons for loss of islet function after transplantation include (1) insufficient transplanted β-cell mass, (2) allograft rejection due to inflammation, (3) recurring autoimmunity, (4) incompatibility of islet implantation site, and (5) the toxicity of the immunosuppressive regiment (Lee, Grossman et al. 2008).

Even as scientists overcome the reasons for failed islet transplantations, the application of this therapy is still limited to a very small subset of the diabetic population due to the supply of donor islets. Using current techniques and practices the number of patients treated by islet transplantations in a typical year are optimistically estimated at 1,000 to 2,000 (Ren, Jin et al. 2007). The need for at least two cadaveric donor pancreases per patient and the worsening trend of the donor to recipient ratio contribute to keeping this number low. This situation is so restricted because islet cells do not significantly expand their population size in culture. One could imagine that donation of a partial pancreas from a living relative may help, and indeed there has been one reported case of successful living donor islet transplantation performed at Kyoto University Hospital in Japan (Matsumoto, Okitsu et al. 2005). The one year follow-up of these patients even indicated that the recipient was still insulin-independent and the donor had recovered quickly with no adverse effects from the partial pancreatectomy (Matsumoto, Okitsu et al. 2006), but given the risk to the living donor during surgery and anesthesia, living donor islet transplantations are only considered for patients with high incidence of life-threatening hypoglycemic unawareness and when cadaveric islets donors are unavailable.

Another problem with islet transplantation is the need for life-long treatment with immunosuppressant drugs. This issue is compounded by the fact that tacrolimus is a nephrotoxic agent, making proper kidney function a prerequisite for islet transplantation (Bertuzzi, Marzorati et al. 2006). Since kidney failure is already a complication of diabetes, this excludes a great number of diabetics from this therapy and increases the

incidence of kidney failure, with 5% of kidney function lost each year among recipients (Ren, Jin et al. 2007). Additionally the immunosuppressive drugs used after transplantation have diabetogenic properties and may account for some of the progressive loss of β-cells (Bertuzzi, Marzorati et al. 2006; Ren, Jin et al. 2007). Despite improvements in islet transplantations, there appear to be insurmountable barriers for widespread application of this therapy.

2.3.1.2 Xenogeneic Islet Trasnplantation

The issue of limited supply of donor pancreases could be relieved if islets derived from animal sources (xenografts) could be made into a viable transplant. Porcine islets have been the subject of intense research, as glycemic regulation is very similar between pigs and humans and porcine insulin was used for many years as a source of exogenous insulin (MacKenzie, Hullett et al. 2003). However, repressing the immune rejection of a xenograft is a formidable challenge, and the mechanisms of xenograft rejection are not entirely understood (MacKenzie, Hullett et al. 2003). In addition, concern of transmission of infectious agents (most notably of the porcine endogenous retrovirus, PERV) from the graft to the host is of paramount concern. In an effort to reduce the risk of disease transmission and improve immune acceptance, encapsulation of islets has been investigated by a number of groups (Lim and Sun 1980; Sambanis 2003; Cui, Barr et al. 2004). Long-term survival of porcine grafts has been challenging and severely limited due to lack of biocompatibility, limited immuno-protective properties, and hypoxia (de Groot, Schuurs et al. 2004). In regard to protecting the host from PERV transmission, however, encapsulation in alginate polylysine capsules was sufficient in preventing the leakage of viral particles or RNA in vitro (Elliott, Escobar et al. 2000*)* and a human subject receiving encapsulated neonatal porcine islets through a Swedish clinical trial showed no evidence of porcine viral or retroviral infection 9.5 years post implantation (Elliott, Escobar et al. 2007). A device based on functional and immune-acceptable

xenogeneic islets would lend itself to wide-spread application, but the incomplete understanding of immune rejection at the fundamental level has so far hampered its development.

Apart from islet transplantation, several other directions are being considered for cell-based therapies of IDD including differentiation of adult or embryonic stem cells into insulin-secreting cells and genetic engineering of autologous non-β-cells.

2.3.2 Differentiation of Stem Cells to β-Cells

Stem cells differentiated into replacement β-cells constitute an attractive approach toward resolving the cell source issue for islet transplantation. By definition, stem cells possess a nearly unlimited regenerative capacity due to the property of self renewal, and may be coaxed to differentiate into specialized cell types. Indeed, the very nature of the disease makes IDD a good target for stem cell therapy, in that just a single protein is deficient and a single cell population is responsible for production of that missing protein.

Stem cells or progenitor cells have been isolated from a wide variety of tissues, including both embryonic and adult sources. These cells are often characterized by the variety of cells that they can give rise to and some of the various sources of stem cells are depicted in Figure 2.3.

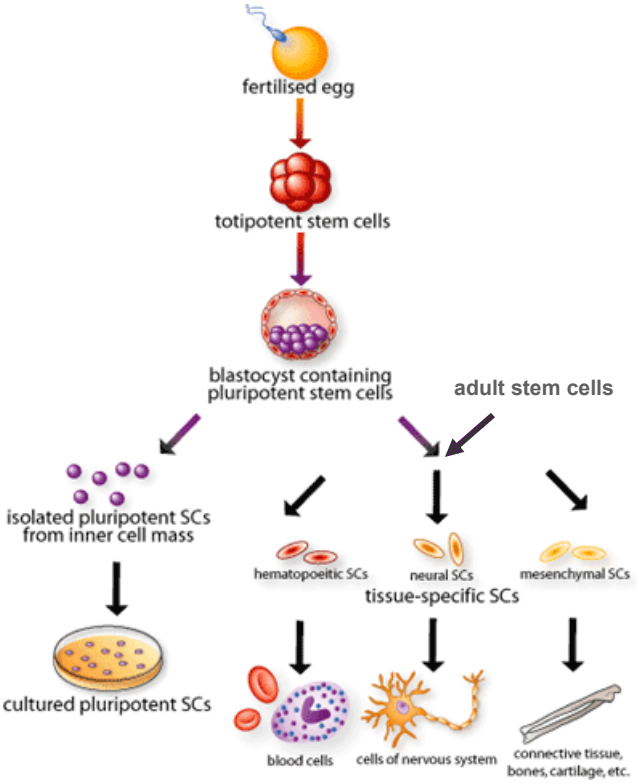


Figure 2.3 The origin, isolation, and specialization of embryonic stem cells. Illustration by Jen Philpott (Chaudry 2004).

Totipotent stem cells can give rise to every cell found in the body including the placental tissue needed to support the growing embryo. Pluripotent stem cells are capable of giving rise to cells from all three germ layers, while further down this scale multipotent stem cells are limited to progeny of a particular lineage found during normal development. Exceptions to the definitions just stated do exist, as certain stem cells and even adult cells have been shown to transdifferentiate—that is, to cross the lines traced by normal developmental lineages. Understandably, the more non-committed a stem cell is to a particular lineage, the more complex the protocol needed to reliably differentiate that cell into the cell type of interest would be, but the higher its amplification potential may be.

2.3.2.1 Differentiation of Embryonic Stem Cells to β-Cells

Pluripotent stem cells include both embryonic stem cells (ESCs) retrieved from the inner cell mass of the developing blastula and a select few adult stem cells, such as those isolated from umbilical cord blood. The generation of insulin-secreting cells from pluripotent ESCs is hampered by the fact that pancreatic-cells appear late during embryonic development. Thus generation of insulin-secreting cells from ESCs involves the sequential activation and deactivation of a considerable number of genes controlling the generation of endoderm lineage cells, the subsequent generation of the precursors of pancreatic cells, and finally the insulin-secreting cells. Despite this difficulty there has been some success in differentiating ESC into insulin producing cells in vitro (Lumelsky, Blondel et al. 2001; Hori, Rulifson et al. 2002; Moritoh, Yamato et al. 2003) and in using the derived cells to normalize glycemia in diabetic mice (Soria, Roche et al. 2000; Kim, Gu et al. 2003). It has been pointed out, however, that many of the insulin-positive cells in ESC cultures may be the product of insulin uptake from the culture medium and not due to endogenous production (Hansson, Tonning et al. 2004). The presence of C-peptide distinguishes de novo insulin synthesis from artifact and there are some genuine insulin-producing cells derived from ESCs.

A major concern with ESCs is the potential of teratoma development arising from undifferentiated cells which may be inadvertently implanted along with the differentiated β-like cells. To protect against this threat, ways to screen stem-cell derived products for teratoma potential have been investigated (Lawrenz, Schiller et al. 2004). Screening alone does not altogether eliminate this risk and as such, genetic engineering of stem cells with genes that allow their in vivo monitoring and targeted destruction if need be are also being investigated (Cao, Drukker et al. 2007). Though these, or other, engineered safety measures enhance the potential of success of stem cell-based therapies, they also enhance the overall complexity. In addition to the threat of cancer development, the β-cell freshly

derived from embryonic stem cells are at risk of allograft rejection or autoimmune destruction, as would be transplanted islets.

2.3.2.2 Differentiation of Adult Stem Cells to β-Cells

Adult stem cells have been better received than embryonic stem cells because their use does not require the destruction of embryos—a major ethical stumbling block for ESC therapy—and they could potentially be retrieved from the patients themselves, thus greatly reducing the risk of rejection, though the possibility of autoimmune rejection inherent to type 1 diabetes cannot be eliminated. As mentioned previously, stem cells from umbilical cord blood are considered adult stem cells, yet are also pluripotent and in vitro differentiation of umbilical cord blood cells into insulin-producing cells has been reported (Sun, Roh et al. 2007). Most adult stem cells, however, are multipotent and are classified by their tissue of origin. For regenerating β-cells, the most obvious adult stem cell source is pancreatic duct cells. These cells seem to be easier to fully differentiate but are more difficult to proliferate (Soria, Bedoya et al. 2005) and there is still considerable debate over whether regeneration of β-cells is due to pancreatic stem cells or replication of existing β-cells. Though there is evidence to suggest that pancreatic ductal tissue may be directed to differentiate into glucose responsive islet tissue (Bonner-Weir, Taneja et al. 2000), more recent work has demonstrated that regeneration of β-cells is due to replication of adult cells and not attributed to pancreatic stem cells (Dor, Brown et al. 2004).

Another obvious source for stem cells used for β-cell regeneration is the liver, as both the liver and pancreas arise from the same cell population in the embryonic endoderm. Adult liver cells have even been made to transdifferentiate into functional insulin-producing cells under the appropriate conditions (Yang 2006). Bone marrow derived stem cells have also been used toward this purpose with some success (Chen, Jiang et al. 2004). The spleen has also been touted as a source for stem cells capable of

differentiation into insulin producing cells. Kodama et al. found that when splenocytes were delivered along with islet transplantation in NOD mice, the mice regenerated islets in the pancreas which were of donor origin, and that when the islet transplant was excised, the mice remained normoglycemic (Kodama, Kuhtreiber et al. 2003). But when others tried to reproduce this work, it was found that the reversal of diabetes was not attributable to the donor spleen cells (Chong, Shen et al. 2006; Nishio, Gaglia et al. 2006; Suri, Calderon et al. 2006).

Though exciting developments will likely be seen in the area of stem cell therapy for IDD, there are still many unknown factors and processes associated with the differentiation process and to date, deriving β-cells from differentiated stem cells has been fraught with difficulty and has not given rise to a reliable method for regenerating islet cell mass.

2.3.3 Transdifferentiation and Metaplasia

Until recently it was thought that differentiated cells could only be produced from embryonic or adult stem cells or progenitor cells. There is now a growing body of evidence that suggests that differentiated cells of one type can be converted to another type through a process called transdifferentiation (or metaplasia) (Thowfeequ, Myatt et al. 2007; Eberhard and Tosh 2008). This phenomenon has understandably generated huge interest because of the potential for adult cells, which could be easily collected, to be converted to other medically important cell types to repair diseased or damaged tissues.

For generating β-cell replacements for IDD, the differentiated adult cells that are investigated for this process are most commonly liver cells and exocrine pancreas cells, as these sources share much of the developmental lineage with the endocrine pancreas. The human PANC-1 and rat ARIP pancreatic ductal cell lines were shown to be able to differentiate into insulin-secreting endocrine cells by expression of Pdx-1 and exposure to

GLP-1 (Hui, Wright et al. 2001). Functional β-like cells have been generated from cultured adult exocrine pancreatic cells treated with leukemia inhibitory factor and epidermal growth factor (Baeyens, De Breuck et al. 2005). Recently, a similar approach to reprogramming exocrine pancreatic tissue in vivo through expression of a combination of transcription factors (Ngn3, Pdx-1, and Mafa) has received significant attention (Zhou, Brown et al. 2008). Successful in vivo transformation of hepatocytes using some of the same transcription factors has also been reported (Ber, Shternhall et al. 2003; Kojima, Fujimiya et al. 2003; Imai, Katagiri et al. 2005; Kaneto, Matsuoka et al. 2005; Kaneto, Nakatani et al. 2005).

The fundamental understanding of how these transformations take place however is still a subject of intense investigation and many questions remain to be answered prior to use of this sort of therapy for human clinical studies.

2.3.4 Genetic Engineering of Autologous Non-β-cells

The use of non-β-cells from the patient themselves relaxes the requirements of availability and immune acceptance but is hindered by the need to recapitulate the complex regulatory system of insulin production and secretion inherent to the β-cell. The first attempt at engineering non-β-cells for insulin production, focused on endocrine cells of the pituitary because they efficiently process proinsulin to insulin and can secrete bioactive insulin in a regulated manner after they are engineered to express proteins commonly referred to as glucose sensors: the phosphorylating enzyme glucokinase (GK) and the glucose transporter GLUT2 (Hughes, Quaade et al. 1993; Motoyoshi, Shirotani et al. 1998). Since pituitary cells are not naturally glucose responsive, and many endogenous products are not compatible with prandial metabolism, unless efforts are made to knock down endogenous hormone expression, over-secretion of the native hormones, such as adrenocorticotropic hormone (ACTH), may upset the metabolic state of the patient. Autologous neuroendocrine tissues are also difficult to obtain or transduce

in vivo. Other, more easily accessible cells, such as muscle (Yin and Tang 2001) and skin (Lei, Ogunade et al. 2007) cells have also been targets for insulin gene therapy. These non-endocrine cells, however, require the use of recombinant insulin that is either bioactive as a single chain (Lee, Kim et al. 2000) or able to be cleaved by the ubiquitous endopeptidase, furin (Groskreutz, Sliwkowski et al. 1994). In the situation of a bioactive single chain there is a lack of C-peptide, a product which normally connects the A and B chains of insulin prior to post-translational modification and is released in equimolar amounts by cells with proper processing of proinsulin to insulin by prohormone convertases (PC). Although C-peptide is not present in pharmaceutical preparations of insulin, C-peptide has many beneficial effects on preventing complications normally associated with IDD (Rebsomen, Khammar et al. 2008; Sima and Kamiya 2008). Furthermore, these non-β-cell sources are still inadequate, as they do not possess the elements necessary for nutrient-regulated secretion, and so, may only be used to fulfill the need for basally secreted insulin. Like β-cells, liver cells (hepatocytes) are glucose sensitive, making them a likely candidate for engineering of non-β-cells. Indeed, success has been seen in animal models in which hepatocytes are genetically engineered to secrete insulin that is transcriptionally regulated by a glucose and insulin sensitive promoter (Huang, Thule et al. 1995; Thule, Liu et al. 2000; Thule and Liu 2000; Olson, Paveglio et al. 2003). Transcriptional regulation, however, is sluggish to turn on and off insulin production and so, cannot provide the acute post-meal insulin release considered necessary for glycemic normalization in higher animals and, eventually, humans. Improvements in the responsiveness of this system have been accomplished through the destabilization of the insulin messenger RNA (Tang and Sambanis 2003).

A better solution may be to engineer insulin production in endocrine cells of the gastrointestinal tract (enteroendocrine cells), as these cells already share a number of the elements needed for regulated secretion and play a role in post-prandial processes.

2.3.5 Enteroendocrine Cells for the Treatment of Diabetes

The importance of enteroendocrine cells (and in particular, L-cells) was first put forward by Creutzfeldt whose primary interest in enteroendocrine cells was for the prospect of using glucagon-like peptide-1 (GLP-1) for the treatment of type 2 diabetes (Creutzfeldt 1974). The incretins GLP-1 (from L-cells) and glucose-dependent insulinotropic polypeptide, GIP (from K cells) are released after a meal to potentiate insulin production from the pancreas.

As with β-cells, enteroendocrine cells are polar, with sensing microvilli on their luminal side and secretory granules docking at the basolateral side, adjacent to capillaries. Enteroendocrine cells secrete incretin hormones in a tightly controlled manner that parallels the secretion of insulin by β-cells, following oral glucose load as shown in Figure 2.4 (Schirra, Katschinski et al. 1996).

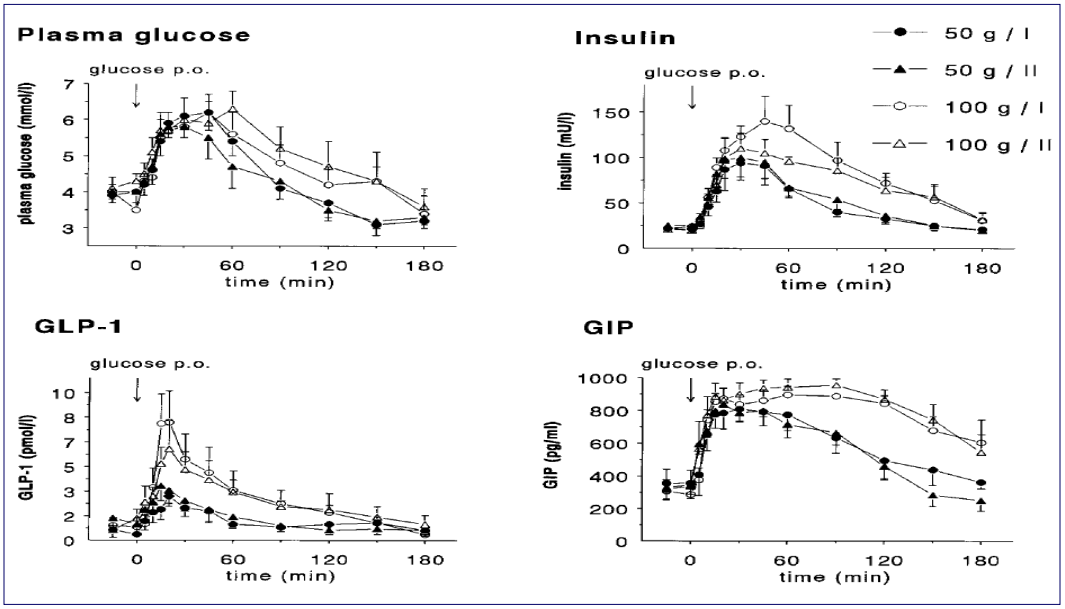


Figure 2.4 Gastric emptying and release of incretin hormones after glucose ingestion in healthy human subjects **(**Schirra 1996)

L-cells produce GLP-1 and GLP-2 and secrete them in parallel in adult humans after oral feeding. GLP-1 and GLP-2 are released in a biphasic manner with a rapid initial increase occurring within 15 minutes, followed by a second increase after about one hour (Burrin, Petersen et al. 2001).

In addition to amenable secretion characteristics and a natural connection to metabolism, enteroendocrine cells are also appealing due to their location within the intestine. The intestinal tract is an attractive target because of its large size and accessibility. In fact the combined mass of the different enteroendocrine cell types make up the largest endocrine organ in the body (Wang, Liu et al. 2004). Despite the advantages, intestinal gene therapy is difficult because of the harsh conditions present in the stomach and intestine, and the intestinal epithelium renews rapidly, with the entire epithelium being replaced every three to five days (Fujita, Cheung et al. 2004). In addition, the epithelium is difficult to transduce because of its intrinsic function as the body's barrier to external threats (Tang, Sambanis et al. 2005).

Because of the unique connection between incretins and insulin, engineering of enteroendocrine cells for regulated insulin secretion arises as an appealing approach for IDD treatment in terms of the dynamic release of insulin, as well as the compatibility of incretins and insulin in glycemic normalization. Work by Cheung et al. in 2000 demonstrated that insulin produced and secreted by genetically modified intestinal K cells prevented these transgenic mice from becoming diabetic after injection with streptozotocin (STZ) (Cheung, Dayanandan et al. 2000). Similarly, transgenic mice which produced human insulin in gastric G cells, displayed meal-regulated increase in the level of transgenic insulin and corresponding decrease in blood glucose levels (Lu, Sternini et al. 2005). These are important proof-of-concept studies which showed that enteroendocrine cell-produced insulin can provide regulation of blood glucose levels.

Despite the straight-forward promise of using enteroendocrine cells for insulin gene therapy, applying genetic engineering methodologies in vivo to enteroendocrine

cells remains a challenging problem. This is due largely in part to the complex anatomy of the intestinal epithelium, which is made up of fingerlike projections called villi and invaginations of the gut mucosa called crypts. Enteroendocrine cells are found at the base of the crypts (represented in Figure 2.5).

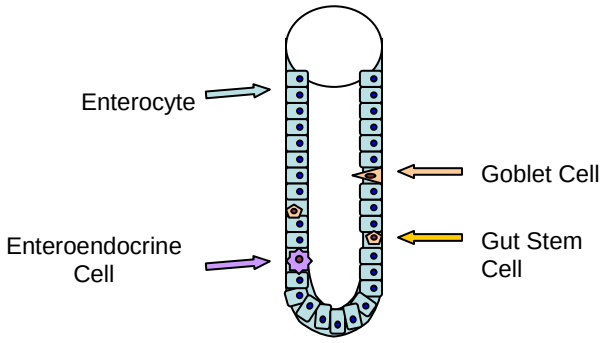


Figure 2.5 Structure of a single colonic crypt. The four cell constituents (enterocyte, goblet cell, gut stem cell, and enteroendocrine cell) are indicated with arrows.

Although L-cells are the most abundant enteroendocrine cell, enteroendocrine cells make up only about 1% of the total cell population of the intestinal epithelium (Wang, Liu et al. 2004) with adsorptive enterocytes being the major cell population. The intestinal epithelium is also a tissue with rapid turnover. Unlike many endocrine cells in various glands throughout the body that differentiate early in life and turnover slowly, enteroendocrine cells actively self-renew and differentiate throughout the life of an animal from a large reservoir of stem cells (Schonhoff, Giel-Moloney et al. 2004). These stem cells are also localized in the base of crypts and serve the function of renewing the entire epithelium (Winton 2001). Recently, the discovery of the intestinal stem cell marker, Lgr5, a transmembrane protein with a large extracellular domain for ligand binding, has opened doors to a number of important studies that were not possible just a couple of years ago (Barker, van Es et al. 2007). Markers suggested previously

included: telomerase (Booth and Potten 2000); Musashi and Hes-1 (Kayahara, Sawada et al. 2003; Potten, Booth et al. 2003); integrins (Beaulieu 1992); BMPR1α and phospho-PTEN (He, Zhang et al. 2004); DCAMKL1 (Giannakis, Stappenbeck et al. 2006); and Eph receptors (Batlle, Henderson et al. 2002), but none of these studies showed definitive proof that the positive-staining cells were functional intestinal stem cells (Montgomery and Breault 2008). This marker can now be envisioned to develop a reliable method of targeting intestinal stem cells for gene delivery, to provide enduring insulin expression within the intestinal epithelium. Stem cells of the small intestine have genome protective measures to ensure the accuracy of the copies made during the many divisions they must undergo in their lifetime. Though this may provide a level of safety when targeted by genetic engineering approaches to derive insulin-producing tissue to treat diabetes (Fujita, Cheung et al. 2004), it may also hinder attempts to efficiently deliver genetic material. Despite the challenges inherent to working with intestinal stem cells, they present a great opportunity; harnessing these stem cells may provide enduring insulin expression.

2.3.6 Autoimmunity in Type 1 Diabetes

Any cell therapy for IDD must be concerned with what would happen to the engineered surrogate β-cells or newly differentiated β-cells upon implantation into the diabetic host. Particularly for subjects with type 1 diabetes, if autoimmunity to β-cells remains, how will this affect the β-cell or β-cell-like tissue? It is reasonable to suspect that regenerated β-cells would be subject to the same processes that caused the initial onset of the disease, but β-cell surrogates may lack the elements that would be required for recognition by autoantigen-reactive T-cells.

Multiple islet molecules are the target of autoimmunity in man and in animal models of type 1 diabetes (Wegmann, Norbury-Glaser et al. 1994; Lieberman, Evans et al. 2003). The autoantigens implicated in type 1 diabetes include insulin, glutamic acid

decarboxylase (GAD), the protein tyrosine phosphatase-related islet antigen 2 (IA-2), and the zinc transporter of the insulin secretory granules (Slc30A8) (Knip and Siljander 2008). Positive expression of a single autoantibody usually represents harmless non-progressive β-cell autoimmunity, whereas the presence of two or more autoantibodies reflects a progressive process that only rarely reverts (Knip and Siljander 2008). It is not clear, however, whether any of the target molecules are essential for the destruction of β-cells and there is no consensus whether or not there is any primary autoantigen. Understandably, insulin remains the most suspected candidate because its encoding gene is only expressed in the cells that are specifically targeted during autoimmune attack (Pasquali, Giannoukakis et al. 2008).

Interestingly, in transgenic nonobese diabetic (NOD) mice that secreted mature insulin via the regulated secretion pathway in pituitary cells, these cells were not targeted or destroyed by cells of the immune system, unlike pancreatic β-cells (Lipes, Cooper et al. 1996). This suggests that expression of insulin alone in non-β-cells is not sufficient to induce autoimmune destruction in an autoimmune model for diabetes. As such it is expected that, although insulin is a suspected autoantigen, insulin-expressing intestinal L-cells will be able to avoid destruction by autoimmune processes.

2.4 Studying L-Cells In Vitro

Due to the complex anatomy of the intestinal epithelium and low percentage of L-cells, establishing pure primary cultures of these enteroendocrine cells is difficult and these cultures often have low viability in vitro. Thus, primary L-cell preparations are short-lived and heterogeneous, making analysis of primary L-cells in vitro very challenging. The fetal rat intestinal cell (FRIC) culture is the only primary cell model that has been developed for in vitro studies of intestinal cells, and though it is a heterogeneous, poorly characterized cell population, it has provided insight about primary epithelial cells in a number of studies (Anini, Hansotia et al. 2002; Ni, Anini et al. 2003).

There are still no standard protocols for isolating adult primary cultures of intestinal L-cells.

A limited number of immortalized cell lines, however, have been developed for studying GLP-1 secretion by L-cells that have proven to be quite useful to date. Two of these continuous cell lines have been derived from intestinal tumors of transgenic mice (the GLUTag and STC-1 cell lines) and a third is derived from a poorly differentiated human cecal carcinoma (the NCI-H716 cell line). The NCI-H716 cells have been used in a number of studies as a model enteroendocrine, L-cell population (Reimer, Darimont et al. 2001; Tang and Sambanis 2003; Tang and Sambanis 2004). These cells are cultured as floating aggregates prior to the induction of differentiation by exposure to specific extracellular matrix proteins found in Matrigel (de Bruine, Dinjens et al. 1993). Once differentiated, these L-cells grow as monolayers and respond to nutrient stimulation in the form of peptones (meat hydrolysates). However, Cao et al. (Cao, Flock et al. 2003) reported that this cell line has aberrant regulation of the proglucagon promoter and, as such, is a poor experimental system to investigate the proglucagon promoter for cell type-specific expression of a gene therapy product.

Brubaker et al. have investigated the regulated secretion of GLP-1 from L-cells using a heterogeneous mouse cell line, STC-1 (Brubaker, Izzo et al. 2003). Similarly, Hira et al. used the STC-1 cell line to investigate cholecystokinin (CCK) release following dietary protein stimulation (Hira, Hara et al. 2003). Boylan et al. used this same cell line transfected with a mammalian selection marker driven by a K cell-specific promoter to generate a more homogeneous K cell population (Boylan, Jepeal et al. 1997). More recently, the STC-1 cell line has been genetically modified to express insulin in K cells under control of the GIP promoter, and these cells were shown to reverse hypoglycemia when transplanted under the kidney capsule (Han, Lee et al. 2007) or subcutaneously (Zhang, Yao et al. 2008) in diabetic mice. In a separate study of a genetically engineered STC-1 line, cells doubly transfected for neomycin resistance and

insulin expression, both under control of GIP promoters did not display glucose-regulated release of insulin or GIP (Ramshur, Rull et al. 2002), though this is likely an artifact of the stable clone selection processes.

GLUTag cells, a murine cell line, are a homogeneous population of L-cells that have been successfully employed by a number or laboratories for the study of L-cells' regulated secretion (Drucker, Jin et al. 1994; Brubaker, Schloos et al. 1998; Cordier-Bussat, Bernard et al. 1998; Nian, Drucker et al. 1999; Dhanvantari, Izzo et al. 2001; Reimann and Gribble 2002). This cell line has been used specifically in electrophysiology studies to explore the stimulated secretion pathway of enteroendocrine cells as well as studies to determine glucagon gene regulation in enteroendocrine cells. In comparing GLUTag cells to primary L-cell cultures and in vivo models, Brubaker et al. found GLUTag cells to respond appropriately to the regulatory mechanisms controlling intestinal GLP-1 secretion, making this a very useful L-cell model (Brubaker, Schloos et al. 1998).

2.5 Tissue Engineered Pancreatic Substitutes

Tissue engineering is an interdisciplinary field that applies the principles of engineering and the life sciences toward the development of biological substitutes that restore, maintain, or improve tissue function (Langer and Vacanti 1993). Tissue engineering may use one of three basic strategies: isolated cells or cell substitutes, tissue inducing substances, or cells placed within matrices. For the purposes of IDD, the first approach is already being applied in islet transplantation. Since β-cells do not significantly expand in cell number *in vivo* the second approach of a tissue inducing substitute is considerably more challenging. As discussed previously, adult stem or progenitor cells may reside within the pancreas and if suitable cues were provided to these cells, potentially though presentation of the appropriate growth factors via a biomaterial, regeneration of β-cell mass would be theoretically possible. Alternatively, it

has been reported that exocrine pancreas tissue can be induced to take on a β-cell phenotype through metaplasia (Zhou, Brown et al. 2008) so a similar approach could be envisioned to target those cells. In all of these cases, if the regenerated β-cells are recognized by the autoimmunity in type I diabetic subjects, a method to prevent autoimmune destruction of the cells would be critical. The biological understanding of stem or progenitor cell differentiation and metaplasia is still in its infancy, however and as such, so is this approach to tissue engineering.

The third approach in which cells are combined with a biomaterial is the most commonly utilized tissue engineering strategy for IDD. This is because when cells displaying class I and II major histocompatibility (MHC) markers are transplanted between unrelated individuals, it normally results in the rejection of those cells. The expression of type II MHC has been minimized for skin tissue by passaging the cells during *in vitro* culture (Thivolet, Faure et al. 1986). Unfortunately, this approach is not possible for cells of the pancreatic islets, as they do not propagate in culture and so hiding the MHC markers by coating with a polymeric membrane or entrapment within a membrane is a more suitable approach to lessening the need for immunosuppressive drugs.

The pore size of the polymeric biomaterial or membrane is designed such that small molecules including metabolites essential to cell survival and function (e.g. oxygen and glucose) and insulin can pass freely, while most immune response elements (antibodies and lymphocytes) are denied entry. Thus the encapsulated cells can sense the surrounding glucose levels and respond with insulin secretion accordingly while being partially hidden from the host's immune system. One commonly used method involves the encapsulation of cells or cell aggregates in alginate microbeads (Lim and Sun 1980) which are typically 400-800 μm in diameter. These microbeads are usually implanted in avascular sites such as the peritoneal cavity. The commonly employed site of islet transplantation (hepatic vasculature) is unavailable for microbead transplantation due to

the dangerous rise in intraportal pressure that would result due to the relatively large size of the microbeads. As such, cells entrapped in large devices are often well beyond the 200 μm distance from the capillaries that is normally seen in the body and thus diffusion of nutrients and especially oxygen may limit the functionality of cells implanted in such devices. Even sublethal levels of hypoxia can have deleterious effects on ATP-dependent cell functions such as insulin secretion (Dionne, Colton et al. 1993; Papas, Long et al. 2000; Wilson and Chaikof 2008).

To reduce mass transport limitations recent efforts have focused on developing conformal coatings of micron and submicron scale in individual cells or cell aggregates (Wilson, Cui et al. 2008), incorporating vasculature within tissue engineered constructs, or transplanting grafts into prevascularized sites. For instance delivery of vascular endothelial growth factor and fibroblast growth factor-2 via a scaffold consisting of nanofibers led to improved engraftment of islets during implantation to the omentum due to an increase in neovasculature within the construct (Stendahl, Wang et al. 2008). Alternatively, digested vascular tissue was added directly to collagen gels of a tissue engineered construct and the presence of the prevascularized collagen was found to improve the viability and metabolic function of encapsulated islets (Hiscox, Stone et al. 2008).

2.6 Animal Models for IDD

Diabetic mouse models have been used extensively in the literature and are essential tools for evaluating the ability of pancreatic substitutes to regulate glucose metabolism in vivo. The two most commonly used models for IDD are the STZ-induced mouse model and the non-obese diabetic (NOD) mouse (Atkinson and Leiter 1999). In the first model, STZ is injected into mice and this drug causes specific ablation of β-cells, thus inducing the onset of a diabetic state in the mouse model. STZ is an agent used in the treatment of metastasizing pancreatic islet tumors and diabetes research. The structure

of STZ is comprised of a glucose molecule with a highly reactive nitrosurea side chain. It is thought that the glucose moiety directs it to pancreatic β-cells where it binds to a membrane receptor to generate structural damage to the cells, though intracellular responses to the drug also assist in cell death (McNeill 1999). Alternatively, the NOD model is an inbred mouse strain commonly used as a model for autoimmunity in diabetes. NOD mice have high incidence of insulitis (infiltration of the pancreas with leukocytes), which in many animals leads to the spontaneous development of IDD when they are between 12 and 30 weeks old (Rees and Alcolado 2005). NOD mice, however, show abnormalities in addition to those observed in the pancreas. Autoimmune inflammation of the thyroid and inflammation of submandibular and lacrimal glands also occur frequently which lends this experimental model to the study of other autoimmune diseases like multiple sclerosis and Sjögren's syndrome. Due to these complications, the STZ is also very useful despite the absence of autoimmune rejection of the β-cells.

In addition to the well established diabetic animal models, the enteroendocrine cells of the mouse are remarkably similar to those in humans (Schonhoff, Giel-Moloney et al. 2004), making it likely that the insights learned from the mouse may contribute to our understanding of enteroendocrine cell insulin treatment for IDD in humans.

CHAPTER 3

INSULIN-SECRETING L-CELLS FOR THE TREATMENT OF INSULIN-DEPENDENT DIABETES[1]

Cell-based treatments for insulin-dependent diabetes (IDD) may provide more physiologic regulation of blood glucose than daily insulin injections, thereby reducing the occurrence of secondary complications associated with diabetes. An autologous cell source is especially attractive for regulatory and ethical reasons, in addition the need for immunosuppresion is ameliorated. Enteroendocrine L-cells, exhibit regulated secretion in response to physiologic stimuli and their endogenous products are fully compatible with prandial metabolism. The aim of this study was to develop a surrogate β-cell based on this potentially autologous cell type and evaluate the function of the resulting genetically modified L-cells. Murine GLUTag L-cells were transfected with a plasmid co-expressing human insulin and neomycin resistance and the stable cell line, GLUTag-INS, was established. Secretion properties of GLUTag-INS cells were investigated in vitro through induced secretion tests using meat hydrolysate or 3-isobutyl-1-methylxanthine and forskolin as secretagogues. GLUTag-INS cells rapidly co-secreted recombinant insulin and endogenous GLP-1 in response to metabolic cues from the surrounding medium and demonstrated efficient processing of proinsulin to insulin.

3.1 Introduction

The use of autologous, non-β-cells for treatment of IDD would eliminate the need for immunosuppresion currently required for other cell therapies and would relax the constraint of tissue supply. Though studies have achieved insulin secretion in non-β-cells, attempts to restore normoglycemia have fallen short of reconstructing the complex

[1] This work has been published: Bara and Sambanis (2008). Biochem Biophys Res Commun **371**(1):39-43

sensory and regulatory machinery unique to β-cells. Pituitary cells were engineered for insulin production because they efficiently process proinsulin to insulin and can secrete insulin in a regulated manner upon expression of GK and GLUT2 (Hughes, Quaade et al. 1993; Motoyoshi, Shirotani et al. 1998). Pituitary products, however, are not compatible with prandial metabolism and aberrant over-secretion of the native hormones may upset the metabolic state of the patient. Neuroendocrine tissues are also difficult to obtain for ex vivo manipulation and difficult to transduce in vivo. More easily accessible targets, such as muscle (Yin and Tang 2001) and skin (Lei, Ogunade et al. 2007) have also been used for insulin gene therapy. These cells, however, require the use of recombinant insulin that is either bioactive as a single chain (Lee, Kim et al. 2000) or able to be cleaved by a ubiquitous endopeptidase (Groskreutz, Sliwkowski et al. 1994). Additionally, these non-β-cell sources do not possess the elements necessary for nutrient-regulated secretion, and so, may only be used to fulfill the need for basally secreted insulin. Like β-cells, hepatocytes possess GK and GLUT2, making them glucose sensitive, but these cells possess no regulated secretion pathway, and attempts to reengineer regulated release have relied on regulation at the level of gene transcription. Although success has been achieved in animal models with genetically engineered hepatocytes expressing insulin under transcriptional regulation by a glucose and insulin-sensitive promoter (Thule and Liu 2000; Olson, Paveglio et al. 2003), this system cannot provide the acute post-meal insulin release considered necessary for glycemic normalization in higher animals and, eventually, humans.

Enteroendocrine cells, exhibit many useful properties that make them appropriate targets for recombinant insulin expression. Like other endocrine cells, they are capable of processing wild type proinsulin. Enteroendocrine cells' function is to secrete hormones called incretin which serve to potentiate insulin secretion from β-cells in the presence of glucose, and their regulated release parallels the secretion of insulin by β-cells, following an oral glucose challenge (Schirra, Katschinski et al. 1996; Kieffer and

Habener 1999). Thus, engineering of enteroendocrine L-cells for insulin expression arises as an appealing treatment for IDD in terms of dynamic release and compatibility of incretins and insulin in glycemic normalization. Furthermore, studies with transgenic mice producing insulin from genetically engineered K cells (Cheung, Dayanandan et al. 2000) and G cells (Lu, Sternini et al. 2005) showed that insulin-expressing enteroendocrine cells can provide regulation of blood glucose levels in transgenic mice.

Previously, our lab engineered a human L-cell line to release B10-mutated insulin in response to nutrient administration (Tang and Sambanis 2003) and showed preferential transduction by adeno-associated virus for L-cells over enterocytes in a co-culture model (Tang and Sambanis 2004). The B10 mutation is a naturally occurring, single point substitution of aspartic acid for histidine at position 10 of the B chain of insulin (Chan, Seino et al. 1987), which results in a superactive hormone. In human subjects containing this point mutation, the physiological effect is that of hyperproinsulinemia, due to the fact that B10-mutated proinsulin can escape through the unregulated secretion pathway in contrast to native proinsulin which is almost exclusively handled by the regulated secretory pathway (Carroll, Hammer et al. 1988). Nevertheless, much of the B10-mutated proinsulin is processed via regulated secretion to become active B10-mutated insulin, which is notably more active than the non-mutated insulin. Native insulin can form dimers and even hexamers, but only the monomeric form of insulin has physiological action. B10-mutated insulin is able to form dimers, but not hexamers (Brange, Owens et al. 1990; Weiss, Hua et al. 1991; Liao, Tang et al. 2001). Thus, part of the reason for B10 insulin's greater activity, may be due to its higher bioavailability. Additionally it has been reported that B10-mutated insulin has a greater binding affinity to the insulin receptor than does native insulin (Schwartz, Burke et al. 1987; Brange, Ribel et al. 1988; Shoelson, Lu et al. 1992). Yet another reason for the higher activity of B10-mutated insulin may be attributed to greater stability (Brems, Brown et al. 1992).

In advancing L-cell mediated insulin therapy to a tissue-engineered treatment for an adult mouse model for IDD, the murine L-cell line, GLUTag, was selected for this study, as it would represent a closer allograft model for mice and GLP-1 secretion from this cell line has been found similar to that of primary cell cultures (Brubaker, Schloos et al. 1998). This chapter describes the genetic modification of murine GLUTag cells for the stable expression of human insulin and the characterization of the newly developed cell line, GLUTag-INS.

3.2 Materials and Methods

All reagents were purchased from Sigma (St Louis, MO) unless otherwise noted.

3.2.1 Cell Culture

GLUTag cells were obtained from the laboratory of Dr. P.L. Brubaker with the permission of Dr. D.J. Drucker (University of Toronto, Ontario, Canada). The cells were cultured in a 37°C/5% CO_2 humidified incubator in T-flasks in complete medium consisting of L-glutamine-free Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (Cellgro, Herndon, VA); cultures were split at a 1:5 ratio when 80% confluency was reached.

3.2.2 Antibody Staining and Microscopy

Cells were washed then fixed in 4% paraformaldehyde in phosphate buffered saline (PBS), permeabilized with 0.5% Triton X-100 in PBS, blocked using 10% horse serum in PBS before adding diluted primary antibodies (either rabbit antihuman PC1/3, PC2, or mouse antihuman insulin). Cells were incubated overnight at 4°C. The following day, cells were rinsed twice with PBS and diluted secondary antibody (either anti-rabbit or anti-mouse IgG-TRITC-conjugate) was added and incubated for 1.25 hours

in the dark at room temperature. Cells were rinsed twice in PBS, coverslipped, and imaged by using the LSM 510 confocal microscope (Carl Zeiss Inc.).

3.2.3 Transfection and Selection of Stable Clone

The transgene for stable insulin expression was constructed by inserting the human B10 mutated insulin gene (Genentech, San Francisco, CA) into the pcDNA3.1(+) vector (Invitrogen, Carlsbad, CA) and the expression cassette is depicted below in Figure 3.1.

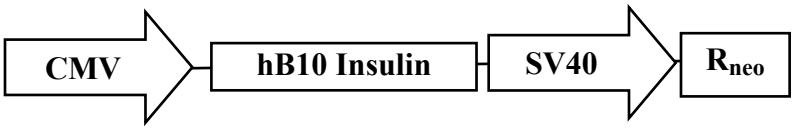


Figure 3.1 Elements of the plasmid used for generation of the stable GLUTag clone expressing human B10-mutated insulin.

The expression cassette directs simultaneous expression of human insulin from the cytomegalovirus (CMV) promoter and neomycin resistance from the simian virus 40 (SV40) promoter. GLUTag cells were plated two days prior to transfection at a density of half-a-million cells per well of a 12-well plate. Cells were then transfected the using FugeneHD (Stratagene, La Jolla, CA) according to manufacturer's protocol at a ratio of 8 µl FugeneHD to 2 µg DNA. Selection of a stable clone was performed by replacing the medium on the day following transfection with complete medium supplemented with 200 µg/ml Geneticin (Invitrogen). The concentration of Geneticin was increased to 600 µg/ml in incremental steps of 200 µg/ml over the course of two days. Selective pressure was maintained for one month with medium changes every one to three days until colonies that were large enough to be seen with the unaided eye formed. Individual colonies were transferred to wells of a 24-well plate. Spent medium from those wells

was assayed for insulin production, the clone with the highest expression was amplified and used in the remainder of the studies and is henceforth referred to as GLUTag-INS.

3.2.4 Induced Secretion Tests

Secretion test were performed on GLUTag-INS cell monolayers in six-well tissue culture plates. One million cells were seeded in each well two to four days prior to induced secretion tests. On the evening prior to the secretion tests, the medium was changed to basal medium (DMEM with 5 mM glucose, without L-glutamine, supplemented with 1% FBS). On the day of the secretion test, parallel cultures were briefly washed with PBS, and then subjected to three consecutive one-hour incubations in basal medium to stabilize basal insulin and GLP-1 secretion. The secretion test was then initiated by incubating the monolayers in basal medium for two hours to establish the basal secretion rate. Samples were taken, the wells were washed twice with PBS, and monolayers were either changed to fresh basal medium for use as non-induced controls or switched to basal medium supplemented with 2% (w/v) meat hydrolysate (MH) or 3-isobutyl-1-methylxanthine (IBMX) and forskolin at 10 μM each to stimulate insulin and GLP-1 secretion in the induced wells. Samples were taken at the end of the two-hour induction period. This scheme is depicted in Figure 3.2.

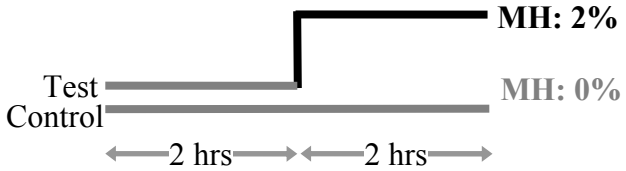


Figure 3.2 Scheme used for changes in culture medium during induced secretion tests.

The time course experiment was performed in much the same way, except that only 2% MH was used as a secretagogue and samples were taken every 20 minutes, upon induction.

3.2.5 Assays

Insulin and GLP-1 concentrations were determined using the human insulin and GLP-1 radioimmunoassay kits (RIA, Millipore, Billerica, MA) according to the manufacturer's protocols.

3.3 Results

3.3.1 PC1/3 and PC2 Expression in GLUTag Cells

To confirm that GLUTag cells possess the enzymes necessary to properly convert wild-type proinsulin to its active form, antibody staining was performed. Figures 3.3.B and 3.3.C depict the staining of GLUTag cells for antibodies to PC2 and PC1/3 respectively while Figure 3.3.A demonstrates that there was no contribution of background staining when cells were treated with the secondary, TRITC-conjugated, antibody alone.

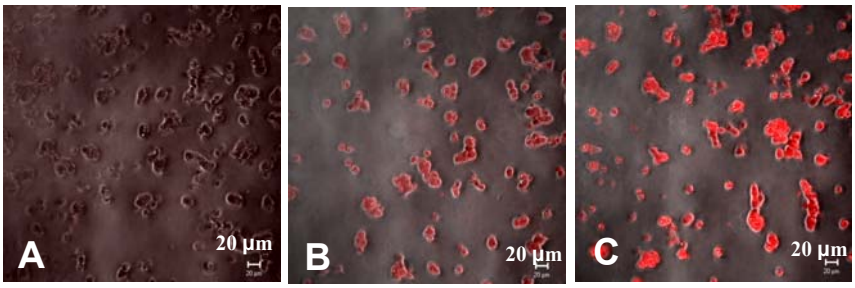


Figure 3.3 Confocal images of PC1/3 and PC2 immunofluorescence staining. Parental GLUTag cells stained in the absence of primary antibody (A) and with antibodies for PC2 (B) and PC1/3 (C). Phase contrast images have been overlayed to show cell outlines.

3.3.2 Generation of a Stable Insulin-Expressing GLUTag Clone

In this work, an L-cell line which stably and predictably expressed insulin was developed by transfection and continued culture under selection pressure. Following this selection period, three colonies were confirmed to produce insulin at significant levels

and displayed good growth characteristics. The colony with the most robust insulin expression was used in all experiments and these cells are referred to as GLUTag-INS cells. Insulin production was verified through RIA of spent medium and by immunofluorescent staining of engineered GLUTag-INS cells (Figure 3.4.A and Figure 3.4.B) and parental GLUTag cells (Figure 3.4.C). To ensure that the parental GLUTag cells do not express insulin, the concentrations of primary and secondary antibodies used to stain parental cells were 10-fold higher than those used to stain GLUTag-INS cells and the cells were observed under a higher magnification.

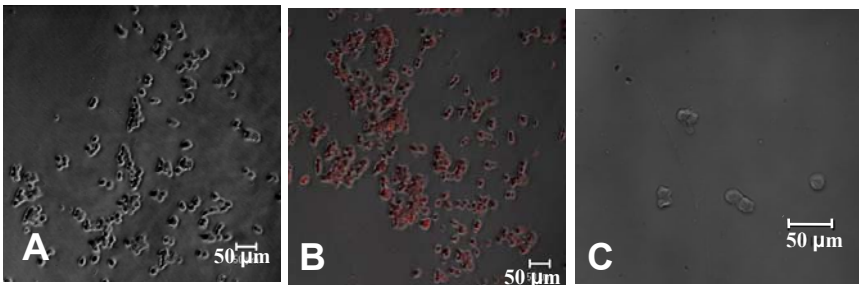


Figure 3.4 Confocal images of insulin immunofluorescence staining. GLUTag-INS cells stained without primary antibody (A), and with an antibody for human insulin (B). Parental GLUTag cells stained were also stained with primary antibody against insulin (C). Phase contrast images have been overlayed to show cell outlines.

3.3.3 Induced Secretion of Insulin and GLP-1 from GLUTag-INS Cells

GLUTag-INS cells were evaluated in vitro using induced secretion tests to determine how these cells regulate the secretion of insulin and GLP-1. Nutrient stimulation for this experiment came in the form of 2% MH supplemented to basal medium, a secretagogue known to induce secretion in a number of studies using enteroendocrine cell lines. Peptones, such as MH, are potent nutrient secretagogues for intestinal L-cells, and act not only by triggering secretion of accumulated hormone, but also by increasing gene transcription (Cordier-Bussat, Bernard et al. 1997; Cordier-Bussat, Bernard et al. 1998; Tang and Sambanis 2003; Gevrey, Malapel et al. 2004; Tang

and Sambanis 2004). IBMX and forskolin, a pair frequently used as putative secretagogues (Dhanvantari, Izzo et al. 2001; Reimann and Gribble 2002; Reimann, Williams et al. 2004), were also used as a positive control, while basal secretion was determined through parallel tests with basal medium. Insulin and GLP-1 induction were calculated by normalizing the amount of insulin and GLP-1, respectively, secreted during the two hours of induced secretion to the amount secreted during the two hour basal period. Results are shown in Figures 3.5 and 3.6. Secretion of insulin for all groups during treatment with basal medium was 424.8 ± 86.4 fmole/(well · 2 hr) (n=27 from three independent experiments). Cells treated with MH and IBMX/forskolin exhibited 220.7% ± 32.4% and 423.7% ± 47.1% induction relative to basal secretion, respectively (Figure 3.5).

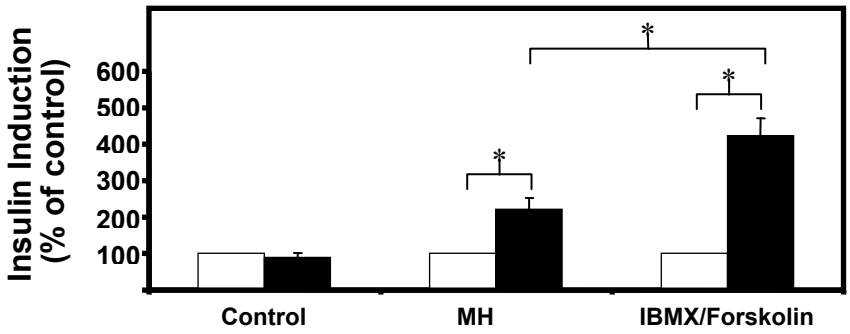


Figure 3.5 Insulin release during induced secretion tests. GLUTag-INS cells were exposed to basal medium for two hours (open bars) followed by another two hours in either basal medium (control), 2% MH, or 10 μM IBMX and 10 μM forskolin (solid bars). Secreted insulin is expressed as a percent of the amount secreted during the initial two-hour period. Mean ± SD, n=9 from three independent experiments. * paired t-test with unequal variances, $p < 0.005$.

For GLP-1, the basal secretion rate was 1301.5 ± 245.1 fmole/(well · 2 hr). GLP-1 induction for cells exposed to MH, and IBMX/forskolin were 178.4% ± 10.3%, and 226.0% ± 50.8% of the basal rates, respectively (Figure 3.6).

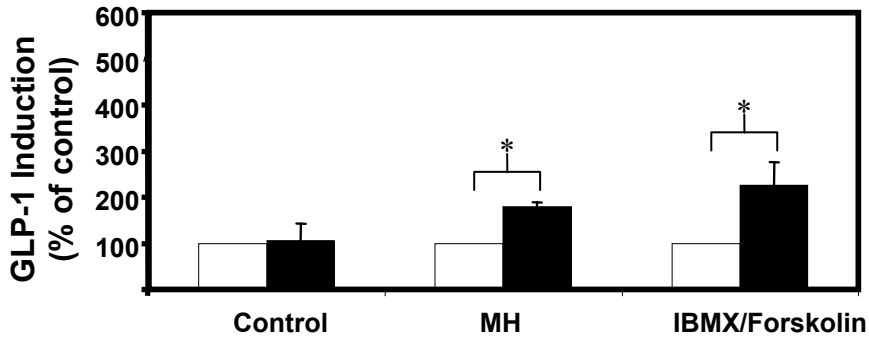


Figure 3.6 GLP-1 release during induced secretion tests. GLUTag-INS cells were exposed to basal medium for two hours (open bars) followed by another two hours in either basal medium (control), 2% MH, or 10 μM IBMX and 10 μM Forskolin (solid bars). Secreted GLP-1 is expressed as a percent of the amount secreted during the initial two-hour period. Mean ± SD, n=9 from three independent experiments. * paired t-test with unequal variances, $p < 0.005$.

3.3.4 Secretion Profile of Insulin from GLUTag-INS Cells

To explore how quickly GLUTag-INS cells respond to nutrient administration, another secretion test was performed to better resolve the time axis, with samples taken every 20 minutes. As seen in Figure 3.7, engineered GLUTag-INS cells released insulin in response to 2% MH in an acute manner, with a significant difference in insulin secretion observed within the first 20 minutes.

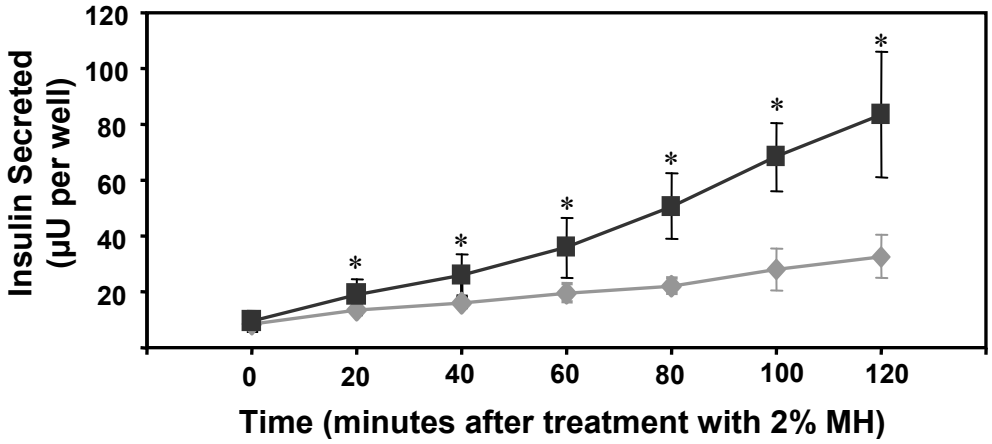


Figure 3.7 Time course of insulin secretion from GLUTag-INS cells. Grey diamonds and black squares indicate insulin secreted by GLUTag-INS cells in basal and induction medium (2% MH), respectively. Mean ± SD, n=9 from three independent experiments. *Significant difference between basal and MH groups at these times, one-way ANOVA $p < 0.05$.

3.3.5 Proinsulin Conversion in GLUTag-INS Cells

To evaluate the efficiency of proinsulin conversion to insulin, the concentrations of proinsulin and insulin accumulated in culture medium were assayed for the experiments of Figures 3.5 and 3.6. Proinsulin conversion was calculated as the percentage of insulin secreted from GLUTag-INS cells relative to total amount of secreted insulin and proinsulin [(insulin*100%)/(insulin + proinsulin)]. Proinsulin conversion was approximately 70% when cells were exposed to basal medium. Incremental increases in proinsulin conversion were noted in groups treated with MH and IBMX/forskolin, 77.6% and 88.3% respectively (Figure 3.8). These rises in proinsulin conversion ($p<0.05$), indicate that GLUTag-INS cells efficiently process proinsulin to insulin, especially in times of high demand.

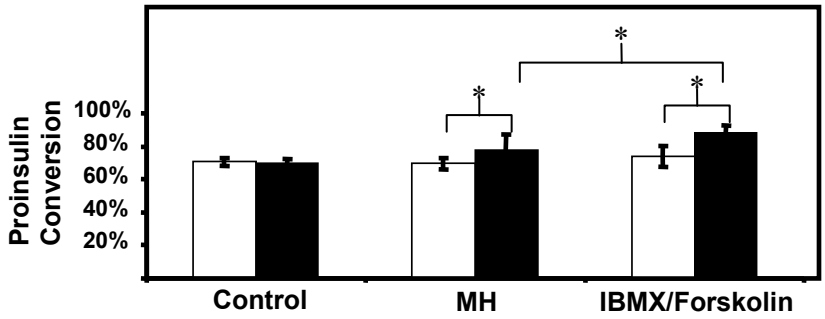


Figure 3.8 Proinsulin conversion of GLUTag-INS Cells in basal and induction medium. GLUTag-INS cells were exposed to basal medium for two hours (open bars) followed by another two hours in either basal medium (control), 2% MH, or 10 μM IBMX and 10 μM Forskolin (solid bars). Proinsulin conversion was calculated as (insulin*100%)/(insulin + proinsulin). Mean ± SD, n=9 from three independent experiments. * paired t-test with unequal variances, $p < 0.05$.

3.4 Discussion

3.4.1 PC1/3 and PC2 Expression in GLUTag Cells

In pancreatic β-cells, both PC1/3 and PC2 are needed to process proinsulin to insulin. Human NCI-H716 L-cells and canine primary L-cell cultures have also been demonstrated to express both PC1/3 and PC2 (Damholt, Buchan et al. 1999; Tang and Sambanis 2004). While in intestinal L-cells PC1/3 is responsible for processing the proglucagon transcript to produce glicentin, GLP-1 and GLP-2, in pancreatic alpha-cells proglucagon is alternately processed by PC2 to produce glucagon. Despite the presence of both PC1/3 and PC2, minimal amounts of glucagon are found in intestinal L-cells (Damholt, Buchan et al. 1999).

Antibody staining for both PC1/3 and PC2 was performed on GLUTag cells and both were found to be expressed in GLUTag cells. The presence of both PC1/3 and PC2 confers L-cells the ability to properly process proinsulin, as evidenced by the presence of immuno-reactive insulin in GLUTag-INS cells and, although not demonstrated in this study, it is reasonably expected that the insulin produced in these cells is also bioactive. Given the growing understanding of C-peptide's role in promoting vascular health

(Vague, Coste et al. 2004), a cell type that expresses equimolar amounts of insulin and C-peptide (from proper processing of wild-type proinsulin) would offer an advantage in treating IDD.

3.4.2 Generation of a Stable Insulin-Expressing GLUTag Clone

For tissue engineering purposes, establishing a cell line which stably expresses insulin is desirable, such that a homogeneous population of insulin-secreting cells can be characterized in vitro and transplanted, as such or in a three-dimensional construct, with a predictable outcome. In this work, this was achieved by transfection and continued culture under selection pressure. A clone was selected through this process which stably expressed insulin under the control of the CMV promoter. This clone was used in all subsequent experiments in this thesis that called for an insulin-expressing L-cell line, and is referred to as GLUTag-INS. In preliminary studies evaluating the function of GLP-1 secretion from GLUTag-INS cells compared to their parental GLUTag cells, it was found that GLP-1 secretion from GLUTag-INS cells is approximately 10-fold lower than that of GLUTag cells. While this may be due to inadvertent selection of this phenotype through the stable clone selection process, it is very likely that the insulin secreted from the GLUTag-INS cells is exerting an autocrine effect on the cells, as it has recently been shown that GLUTag cells do express insulin receptors (Lim, Huang et al. 2008). In this study it was seen that while acute exposure to insulin increased secretion of GLP-1 and proglucagon expression, chronic hyperinsulinemia resulted in suppression of GLP-1 secretion and proglucagon expression. Indeed the GLUTag-INS cell line would represent a model of chronic hyperinsulinemia of L-cells and based on this finding, one would expect lowered secretion of GLP-1.

3.4.3 Induced Secretion of Insulin and GLP-1 from GLUTag-INS Cells

For in vitro characterization of GLUTag-INS cells, induced insulin secretion tests were performed, to investigate if these cells would regulate the secretion of insulin as they do GLP-1, despite the use of the constitutive CMV promoter to drive the transcription of insulin. GLUTag cells are commonly reported to be glucose sensitive (Reimann and Gribble 2002; Gribble, Williams et al. 2003; Reimann, Williams et al. 2004; Reimann, Maziarz et al. 2005; Reimann, Ward et al. 2006), but induction occurs at a subphysiological level of 0.5 mM, with no significant difference in secretion between 5 mM and 25 mM glucose concentrations (Reimann and Gribble 2002). In tests with GLUTag-INS cells using physiologically relevant glucose concentrations, no significant change in insulin secretion was measured when cells were exposed to a step change from 5 mM to 20 mM glucose (data not shown), likely due to the hypersensitivity inherent to the cell line.

With approximately 2.4 million cells per well, the level of insulin expression demonstrated here by GLUTag-INS cells is on par with values reported for other insulin-secreting non-β-cells, but is about five-fold lower than that reported for β-cell lines. The basal rates of insulin secretion for engineered AtT20 cells [32], engineered NCI-H716 cells (Tang and Sambanis 2003), and GLUTag-INS cells are 60, 79, 86 fmole/(10^6 cells · hr) respectively, while the basal rate of secretion for βTC3 cells (Mukundan, Flanders et al. 1995) is 384 fmole/(10^6 cells · hr).

As the basal medium used in this study was DMEM with 5 mM glucose and 1% FBS, a higher rate of basal GLP-1 secretion was expected than that seen in electrophysiology studies in which the basal medium is typically replaced with a nutrient-free buffer. For instance, the basal rate of GLP-1 secretion from GLUTag-INS cells measured in this study is estimated to be 7.1-fold greater than that reported by Reimann and Gribble for GLUTag cells in Krebs Ringer Buffer, when corrected for differences in culture size (Reimann and Gribble 2002). Though there may be inherent differences in

the amount of GLP-1 secreted from parental GLUTag cells and GLUTag-INS, much of the difference in basal GLP-1 secretion rates is expected to be due to medium differences. This trend has also been noted for insulinoma cells: insulin secretion rates in nutrient-free PBS, without or with the addition of 16 mM glucose, were significantly lower compared to insulin secretion rates in nutrient-rich DMEM, again, without or with the addition of 16 mM glucose (Papas and Jarema 1998).

The basal rate of GLP-1 secretion, however, is three-fold greater than the basal insulin secretion, which may explain why the induction fold was not as high for GLP-1 as it was for insulin (as induction folds are determined through normalization to the basal secretion rate). It is interesting to note that the relative induction folds in response to IBMX and forskolin were 226.0% for GLP-1 and 423.7% for insulin, indicating that while MH caused nearly maximal secretion of GLP-1, quite a bit more insulin was secreted in response to IBMX and forskolin. It has been reported that forskolin results in strong activation of the CMV promoter (Loser, Jennings et al. 1998) so the higher induction of insulin relative to GLP-1 during treatment with IBMX /forskolin may be the result of additional activation of the CMV promoter controlling insulin expression in GLUTag-INS cells. GLP-1 expression, controlled by the proglucagon promoter, may have also been affected by forskolin, as it is an activator of cAMP, which is known to activate the proglucagon promoter (Brubaker, Schloos et al. 1998), though the relative strength of each affect is unknown.

The secretory properties of GLUTag-INS cells demonstrate that the insulin secretion response of this engineered cell line to various nutrient and non-nutrient secretagogues is in line with what has been observed for secretion of GLP-1 from parental GLUTag cells. In previous studies in which an insulin-EGFP fusion protein was transiently expressed in human L-cells, engineered insulin-EGFP and endogenous GLP-1 co-localized in secretory granules (Tang and Sambanis 2003). The similarity in the

secretion of insulin and GLP-1 from GLUTag-INS cells suggests that co-localization of insulin and GLP-1 occurs in GLUTag-INS cells as well.

3.4.4 Detailed Secretion Profile of Insulin from GLUTag-INS Cells

To demonstrate the speed at which GLUTag-INS cells respond to nutrient administration, a time course experiment was performed and engineered GLUTag-INS cells were shown to release insulin acutely in response to 2% MH, with a significant difference in insulin secretion observed within the first 20 minutes. This response is in agreement with observations of parental GLUTag cells, which respond rapidly to 10 mM L-glutamine by enhanced secretion of GLP-1 (Reimann, Williams et al. 2004).

3.4.5 Proinsulin Conversion in GLUTag-INS Cells

The rises in proinsulin conversion upon stimulation with medium containing 2% MH or 10 μM IBMX and 10 μM Forskolin indicate that GLUTag-INS cells efficiently process proinsulin to insulin, especially in times of high demand.

CHAPTER 4

DEVELOPMENT AND IN VITRO CHARACTERIZATION OF A TISSUE ENGINEERED PANCREATIC SUBSTITUTE BASED ON RECOMBINANT INTESTINAL ENDOCRINE L-CELLS

A tissue engineered pancreatic substitute (TEPS) consisting of insulin-producing cells appropriately designed and encapsulated to support cellular function and prevent interaction with the host's immune system may provide physiological blood glucose regulation for the treatment of insulin-dependent diabetes (IDD). The performance of agarose-based constructs which contained either a single cell suspension of GLUTag-INS cells, a suspension of pre-aggregated GLUTag-INS spheroids, or GLUTag-INS cells on small intestinal submucosa (SIS), was evaluated in vitro for total cell number, weekly glucose consumption and insulin secretion rates (GCR and ISR), and induced insulin secretory function. The three types of TEPS studied displayed similar number of cells, GCR, and ISR throughout four weeks of culture. However, the TEPS which used SIS as a substrate for the GLUTag-INS cells, was the only TEPS that was able to retain the induced insulin secretory function of non-encapsulated GLUTag-INS cells. Though improvements in the expression level of GLUTag-INS cells and/or the number of viable cells contained within the TEPS are needed for successful treatment of a murine model of IDD, this study has revealed a potential method for promoting proper cellular function of recombinant L-cells upon incorporation into an implantable three-dimensional TEPS.

4.1 Introduction

As discussed in CHAPTER 2, enteroendocrine cells, exhibit many useful properties that make them attractive targets for cell-therapy for IDD. The incretins secreted by enteroendocrine cells are released in a tightly regulated manner that closely parallels the secretion of insulin by β-cells (Schirra, Katschinski et al. 1996; Kieffer and Habener 1999) and are compatible with insulin in glycemic normalization and enteroendocrine cells are able to process wild type proinsulin into mature insulin and C-peptide. Indeed, studies with transgenic mice, expressing insulin in enteroendocrine cells demonstrate that insulin-producing enteroendocrine cells can provide regulation of blood glucose levels in vivo (Cheung, Dayanandan et al. 2000; Lu, Sternini et al. 2005), but much work remains in translating enteroendocrine cell insulin therapy into a viable therapeutic modality for adult models of IDD and eventually humans.

The most direct application suggested by these transgenic mice for treating IDD in an adult animal would be to genetically engineer the appropriate enteroendocrine cells in their natural environment, that is, in vivo gene therapy. Besides significant difficulties inherent to delivering and stably expressing recombinant insulin in L-cells in vivo, a major challenge of in vivo gene therapy is in carefully titrating the treatment, so as not to overdose the patient with the transgene, as this might result in sustained over-production of insulin, an error that could be fatal. Performing the necessary genetic modifications in a closely controlled manner outside of the body would greatly enhance safety. A TEPS would not only provide the safety and quality control of in vitro gene therapy, but could additionally allow for retrieval of the construct and cells, if necessary, and for the non-invasive monitoring of the implant by, e.g., nuclear magnetic resonance techniques (Constantinidis and Sambanis 1998; Stabler, Long et al. 2005; Stabler, Long et al. 2005).

In CHAPTER 3, we modified the murine L-cell line GLUTag, to exhibit stable expression of human insulin such that large numbers of L-cells secreting insulin in a uniform manner could be cultured and used for the development of a TEPS based on such

cells. This chapter describes the development and characterization of a TEPS which supports the function of GLUTag-INS cells and facilitates steady performance of the TEPS in vitro for extended periods of time. We hypothesized that the presence of an extracellular matrix derived from the small intestine would be representative of the natural environment of L-cells and thus would improve the function of GLUTag-INS cells compared to TEPS which lack this support. SIS is a cell-free extracellular matrix derived from the porcine intestine, and as such may represent a natural setting for intestinal L-cells (Badylak 2004). It has been frequently used for the development of a tissue engineered intestine (Chen and Badylak 2001; Demirbilek, Kanmaz et al. 2003; Wang, Watanabe et al. 2003; De Ugarte, Choi et al. 2004; Wang, Watanabe et al. 2005) and thus may represent an intuitive reimplantation site for engineered autologous L-cells. Furthermore, increased cell-cell interaction within a TEPS may enhance function over TEPS without such interactions. These hypotheses were tested by comparing agarose constructs which housed either a single cell suspension of GLUTag-INS cells, a suspension of pre-aggregated GLUTag-INS spheroids, or GLUTag-INS cells on small intestinal submucosa (SIS). Spheroids represent a unique cellular environment and may influence the survival of a number of cell types (Bates, Edwards et al. 2000). The design of all TEPS called for agarose encapsulation to prevent direct contact of implanted cells with the host during future in vivo studies with diabetic mice. Indeed, the presence of a hydrogel barrier preventing direct contact with the host has proven sufficient in providing immunoprotection to other murine allografts in vivo (Black, Constantinidis et al. 2006). Agarose was selected for this study because it can be easily manipulated to encase the SIS and gels uniformly by cooling from 42°C to 37°C, as opposed to alginate which requires the addition of a divalent cation solution to induce cross-linking. In addition, natural agarose of sufficient purity and low endotoxin content is available commercially and was used in this study.

4.2 Materials and Methods

All reagents were purchased from Sigma (St Louis, MO) unless otherwise noted.

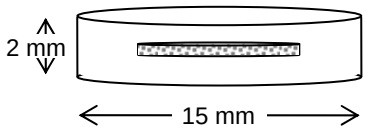
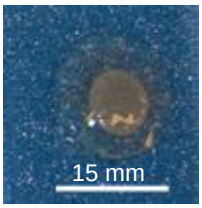
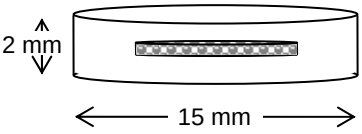
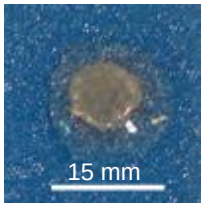
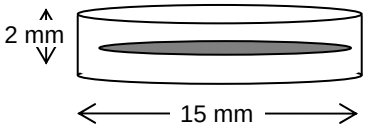
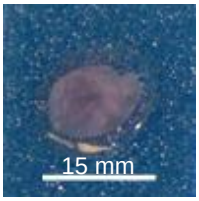
4.2.1 Cell Culture

GLUTag-INS cells were derived from the GLUTag cell line to stably express human insulin under the control of a CMV promoter (Bara and Sambanis 2008) and were cultured as described in CHAPTER 3.

4.2.2 Construct Design and Fabrication

The performance of GLUTag-INS cells, cultured within three-dimensional constructs, suitable for intraperitoneal implantation into a mouse, was evaluated in vitro. The three tissue-engineered pancreatic substitutes (TEPS) tested are depicted in Table 4.1. These were: (1) single cells entrapped in an inner agarose disk, which was then encased in a cell-free agarose disk; (2) cells pre-aggregated into spheroids and entrapped in an inner agarose disk, which was then encased in a cell-free agarose disk; and (3) cell-seeded SIS within a cell-free agarose disk.

Table 4.1 Tissue Engineered Pancreatic Substitutes Evaluated in this Study

Description	Schematic	Picture
GLUTag-INS single cells in inner agarose disk encased in cell-free agarose		
GLUTag-INS cells pre-aggregated into spheroids in inner agarose disk encased in cell-free agarose		
GLUTag-INS cells on SIS encased in cell-free agarose		

The total size and shape of the disks was based on the work by Stabler et al. (Stabler, Long et al. 2005) and was 15 mm in diameter and 2 mm in height (350 µl in volume). The inner disk in (1) and (2) was 1 mm in height and 5.5 mm in diameter (100 µl in volume). The initial cell number in each TEPS was similar for the three designs tested. To fabricate the TEPS, SIS was first seeded with GLUTag-INS cells and cultured prior to encapsulation. Circular pieces of SIS were seeded by immobilized under a sterile cloning cylinder and incubation with a cell suspension containing 2 x 10^6 cells for 1 hour. Using gentle manipulation this process was repeated on the other side. Cell-seeded SIS was then cultured for seven days in a six-well plate, suspended in 3 ml of medium until encapsulation. During this culture, GLUTag-INS cells were also grown in parallel as monolayers for use in the other two constructs. Spheroids of GLUTag-INS cells were allowed to form by culturing freshly trypsinized GLUTag-INS cells in a spinner flask

using the conditions reported by Papas et al. (Papas, Constantinidis et al. 1993). Briefly, 100 ml of cell suspension, at a density of 1.2 x 10^6 cells/ml medium, was cultured in a 125 ml spinner flask at 60 rpm overnight until spheroids with an average diameter of 100 μm formed.

The following day, the fabrication of all three TEPS was completed by encapsulation in 2% SeaPlaque agarose (Cambrex, East Rutherford, NJ). The inner agarose disks containing either freshly trypsinized single cell suspension or a suspension of spheroids were formed by transferring 100 μl of the cell or spheroid agarose suspension to a sterile Lexan mold (McMaster-Carr, Atlanta, GA) and allowing to gel for 5 minutes at room temperature. Both suspensions had an overall cell density of 3 x 10^7 cells/ml agarose. Cell number per ml of settled spheroid volume was determined by citric acid crystal violet nuclei count and was 1.84 x 10^8 cells/ml spheroids. Encapsulation in the outer agarose layer was performed in the same manner for all constructs. Briefly, the sterile Lexan mold was half-filled with agarose, the cell-containing insert was transferred to the center of the mold, and the remaining agarose was added to fill the mold and allowed to gel for 5 minutes at room temperature.

4.2.3 Viability Assessment of GLUTag-INS cells cultured on SIS

Seven days after seeding the SIS with GLUTag-INS cells, a sample of the cell-seeded SIS was imaged by staining with the Live/Dead Viability/Cytotoxicity Kit for mammalian cells (Invitrogen, Carlsbad, CA) and confocal microscopy. This kit is composed of two reagents: ethidium homodimer-1 (EthD-1) and calcein AM. EthD-1 is excluded by the intact cell membrane, but enters through damaged membranes and upon binding to nucleic acids, undergoes a 40-fold enhancement of red fluorescence. Calcein AM is a virtually nonfluorescent cell-permeant molecule that is acted on by the ubiquitous intracellular esterase activity to produce the intensely fluorescent calcein (green). The excitation/emission maxima for EthD-1 and calcein are ~495/635 nm and

495/515 nm respectively. Samples were excited with a combination of 543 nm and 488 nm lasers and long pass (560 nm cutoff) and band pass (500-550 nm cutoffs) filters were used to acquire the emission signals.

4.2.4 TEPS Culture and In Vitro Evaluation

The TEPS described above were cultured in six-well non-tissue culture-treated plates containing 3 ml growth medium placed on a rocking platform in a 37°C/5% CO_2 humidified incubator.

Glucose consumption rate (GCR) and insulin secretion rate (ISR) were determined weekly by initially rinsing the well and TEPS twice with PBS, adding 3 ml fresh medium, sampling for initial metabolite concentrations, incubating for 20 to 24 hours, and sampling again for final concentrations. ISR and GCR measurements were made on the same TEPS at all time points, and parallel constructs were frozen at -80°C for determination of total double-stranded DNA content, which was used to estimate total cell number.

Induced insulin secretion tests were also performed on all three types of TEPS. On the evening prior to the secretion tests, the medium was changed to basal (DMEM with 5 mM glucose, supplemented with 1% FBS). On the day of the secretion test, constructs were briefly washed with basal medium three times. During the secretion test all constructs were incubated in basal medium for two hours and induction medium (basal medium supplemented with 2% (w/v) meat hydrolysate (MH)) for another 2 hours. Constructs were washed with basal medium between periods and medium samples were reserved at the end of each period.

4.2.5 Assays

Samples were assayed for glucose and insulin concentration by the Trinder assay (Diagnostic Chemicals Limited, USA, Oxford, CT) and human insulin RIA kits

(Millipore, Billerica, MA) respectively, according to the manufacturer's protocols. To prepare the samples for double-stranded DNA quantification, the frozen constructs were thawed and dehydrated, then serially digested with proteinase K and agarase. Double stranded DNA was then measured with the picoGreen kit (Invitrogen, Carlsbad, CA) according to manufacturer's protocol.

4.3 Results

4.3.1 Viability and Total Cell Number over Time

Cell viability was assessed by LIVE/DEAD staining for cells on SIS only prior to agarose encapsulation, as the thickness of the agarose prohibited any further confocal visualization. After seven days of culture, GLUTag-INS cells on SIS had formed a nearly confluent layer with high cellular viability (Figure 4.1).

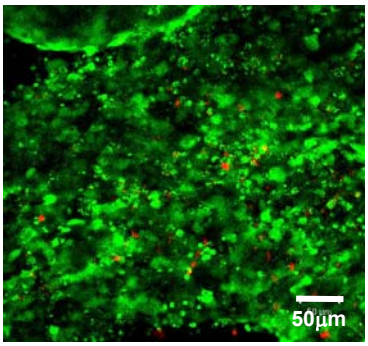


Figure 4.1 Live/Dead staining of GLUTag-INS cells cultured on SIS for 7 days reveals that the SIS is nearly confluent with viable cells prior to encapsulation.

Post-agarose encapsulation, the total cell number (both live and dead) in each TEPS was estimated based on a total DNA content, at given time points using at least three different constructs (Figure 4.2). Each TEPS contained approximately the same number of cells, 3 x 10^6 cells, when encapsulated (day 0). While the SIS-containing and single cell-containing TEPS showed an increase in the total cell number by day 28

($p<0.05$), with little increase during the first three weeks, spheroid-containing TEPS did not display this increase in total cell number. SIS-containing TEPS had 3.76 ± 0.47 and $4.46 \pm 0.34 \times 10^6$ cells on days 7 and 28 respectively. Likewise, TEPS containing single cell suspensions had 3.66 ± 0.45 and $5.58 \pm 1.44 \times 10^6$ cells on days 7 and 28 respectively, but TEPS containing spheroid suspensions had $4.38 \pm 0.04 \times 10^6$ cells on day 7 and only $4.80 \pm 1.38 \times 10^6$ cells on day 28.

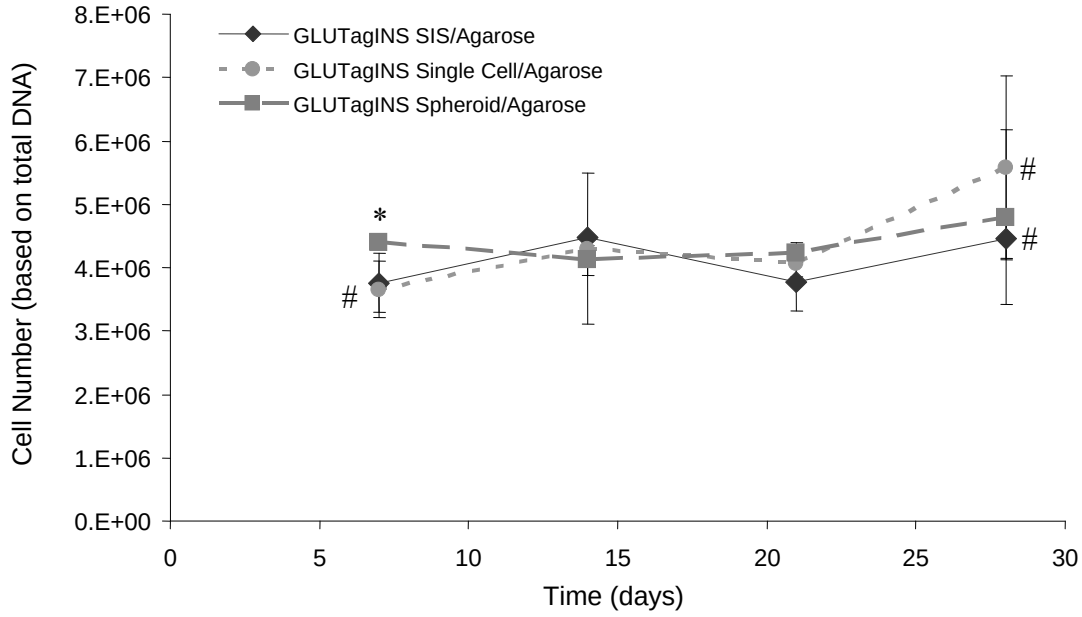


Figure 4.2 Total cell number vs. time of three types of TEPS. TEPS were frozen at the indicated time points and later assayed for total DNA content. Cell numbers were calculated based on a standard curve of cell number vs. DNA amount constructed with GLUTag-INS cells on tissue culture plastic (n=3). Points are shown as mean ± SD. *There is no difference cell number among groups except on day 7, when the spheroid-containing group is significantly higher than the other two groups ($p<0.05$). # Both SIS- and single cell-containing TEPS showed statistically significant increase in total cell number from day 7 to day 28 ($p<0.05$), but no difference was measured for the spheroid-containing group.

4.3.2 Glucose Consumption Rates

GCR is an indicator of the overall number of metabolically active cells contained within the TEPS. GCR was selected over alamarBlue® (Invitrogen, Carlsbad, CA) as GCR, contrary to alamarBlue®, changed minimally, if at all, upon agarose encapsulation of GLUTag-INS cell-seeded SIS. GCRs of the TEPS were determined by assaying the glucose concentration in samples of spent medium (Figure 4.3). As expected, the GCRs of the single cell-containing TEPS increased from day 7 to day 28 as the total cell number increased (Figure 4.2). Though the SIS-containing TEPS also saw a rise in total cell number, this increase was much smaller and, as such, was not reflected in a statistical increase in GCR from day 7 to 28. Also, the consistently high GCR levels of the SIS- and spheroid-containing groups indicate that despite low or no increase in total cell number, the constructs are still supporting viable cells during the month of in vitro culture. Comparisons between groups on each day revealed that while the single cell-containing group had a lower GCR than both spheroid- and SIS-containing groups on days 7 and 14 ($p<0.05$), the temporal GCR increase of the single cell-containing TEPS group erased this difference with the spheroid group on day 21 and with both other groups by day 28.

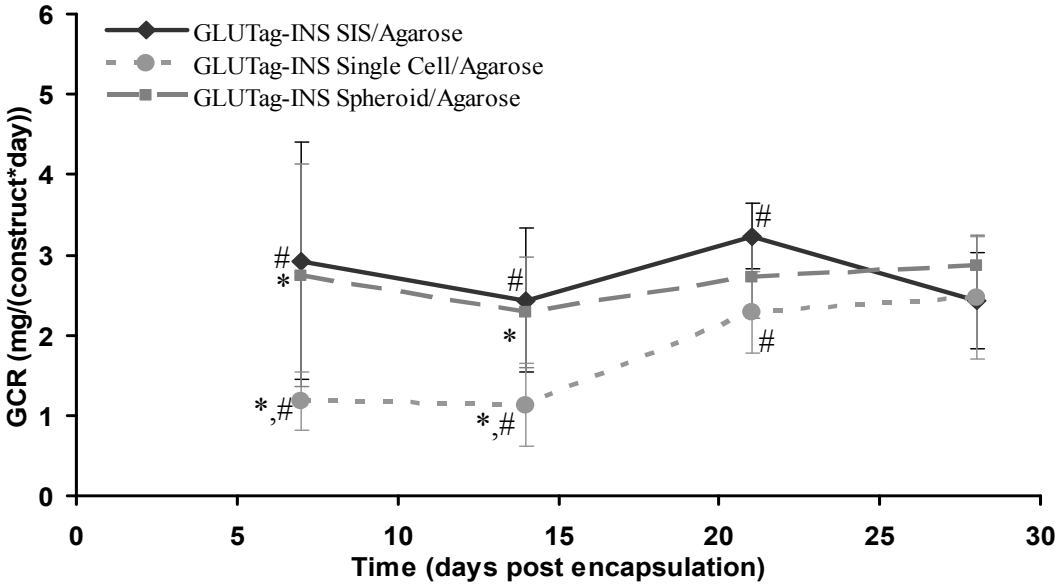


Figure 4.3 Glucose consumption rate (GCR) of GLUTag-INS cells in three types of TEPS over time. The same constructs were examined throughout the course of four weeks of in vitro culture after encapsulation, and glucose consumption was determined by assaying spent culture medium once a week (n=6). Points are shown as mean ± SD. SIS- and spheroid-containing groups had significantly greater GCR than the single cell-containing TEPS (#,* One-way ANOVA, $p<0.05$) on days 7 and 14, though by day 21 the single cell-containing group showed an increase in GCR to match that of spheroid-containing TEPS. By day 28 there were no differences in GCR among groups.

4.3.3 Insulin Secretion Rates

As GCR provides insight into the number of metabolically active cells in the TEPS, insulin secretion rate (ISR) alludes to the number of secreting cells, and also provides insight into a key aspect of TEPS utility, that is, its ability to secrete insulin. There was considerable variation in the ISR profiles of the TEPS (Figure 4.4), with seemingly random differences appearing on throughout the course of the experiment. On day 7 there were no differences among groups, but on day 14 the single cell-containing group had a higher ISR than the spheroid-containing group. This difference was reversed on day 21 with the spheroid-group having the greater ISR. By day 28 there was no difference between sheroid- and single cell-containing groups, but the SIS group was

higher than the single cell-containing group ($p<0.05$). When comparing the average at all time points of each group, the SIS-containing group was found to have higher ISRs than both the single cell and spheroid groups (one-way ANOVA $p<0.05$), but there was no statistical difference between the single cell suspension and spheroid suspension groups ($p>0.05$).

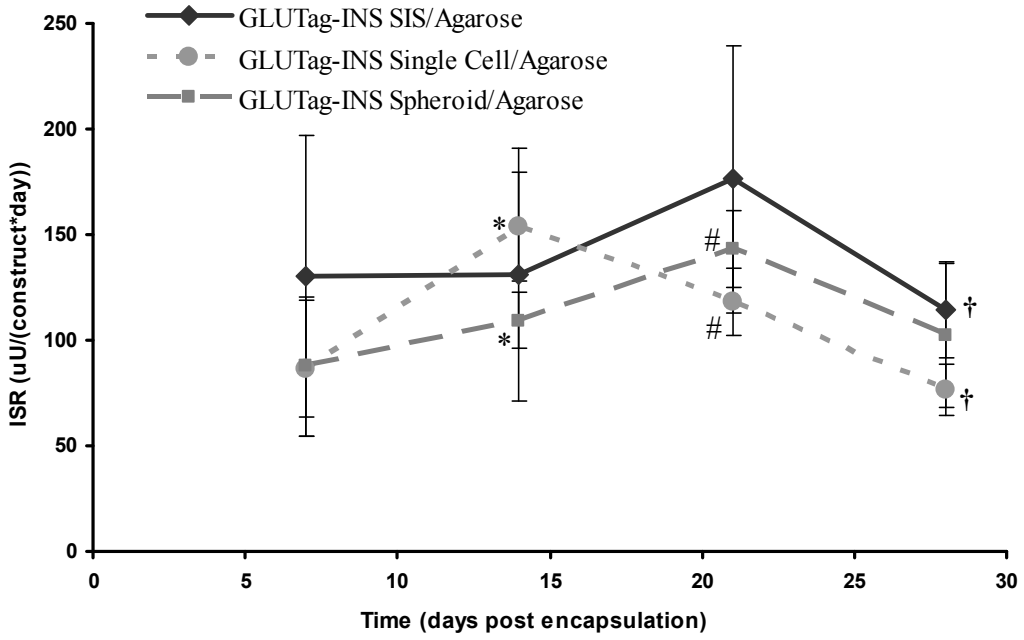


Figure 4.4 Insulin secretion rate (ISR) of GLUTag-INS cells in three types of TEPS over time. The same constructs were examined throughout the course of four weeks of in vitro culture after encapsulation and the average insulin secretion rate over one week was determined by assaying spent culture medium in samples collected at the same frequency (n=6). Points show the mean ± SD. *,#,† $p<0.05$. With ISR averaged for each group over all time points, one-way ANOVA revealed that the SIS-containing TEPS had a significantly greater ISR than both spheroid- and single cell-containing TEPS ($p<0.05$) but no difference was observed between the latter two groups ($p=0.942$).

4.3.4 Induced Insulin Secretion

Selection of SIS as a potential substrate for an implantable TEPS arose from initial testing in which the basal and induced ISRs of GLUTag-INS cells grown on SIS were compared to those of cells grown on conventional tissue culture plastic (Table 4.2). For these tests, insulin secretion was measured during a two hour basal period and again during a two hour induced period in which the cells experienced a step up in the concentration of meat hydrolysate (MH) from 0% to 2%.

Table 4.2 Comparison of Insulin Secretion Rates of GLUTag-INS Cells on Tissue Culture Plastic and SIS.

	Day 2			Day 7		
	ISR ($\mu U/(10^6$ cells · hr))			ISR ($\mu U/(10^6$ cells · hr))		
	Basal	Induced	IF	Basal	Induced	IF
Tissue Culture Plastic	6.6 ± 1.19	10.0 ± 1.29 *,#	153%	11.9 ± 1.69 †	40.2 ± 2.17 *,‡	339%
Unencapsulated SIS	6.0 ± 0.60	18.6 ± 2.79 *,#	310%	7.6 ± 2.01 †	26.1 ± 1.05 *,‡	341%

Rates were normalized to the total number of cells measured by DNA content at each time point. Values are mean ± SD, n=3. *Different than basal value on the same day for the same group ($p<0.05$). #,†, ‡ Different than other group at a given day and condition ($p<0.05$).

The main difference between the two time points (day 2 and day 7) is presumably cell surface density. Though the basal and induced ISRs of GLUTag-INS cells on SIS are generally reduced compared to those on plastic (except for the induced rates on day 2), it is important to note that the induction-fold (IF) is maintained. Again, to better evaluate the performance of agarose-based constructs, induced insulin secretion tests were performed on days 7 and day 14 post encapsulation (Figure 4.5). Induced insulin secretion is expressed as the percentage of insulin secreted during MH stimulation relative to that secreted during the basal period. GLUTag-INS cells on SIS in agarose

exhibited induced release of insulin in response to nutrient stimulation on days 7 (182.2%) and 14 (162.4%), but the GLUTag-INS cells within the other two types of TEPS did not exhibit this induced response.

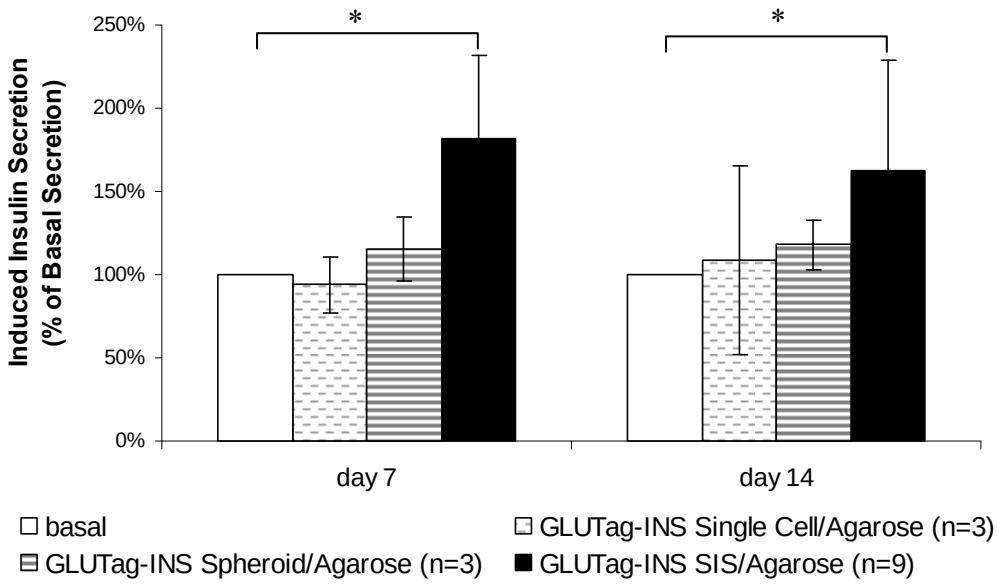


Figure 4.5 Induced insulin secretion tests were performed on the same constructs on days 7 and 14 relative to encapsulation in 2% agarose. Constructs were incubated in basal medium for two hours, then the medium was changed to basal medium containing 2% MH for two hours. Induced insulin secretion is expressed as the percentage of insulin secreted during MH stimulation relative to that secreted during the basal period (mean ± SD). On days 7 and 14, the SIS-containing TEPS had a significantly higher induced secretion response over basal conditions ($p<0.05$), but the other two groups failed to display significant induction on both days. Also on day 7, the SIS group was significantly different than both single cell and spheroid groups, but these differences were lost on day 14.

4.4 Discussion

In this study we evaluated the performance of GLUTag-INS cells within three types of agarose-based TEPS during four weeks of in vitro culture. GLUTag-INS cells are murine L-cells which have been engineered to constitutively express human insulin (Bara and Sambanis 2008). Insulin's secretion from these cells, however, is dictated by

the regulated release pathway and responds to nutrient stimulation. A TEPS based on engineered L-cells may be a more likely therapeutic option for humans than delivery of the cells by themselves, as it accomplishes partial immune protection, localization and retrievability of the implant, which all contribute to superior safety and quality control.

The use of SIS was evaluated here for the potential to improve the regulated secretory function of engineered L-cells, as the origin of this extracellular matrix closely matches the native environment of L-cells. In addition, several studies have reported that the use of SIS improves the in vitro culture and function of islets (Lakey, Woods et al. 2001; Woods, Walsh et al. 2004; Tian, Xue et al. 2005). Though GLUTag-INS cells are an immortalized cell line, derived from an intestinal tumor, and may not be as peculiar to matrix composition as primary cells (like islets), SIS may also maintain or enhance the phenotype of insulin secretion from these engineered L-cells. This is especially important for cells implanted in potentially unfavorable environments, such as the peritoneal cavity, where a drop in dissolved oxygen concentration from 0.20 mM under normal incubator conditions to 0.06 mM (Gross, Constantinidis et al. 2007) may cause a decline in insulin secretory function. In this study, hydrated sheets of SIS were investigated, as this configuration may permit the polarization of the cells. Use of hydrated sheets of SIS, however, required the fabrication of constructs of significantly larger dimensions than alginate microbeads used frequently in TEPS and islet encapsulation (Lim and Sun 1980). SIS did indeed help to maintain a similar induction-fold of insulin to that of cells grown on tissue culture plastic (Table 4.2).

It was also observed that densely confluent monolayers of GLUTag-INS cells had improved secretory function over sub-confluent monolayers. This improvement was reflected in increases in both basal and induced secretion rates as well as the induction factor (comparison of monolayers on day 2 vs. 7 in Table 4.2) and may be attributable to increased cell-cell interaction among GLUTag-INS cells. As such, we hypothesized that

incorporating pre-aggregated spheroids into a TEPS would improve the function of the TEPS over similar constructs containing only single cells.

Though several statistically significant differences were noted in the GCR and ISR results, these differences were nonetheless small. Furthermore, because the differences among groups in the GCR data were not reflected in the ISR data, it is likely that the differences noted in the GCR on days 7 and 14 are due to lower oxygen concentrations seen by the centrally located cells within the SIS- and spheroid-containing TEPS. Indeed, murine insulinoma βTC3 cells have been reported to be more oxygen sensitive with regard to GCR than with regard to ISR (GCR increased at oxygen concentrations below 25 mmHg, while the ISR decreased below 7 mmHg (Papas, Long et al. 1996)), and this may also be the case with GLUTag-INS cells. Again, though the differences in ISR among groups were small, the SIS-containing TEPS did, on average, slightly out-perform the single cell- and spheroid-containing TEPS. Importantly, each type of construct showed no statistical change in ISR during the four weeks in culture, indicating a stable system which is favorable for ensuring that the TEPS does not result in hypoglycemia following implantation and cell proliferation in vivo. In other systems using insulinoma cells, a tetracycline-responsive element has been incorporated in order to provide an external handle on cell growth following implantation (Efrat, Fusco-DeMane et al. 1995). With GLUTag-INS cells in the agarose constructs, however, this type of control does not appear to be needed, as ISR is stable during four weeks in culture.

The induced secretion response of the three types of TEPS tested here revealed the most significant difference between the configurations tested. While agarose encapsulation weakened the secretory response of SIS-containing TEPS compared to non-encapsulated SIS, possibly due to diffusional limitations of insulin and macromolecular constituents of MH, incorporation of SIS did allow for retention of the induced secretion response which was not observed in the single cell- and spheroid-

containing TEPS. As noted, the induction-fold of agarose-encapsulated SIS (182.2% on day 7) was lower than unencapsulated SIS (341% on day 7), it was however, fairly close to the value reported for monolayers on tissue culture plastic (218.6%) (Bara and Sambanis 2008). The overall levels of basal and induced secretion of GLUTag-INS cells on SIS were somewhat lower than cells grown on plastic, however, and this drop was only further exacerbated by encapsulation in agarose. It has been reported that βTC3 insulinoma cells also exhibit a decline in insulin secretion upon encapsulation, although of a lower magnitude than that observed in this study (Mukundan, Flanders et al. 1995).

With regard to the level of insulin secretion provided per construct, the TEPS described here fall short of the therapeutic threshold for a diabetic mouse. In one study, a mass of genetically engineered K-cells (STC-1-14), secreting 78.6 μU/(10^6 cells · day) was implanted subcutaneously, and hyperglycemia was not reversed until about 24 days after implantation, indicating that some tumor growth was needed prior to reaching a therapeutic insulin secretion value (Zhang, Yao et al. 2008). GLUTag-INS cells secrete a greater amount of insulin than the K-cells reported by Zhang et al., even at their basal rate of secretion, 285.6 μU/(10^6 cells · day) versus 78.6 μU/(10^6 cells · day) for STC-1-14 cells, and as such, there is little doubt that GLUTag-INS cells would be capable of regulating the blood glucose levels of diabetic mice if a sufficient number of viable cells were implanted. To better understand what that number would be we can look to studies involving the insulinoma cell line, βTC-tet. Intraperitoneal implantation of 9 x 10^6 βTC-tet cells entrapped in calcium-alginate microbeads was sufficient to reverse diabetes in eight-week old NOD mice within a week of implantation (Black, Constantinidis et al. 2006). Using the insulin secretion rates for βTC-tets reported by Simpson et al. (Simpson, Khokhlova et al. 2005), and assuming that the cells secrete at the stimulated rate for 50% of the time and the non-stimulated rate the other 50% of the time, this is the equivalent of a 227 mU/day. Another estimate for the therapeutic dose of insulin for a diabetic mouse is 200 mU/day as provided by the subcutaneous LinBit implants (Wang

1991). Thus, given the level of secretion for non-encapsulated GLUTag-INS cells reported for day 7 in Table II and making the same assumption that cells are secreting at their stimulated rate for 50% of the time and the non-stimulated rate the other 50% of the time, it would take 3.20×10^8 GLUTag-INS cells to treat a mouse (i.e., produce 200 mU insulin/day) compared to the 3×10^6 cells encapsulated in the TEPS evaluated here. Alternatively if the specific rate of insulin secretion of GLUTag-INS cells could be increased by further genetic engineering and/or manipulation of the cellular microenvironment then this number may be reduced significantly.

The actual amount of insulin needed by per mouse (as with humans) may vary significantly depending on a number of factors including: site of delivery, metabolism, diet, and peripheral insulin resistance. This underscores the importance of using cells which exhibit controlled release like that of islets for IDD cell therapy. Due to the restrictions of allogeneic or xenogeneic transplantation, which include limited supply and immunogenicity, a potentially autologous cell source, such as engineered L-cells, is especially attractive.

CHAPTER 5

IN VIVO EVALUATION OF TISSUE ENGINEERED PANCREATIC SUBSTITUTES BASED ON RECOMBINANT L-CELLS

Although insulin treatments are available for diabetic patients, there are many disadvantages to insulin injections. Furthermore, insulin therapy cannot prevent the development of severe secondary complications such as heart disease, blindness, and peripheral nerve damage commonly associated with diabetes. Implantation of insulin-secreting cells constitutes a promising alternative approach. With the dramatically increasing incidence of diabetes, newer and better treatments are greatly needed.

The objective of this aim was to implant insulin-secreting TEPS containing GLUTag-INS cells intraperitoneally in diabetic mice and to evaluate their function during and after implantation in the allogeneic host. GLUTag-INS cells characterized in CHAPTER 3 were encapsulated in two different geometries—the SIS/agarose based TEPS described in CHAPTER 4 and calcium-alginate microbeads. Both types of constructs are referred to as TEPS and were implanted intraperitoneally in diabetic mice. In vivo efficacy of GLUTag-INS containing TEPS was compared to controls, fabricated in the same manner but containing either no cells or parental GLUTag cells (negative controls) or insulin-secreting mouse insulinoma cells (positive control). Non-fasting blood glucose and weight were determined throughout the duration of the experiment and plasma insulin levels, liver glycogen content, and histology of the agarose constructs were assessed post-mortem.

5.1 Introduction

The goal of the work in this chapter was to evaluate the in vivo performance of TEPS based on genetically modified GLUTag-INS cells. Rodent models are the most

commonly used in vivo test bed for diabetes research due to cost, ethical considerations, and ease of use. Here, we use the STZ mouse model, in which STZ is administered to chemically induce the onset of IDD. As introduced in CHAPTER 2, another commonly used model for IDD, the NOD mouse, may have autoimmunity to β-cells and/or the insulin produced by β-cells. Due to this complication, the NOD model may not be appropriate for the proof-of-principle experiments proposed in this chapter.

A number of implantation sites have been used in the past to implant insulin-secreting cells, either alone or as part of a bioartificial pancreas. These include the intraperitoneal (Stabler, Long et al. 2005; Black, Constantinidis et al. 2006), subcutaneous (Tatarkiewicz, Hollister-Lock et al. 1999), subrenal (Carlsson, Palm et al. 2000; Han, Lee et al. 2007), intrasplenic (Soria, Roche et al. 2000), and intrahepatic (Matsumoto, Okitsu et al. 2005) implantation sites. Alternatively, intravascular devices are connected to the vasculature directly and perfused by the host's blood. For a diffusional device of large dimensions such as ours, the intraperitoneal space was the location of choice.

When assessing a TEPS, the physiological effects typically evaluated are weight gain or loss, blood glucose levels, and often blood insulin and HbA(1)c concentrations (Salvay, Rives et al. 2008; Zhao, Amiel et al. 2008). Because insulin plays such a major role in maintaining normal metabolism and physiology, there are a number of other possible assessments that may be performed to gain a better understanding of what is happening in vivo including liver glycogen storage (Ito, Bujo et al. 2005) and lipid profile (Olson, Paveglio et al. 2003). Additionally evaluating the implant itself after explantation for insulin secretion, and immunohistochemical analysis are very important in determining the overall performance of a TEPS in vivo.

In this chapter two geometries of TEPS were implanted intraperitoneally in diabetic mice: calcium-alginate microbeads and an agarose disk construct containing cell-seeded SIS. Non-fasting blood glucose concentrations and body weights were

determined throughout the duration of the experiment for mice implanted with both types of constructs, while mice implanted with the agarose disks were also tested for plasma insulin levels, liver glycogen content, and histology of the explanted constructs post-mortem.

5.2 Materials and Methods

All methods were approved by the Georgia Institute of Technology Institutional Animal Care and Use Committee.

5.2.1 Fabrication of TEPS

SIS-containing TEPS of disk geometry were fabricated as described in CHAPTER 4. Briefly, pieces of SIS (14 mm in diameter) were seeded with cells and cultured for one week prior to encapsulation in 2% SeaPlaque agarose. To encapsulate, the sterile Lexan molds were half-filled with warm alginate, the cell-containing or cell-free SIS was positioned centrally within the mold and the remaining agarose was added to fill the mold. The final dimensions of the disk-shaped constructs were 15 mm in diameter and 2 mm in height (or 350 μl in volume).

TEPS comprised of calcium-alginate microbeads were fabricated as described by Simpson et al. (Simpson, Stabler et al. 2004). Cell monolayers were harvested with trypsin-EDTA and suspended in 2% sodium alginate (high mannuronic acid content (LVM) alginate; FMC BioPolymer, Drammen, Normay) at a density of 3×10^7 cells/ml. Beads with a diameter of ~500 μm were generated using an electrostatic bead generator (Nisco, Basel, Switzerland) and alginate was crosslinked in a bath of 100 mM $CaCl_2$.

Prior to implantation, insulin secretion from both types of TEPS was evaluated by overnight incubation in culture medium, and insulin content in the medium samples was assayed by RIA (Millipore, Billerica, MA).

5.2.2 Induction of Diabetes

Diabetes was induced in normal, C57BL/6J mice by giving a single injection of 200 mg/kg STZ while mice were under isoflurane anesthesia. Anesthesia was induced by isoflurane at a dose of 5% delivered in 1 liter/min oxygen by a precision vaporizer (via inhalation in a chamber with a scavenger). Anesthesia was maintained with isoflurane at a dose of 1.2 to 1.5% in 300 ml/min oxygen, delivered through a nose cone with scavenger. Depth of anesthesia was verified by lack of pedal withdrawal reflex and slow irregular respirations. Mice that withdrew the foot after toe was pinched were given more isoflurane in the smallest increment the vaporizer allowed. After an adequate plane of anesthesia was confirmed, the STZ was administered by intraperitoneal injection. Following at least 24 hours, the diabetic state was determined by blood glucose monitoring with the TrueTrack Smart System® (Home Diagnostics, Inc, Ft Lauderdale, FL) using blood collected at the tail tip site. Onset typically occurred three days after injection and was defined as those mice with two consecutive non-fasting blood glucose levels of more than 250 mg/dl.

5.2.3 Implantation of Artificial Pancreases

The experimental groups for each experiment (alginate microbead or SIS/agarose disk TEPS implantation) are described in Table 5.1. To prepare the mice for implantation surgery, isoflurane anesthesia was induced and maintained as described earlier, via inhalation in a chamber and through a nose cone respectively. After an adequate plane of anesthesia was confirmed, hair was removed from the abdomen and the skin prepared with successive applications of chlorhexadine and alcohol. Mice were then covered with a sterile drape with a fenestration over the abdomen.

For the disk-shaped artificial pancreases, a midline abdominal incision was made through the skin and muscle with surgical scissors and the implant was positioned in the peritoneal cavity on top of the viscera. The muscle layer was closed with 6-0 absorbable

suture in a simple interrupted pattern. For artificial pancreases of bead geometry, implantation was accomplished by a small incision through the skin and injection with a 16 gauge needle through the muscle layer. In each type of surgery, the skin was closed with mouse-sized wound clips. Immediately following surgery, mice were kept warm with a heat lamp and an injection of buprenorphine (0.05 mg/kg,) was administered as soon as the mice recovered from the isoflurane anesthesia.

Table 5.1 Experimental Groups Used in Each In Vivo Study

Mouse Type	Construct Type (Cells Seeded)	Group
Healthy	**None**	+ Control
Diabetic	**βTC-tet cells**	
	None	- Control
	GLUTag cells	
	GLUTag-INS cells	Test

5.2.4 Monitoring Body Weight and Blood Glucose

Mice were observed daily and body weights were recorded. When appropriate, blood glucose was also assessed by collection of a drop of blood at the tip of the tail and rapid measurement with a TrueTrack Smart System® hand-held glucose monitor and disposable testing strips (Home Diagnostics, Inc).

5.2.5 Plasma Insulin Levels

After determination of graft failure or at a specified time after implantation, mice were euthanized. Immediately following euthanization by CO_2 inhalation, approximately 1 mL of blood was obtained by cardiac puncture. Blood was allowed to clot at room temperature, and then centrifuged at 1600 g at 4°C to isolate the plasma. Plasma was frozen at -80°C until assayed by Human Ultra-sensitive RIA (Millipore).

5.2.6 Retrieval of Artificial Pancreases

Upon termination and immediately following blood draw by cardiac puncture, the constructs were retrieved. Retrieval was initiated by injection of Hank's balanced saline solution (HBSS) to loosen the constructs from surrounding tissues then a midline abdominal incision through skin and muscle was made with surgical scissors. The disk constructs were located visually and gently lifted out of the peritoneal cavity using a sterile spoon. Microbeads were retrieved by aspiration of the injected saline solution and peritoneal fluids containing the microbeads. Approximately 0.2 ml of beads were able to be retrieved.

5.2.7 Insulin Secretion from Implants

Explanted constructs were placed in complete culture medium and returned to incubator conditions overnight to assess the insulin secretion capability post implantation. Times were noted when each construct was placed into culture and upon sampling the following day. Insulin concentration was determined using the Human RIA (Millipore).

5.2.8 Live/Dead Staining and Confocal Microscopy

All images were taken on the LSM 510 confocal microscope (Carl Zeiss Inc.). The Live/Dead viability/cytotoxicity kit (Molecular Probes) was used to stain samples for viability assessment on SIS as described in CHAPTER 4.

5.2.9 Immunohistochemistry of Constructs

Following overnight incubation, the remaining constructs were fixed in 30% sucrose overnight, snap frozen in OCT, and stored at -80°C until 7 μm thick sections were sampled using the cryostat. Slides with frozen sections were stored at -80°C until used for immunohistochemistry (IHC).

To prepare the samples for IHC, frozen slides were brought to room temperature, fixed in cold acetone for five minutes, washed twice in PBS, blocked in 2% serum for 20

minutes, and blocked with Avidin D for 15 minutes. Then the diluted primary antibodies were applied to the sections. To determine if explanted constructs contained cells that contained insulin, insulin staining with a monoclonal anti-insulin antibody (product number I 2018, Sigma) was used at a 1:100 dilution. On other samples, the immune response was investigated using a CD68 antibody for macrophages (product number ab53444, Abcam, Cambridge, MA) and a neutrophil antibody (product number ab2557, Abcam) at dilutions of 1:200 and 1:100 respectively. Sections were incubated with primary antibody overnight at 4°C in a humid chamber. The following day, slides were washed twice with PBS, incubated with the appropriate biotinylated secondary antibody for 30 minutes. To reveal the staining, slides were treated with the ABC-alkaline phosphatase kit.

The procedure for use of the ABC-alkaline phosphatase kit was as follows. Slides were blotted and washed two times in PBS to remove excess antibody and a working solution of ABC-alkaline phosphatase complex (Vector #AK-5000) was applied to the samples for one hour at room temperature. Slides were again blotted and rinsed in PBS prior to incubation with 100 mM tris (pH 8.2) for five minutes. The alkaline substrate solution (Vector Red #SK-5100) containing levamisole (Vector #SP-5000) was then applied to the sections and incubated in the dark for 30 minutes. The reaction was stopped by blotting and rinsing twice in PBS. Finally slides were counterstained with hematoxylin, coverslipped and imaged.

5.2.10 Liver Glycogen Storage

Liver tissue was also harvested at time of euthanization. Livers were formalin-fixed and paraffin-embedded. Sections, 5 μm thick, were sampled from select livers and

glycogen content of the fixed tissue was qualitatively determined by Periodic Acid-Schiff (PAS) staining. The periodic acid selectively oxidizes the glucose residues, creating aldehydes that then react with the Schiff reagent to create a purple-magenta color. Briefly, the sections were deparaffinized and rehydrated in distilled water, rinsed once more in distilled water, then immersed in 0.5% periodic acid for five minutes. Slides were washed three times in distilled water and covered in Schiff's solution for 15 minutes. Slides then were treated with 0.55% potassium metabisulfate two times for one minute each to remove excess stain and washed in running tap water for 10 minutes to develop the color. Following PAS staining, slides were counterstained with hematoxylin, rinsed with tap water, and dehydrated by immersion in 95% and 100% alcohol twice each. Finally slides underwent two changes of xlene and were coverslipped with synthetic resin.

5.3 Results

5.3.1 Monitoring Body Weight and Blood Glucose

5.3.1.1 Implantation of Constructs of Disk Geometry

Beginning several days prior to STZ administration mice were weighed daily and their blood glucose level was sampled regularly. Body weight and blood glucose as a function of time for mice implanted with disk-shaped constructs have been plotted for each group of mice in Figures 5.1 and 5.2 respectively on the following pages. Day 0 is the day of implantation and both day 0 and the day on which STZ was administered are demarked with a vertical black line.

As expected, all mice becoming diabetic following STZ injection started losing weight at this point while the healthy control group, which received only buffer, continued to gain weight. It is also no surprise that following this major surgery a slight, but noticeable drop in body weights of all groups was observed. Upon recovery in the

subsequent four days the non-diabetic mice gained weight ($p<0.01$), but no differences in weight were noted for any other group, though the weight gain between days 1 and 4 of the βTC-tet group nearly gained significance ($p=0.053$).

Prior to STZ administration, all mice maintained their blood glucose concentrations within a narrow range of healthy, non-diabetic values (130 to 200 mg/dl). While this range was maintained for the healthy control group throughout the duration of this experiment, the mice receiving STZ saw a steep increase in blood glucose levels within two to four days following injection and then maintained hyperglycemic blood glucose levels above 250 mg/dl. Mice treated with insulin-secreting βTC-tet cells did have lowered blood glucose on day 2 post implantation compared to the pre-implantation value (paired t-test, $p<0.05$). A decline in blood glucose was also noted for GLUTag and sham (cell-free) treated negative controls on days 2 and 4 respectively ($p<0.05$). Though these drops were statistically significant, they were still well within the diabetic range of blood glucose values, and as these are measured during the non-fasting state, some variation is to be expected.

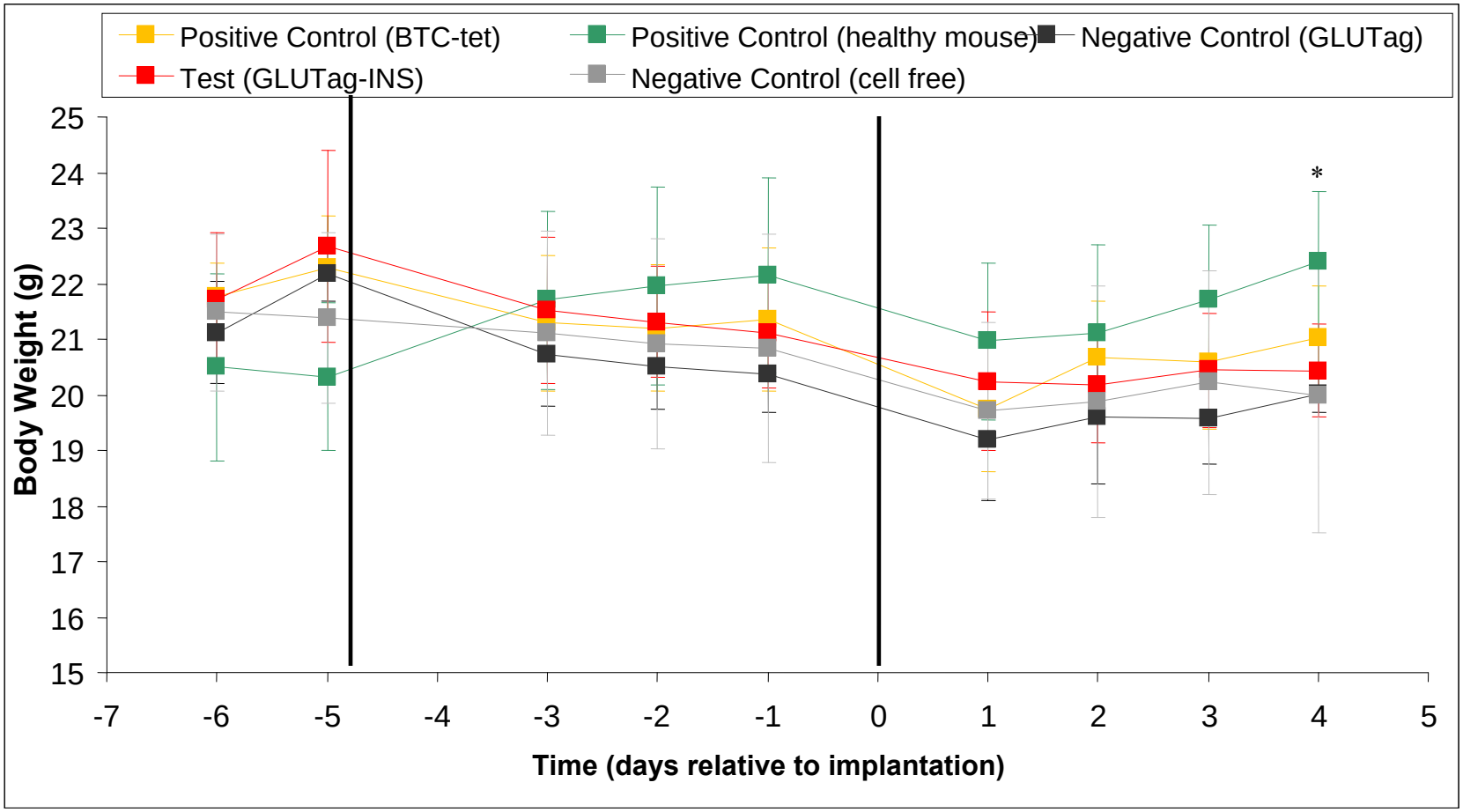


Figure 5.1 Body weights of mice implanted with disk-shaped constructs. STZ was administered on day -5 to all groups except the healthy positive control (injected with buffer only). Constructs were implanted on day 0. The condition of the mouse and the type of cells seeded on the SIS embedded within the agarose is indicated in parenthesis within the legend. *Healthy group greater than all others ($p<0.05$).

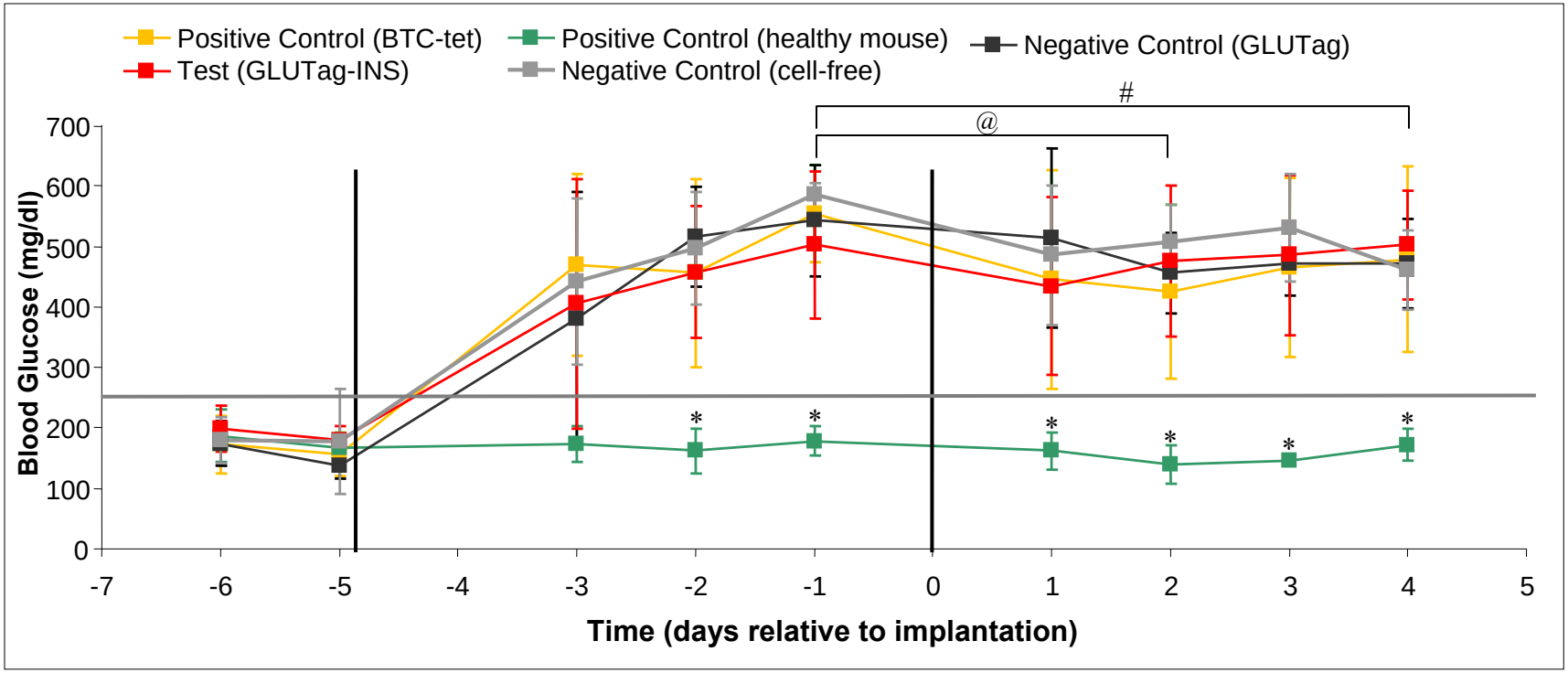


Figure 5.2 Blood Glucose levels in mice implanted with disk-shaped constructs. STZ was administered on day -5 to all groups except the healthy positive control (injected with buffer only). Constructs were implanted on day 0. The type of cells seeded on the SIS embedded within the agarose is indicated in parenthesis within the key. * Blood glucose of the healthy group was significantly lower than all other groups (One-way ANOVA, $p<0.005$). @ βTC-tet and GLUTag groups had lower blood glucose values on day 2 compared to day -1 (paired t-test, $p<0.05$). # The cell-free negative control had lower blood glucose on day 4 compared to day -1 (paired t-test, $p<0.05$).

5.3.1.2 Implantation of Constructs of Bead Geometry

Body weight and blood glucose concentrations of mice implanted with calcium-alginate microbeads have been plotted for each group of mice as a function of time in Figures 5.3 and 5.4 respectively on the subsequent pages. Day 0 is the day of implantation and both day 0 and the day on which STZ was administered are demarked with a vertical black line.

As expected, all mice becoming diabetic following STZ injection started losing weight at this point while the healthy control group, which received only buffer, continued to gain weight. In contrast to the implantation of disk-shaped constructs, mice undergoing microbead implantation did not display the drop in body weight following implantation, presumably because the surgery in this case is minor, resulting in an easier recovery for the mice with sooner return to normal food ingestion.

Prior to STZ administration, all mice maintained their blood glucose concentrations within a narrow range of healthy, non-diabetic values (130 to 200 mg/dl). While this range was maintained for the healthy control group throughout the duration of this experiment, the mice receiving STZ saw a steep increase in blood glucose levels within two to three days following injection to a hyperglycemic range (above 250 mg/dl). While mice in the negative control group maintained this level of hyperglycemia, mice treated with insulin-secreting βTC-tet cells saw a quick decline in blood glucose levels and both mice became hypoglycemic by day 2 or 3. Though one mouse treated with GLUTag-INS cell saw a decline in blood glucose to below the diabetic threshold, the group as a whole did not have a reduction in blood glucose levels.

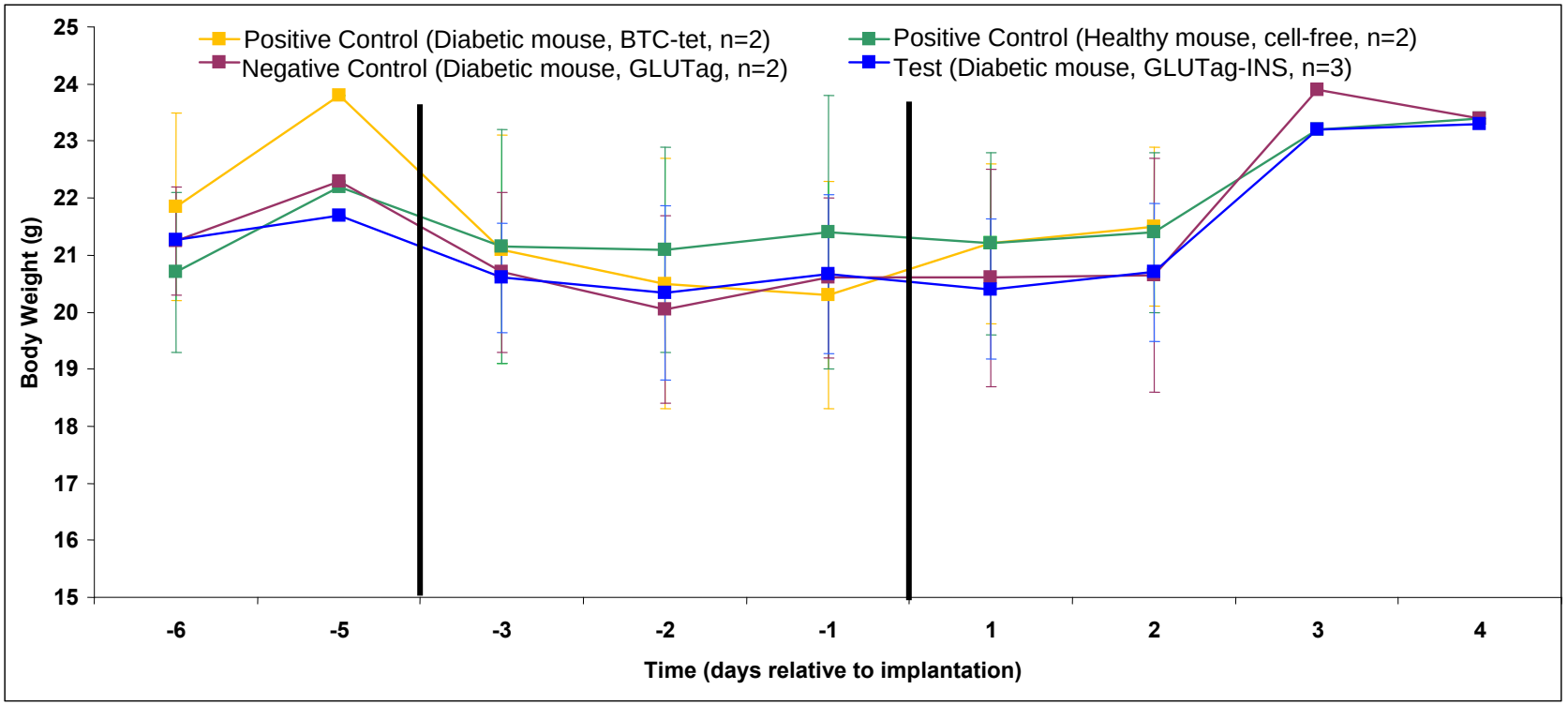


Figure 5.3 Body weights of mice implanted with microbead constructs. STZ was administered on day -4 to all groups but the healthy positive control (injected with buffer only) and constructs were implanted on day 0. The condition of the mouse and the type of cells encapsulated within the alginate microbeads is indicated in parenthesis within the legend.

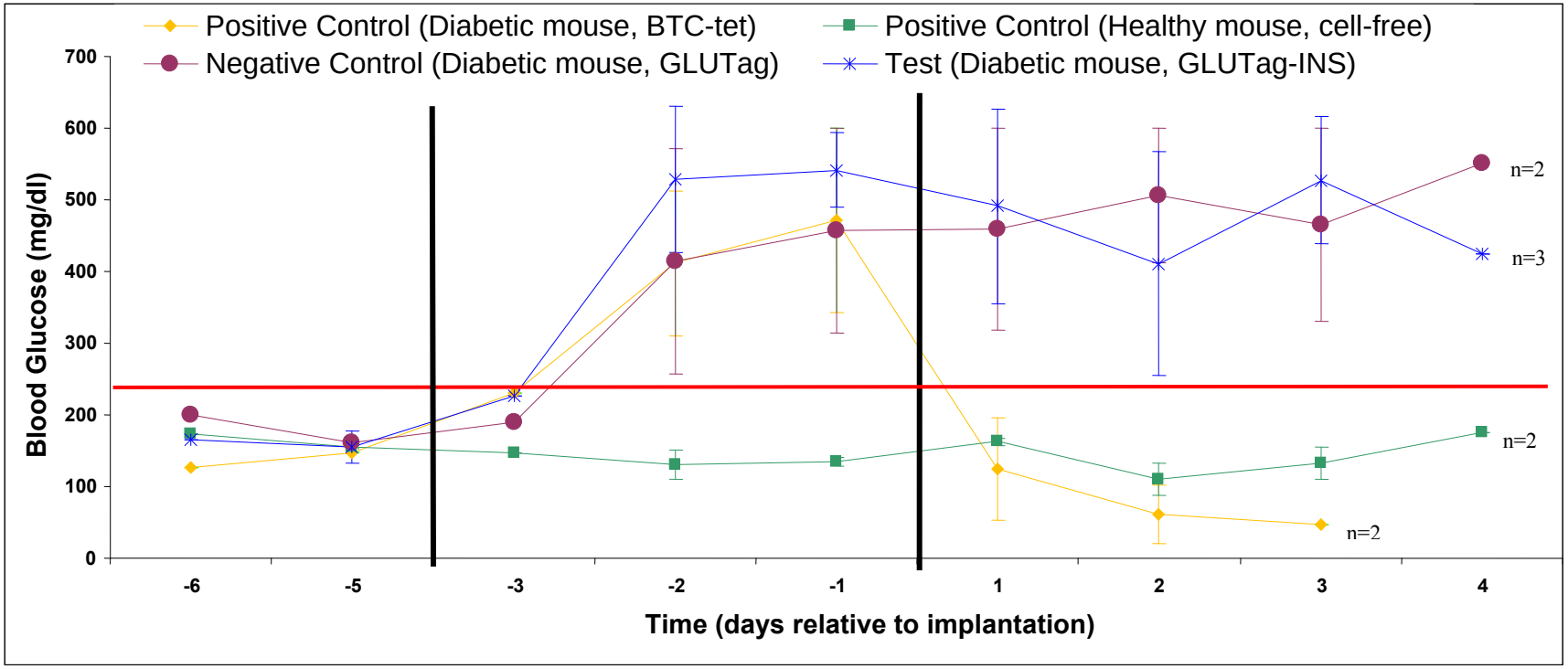


Figure 5.4 Blood glucose levels in mice implanted with microbead constructs. STZ was administered on day -4 to all groups but the healthy positive control and constructs were implanted on day 0. The condition of the mouse and the type of cells encapsulated within the alginate microbeads is indicated in parenthesis within the legend.

The assessments of implant function described in sections 5.4.2 to 5.4.8 were conducted only on mice receiving constructs of the disk geometry.

5.3.2 Plasma Insulin Levels

Despite the lack of effect of insulin-expressing cell-containing TEPS of disk geometry on body weight or blood glucose levels, human insulin was detected (using the human ultrasensitive RIA kit) in the plasma of GLUTag-INS cell treated mice (5.1 ± 1.8 μU/ml, Figure 5.5 B). This level was significantly higher than the level detected in sham treated mice ($p<0.05$). The level measured from healthy mice in Figure 5.5 B is apparently due to the cross-reactivity of murine insulin with the human RIA. As this level is similar to the level detected in sham-treated mice, the cross-reactivity of murine insulin on the human ultrasensitive kit is quite low. It is also interesting to note that the mice treated with murine βTC-tet cells had significantly elevated plasma levels of murine insulin (detected using the rat RIA) compared to healthy mice and GLUTag- and sham-treated diabetic mice ($p<0.05$) (Figure 5.5A).

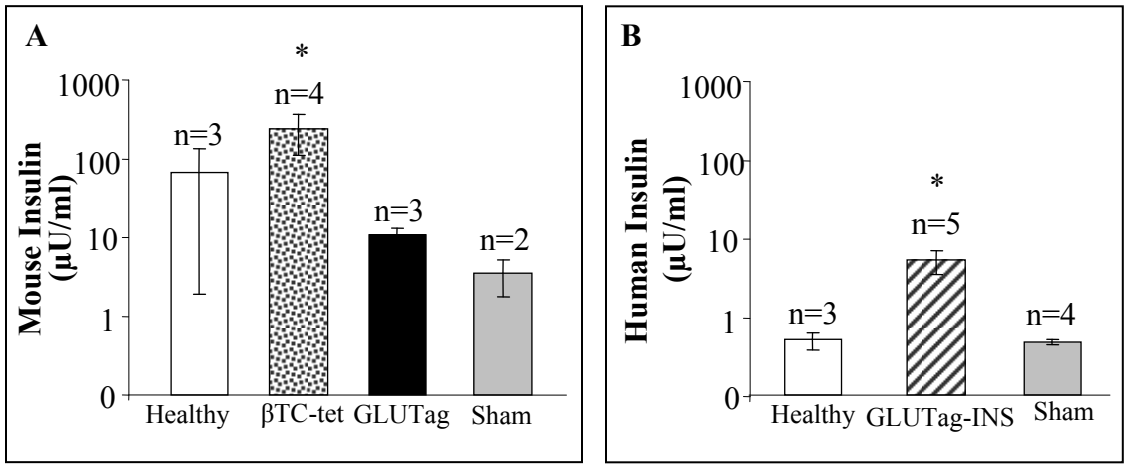


Figure 5.5 Blood plasma levels of murine (A) and human (B) insulin in mice implanted with disk-shaped constructs. The normal value of healthy mice detected with each kit is the far left bar (open) in each graph while the far right bar (grey) in each graph is the value detected for sham treated diabetic mice. Insulin concentration (μU/ml) is shown on a log scale. * Different than all other groups in graph (One-way ANOVA $p<0.05$).

5.3.3 Insulin Secretion from Implants

In order to quantify the insulin secretory function of the constructs both pre-implantation and post-explantation, constructs were incubated with complete culture medium overnight and the accumulated insulin was assayed. These values are compared in Table 5.2. While insulin was detected under both circumstances, the amount measured following explantation was markedly lower than the pre-implantation values for both GLUTag-INS and βTC-tet-containing constructs.

Table 5.2 Insulin Secretion from Implants Pre-implantation and Post-explantation.

	GLUTag-INS	**βTC-tet**
Pre-Implant	216.7 ± 13.6 μU/(construct·day)	65.2 ± 11.9 mU/construct/day
Post-explant	56.2 ± 7.9 μU/(construct·day)	2.0 ± 1.8 mU/construct/day
% of pre-implantation	25.9 ± 3.4%	2.8 ± 2.3%

5.3.4 Construct Retrieval and Inspection

Visual inspection of the constructs following retrieval after only four days of being implanted revealed many structural flaws in the encapsulating agarose layer as seen for a selection of the constructs in Figure 5.6. The breakages did not appear to be correlated to whether or not the constructs contained cells or were cell-free. The pink observed in some explanted constructs was the result of incubation in phenol red-containing culture medium and the incomplete removal of the dye after rinsing in PBS.

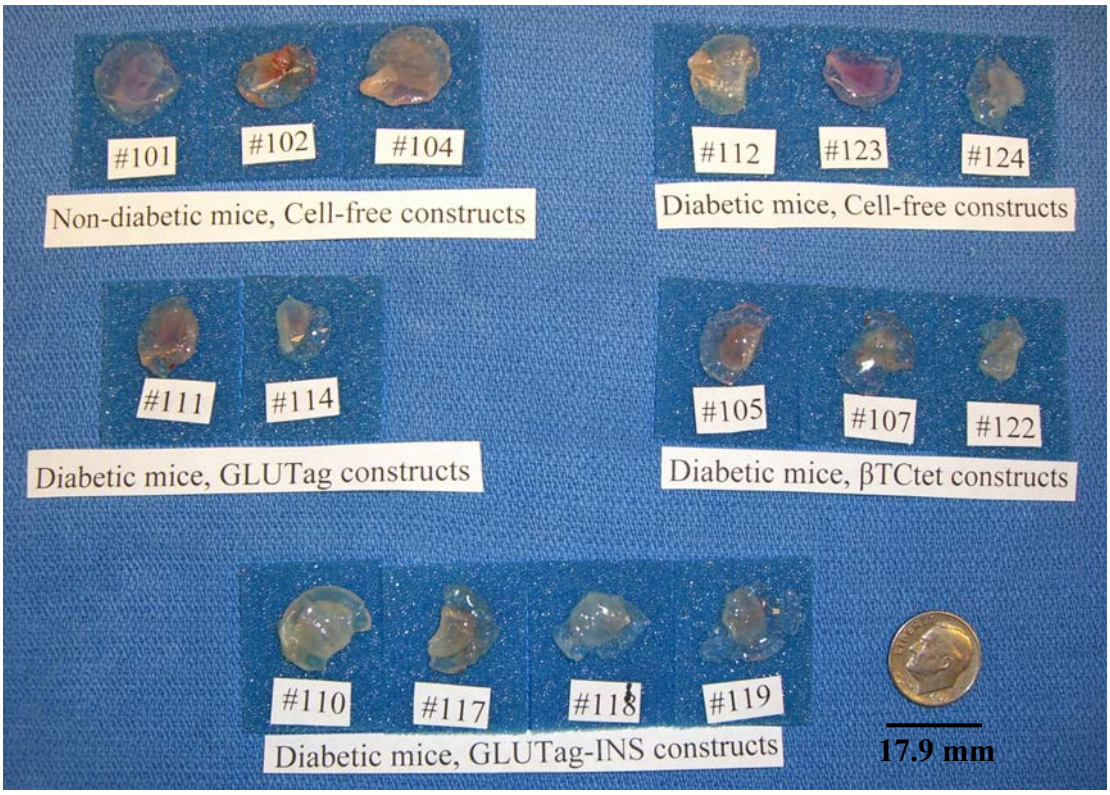


Figure 5.6 Appearance of disk-shaped constructs post-explantation.

5.3.5 Live/Dead Staining and Confocal Microscopy

Though the Live/Dead staining of retrieved βTC-tet-, GLUTag-, and GLUTag-INS-containing constructs (Figures 5.7.C -E) revealed large amounts of green fluorescence, which was not present in non-implanted cell-free SIS (Figure 5.7.A), a similarly implanted cell-free construct (Figure 5.7.B) also displayed this green staining. Thus the viable cells detected with this method could very easily have originated from the host and implanted cells are not distinguishable from host cells using this assay.

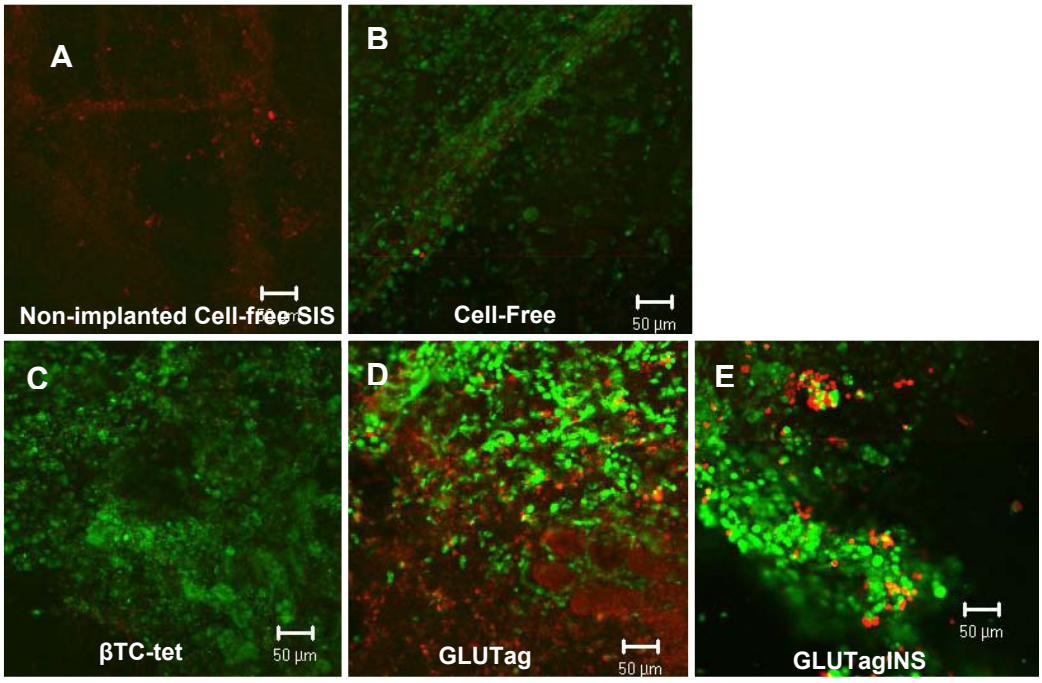


Figure 5.7 Confocal images of Live/Dead stained SIS removed from implanted disk-shaped constructs.

5.3.6 Immunohistochemistry of Constructs for Host Immune Response

The influence of the host's immune response toward the implants was qualitatively investigated using immunohistochemistry. Antibody staining against macrophages (Figure 5.8) and neutrophils (Figure 5.9) revealed that a strong reaction occurred against GLUTag-INS-containing constructs and to a lesser degree cell-free constructs. No staining was observed for non-implanted negative controls.

Because neutrophil staining was positive after four days of implantation, thus indicating a chronic inflammation, samples of non-implanted constructs and agarose were evaluated for endotoxin content using the QCL-1000 Endpoint Chromogenic LAL Assay (Lonza). All samples containing agarose, even in the absence of SIS and cells, were found to have nearly 0.6 EU/ml, which exceeded the FDA cut-off of 0.5 EU/ml.

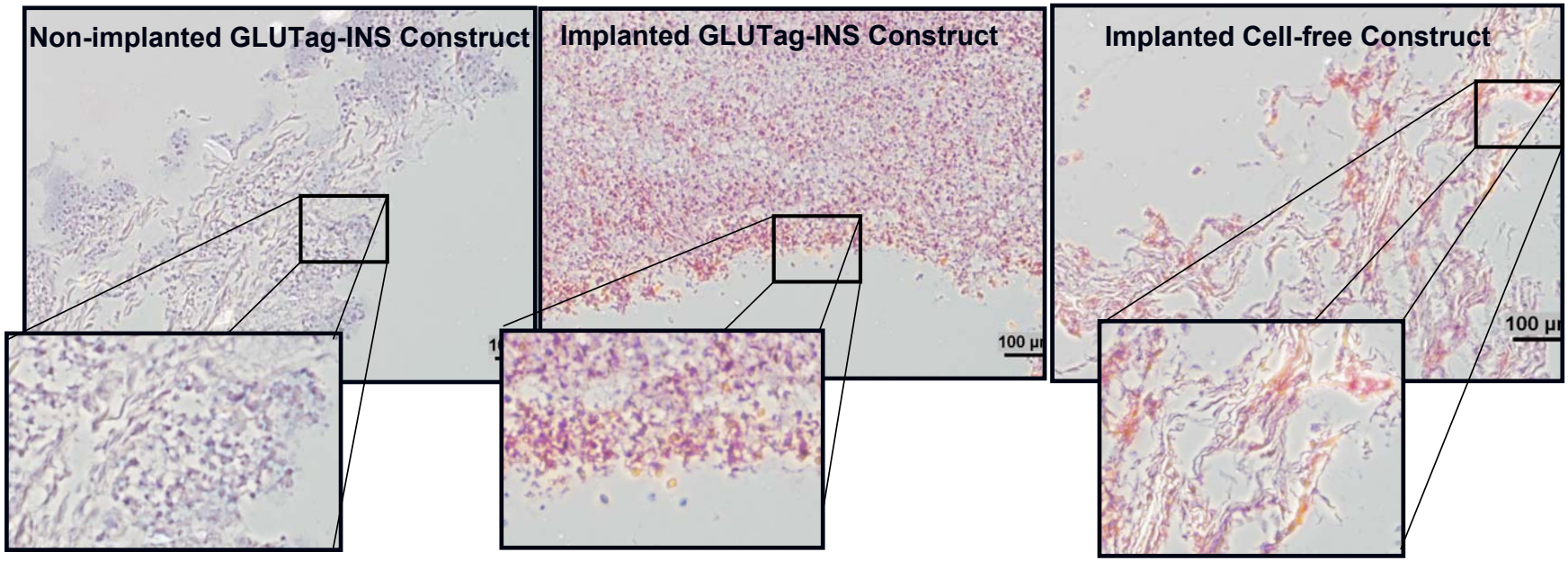


Figure 5.8 Macrophage staining of constructs using a CD68 antibody. Antibody revealed with ABC-Alkaline phosphatase and Vector Red substrate and counterstained with hematoxylin.

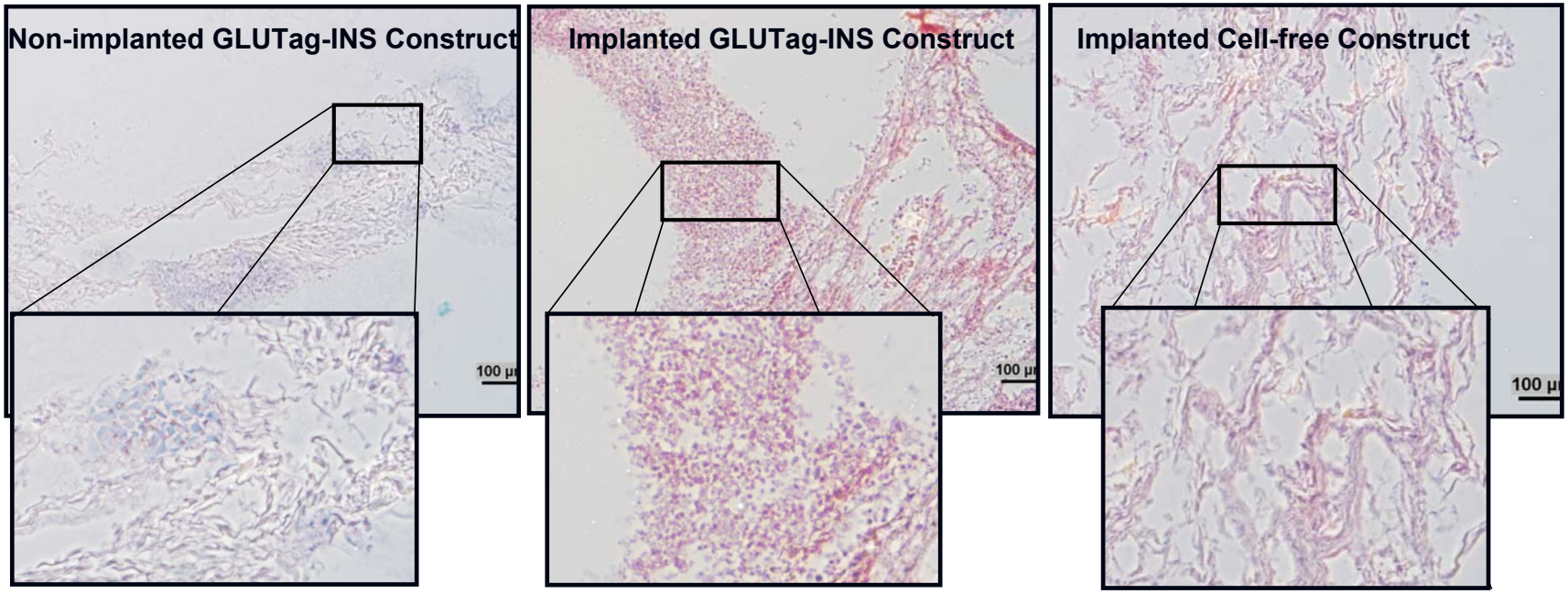


Figure 5.9 Neutrophil staining of constructs using a NIMP-R14 antibody. Antibody revealed with ABC-Alkaline phosphatase and Vector Red substrate and counterstained with hematoxylin

5.3.7 Insulin Immunohistochemistry of Constructs

Insulin immunohistochemistry was attempted on non-implanted GLUTag-INS constructs (positive control), non-implanted cell-free constructs (negative control), and implanted GLUTag-INS and cell-free constructs (tests) (Figure 5.10). While weak staining was observed for non-implanted GLUTag-INS constructs and not on non-implanted cell-free constructs, more staining was seen unexpectedly on both implanted samples. In an attempt to get a sharper image of the insulin antibody staining by fluorescent detection, a different secondary antibody was used which was TRITC conjugated. Despite the change in the secondary antibody and detection system, the same results were obtained, indicating that the primary antibody used had unexpected cross-reactivity to an epitope of a host cell. As insulin expression from the constructs post-explantation had been demonstrated by secretion assay, staining was not repeated using another primary anti-insulin antibody.

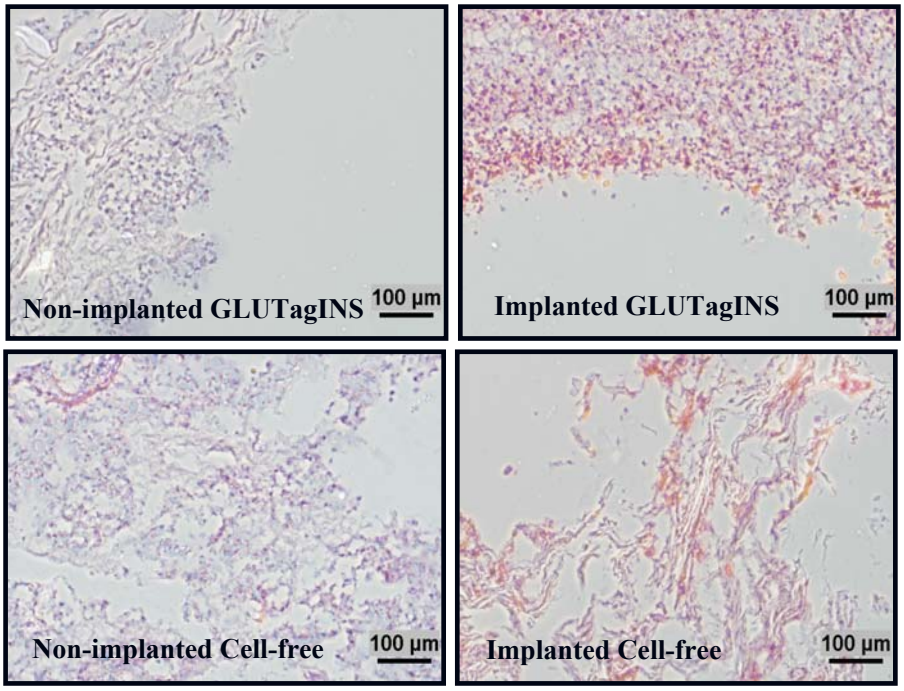


Figure 5.10 Insulin staining of constructs using a monoclonal insulin antibody. Antibody revealed with ABC-Alkaline phosphatase and Vector Red substrate and counterstained with hematoxylin.

5.3.8 Liver Glycogen Storage

Another measure of how the implants may have affected the mice's metabolism was the amount of glycogen stored within liver cells. Qualitatively examined here by PAS staining of formalin-fixed livers, it was seen that healthy, non-diabetic mice showed the greatest amount of stored glycogen (Figure 5.11 A). Sham treated diabetic mice and diabetic mice treated with GLUTag-INS cells showed similar levels of glycogen storage (Figure 5.11 B and D). A slightly lower level of staining was noted for GLUTag cell-treated mice (Figure 5.11 E). Though βTC-tet-treated mice (Figure 5.11 C) showed more staining than the other diabetic groups, it was nonetheless lower than the amount seen in healthy mice. As with the staining seen in healthy controls, localized deep staining was present in many cells of the βTC-tet-group, a trait not evident in the other groups.

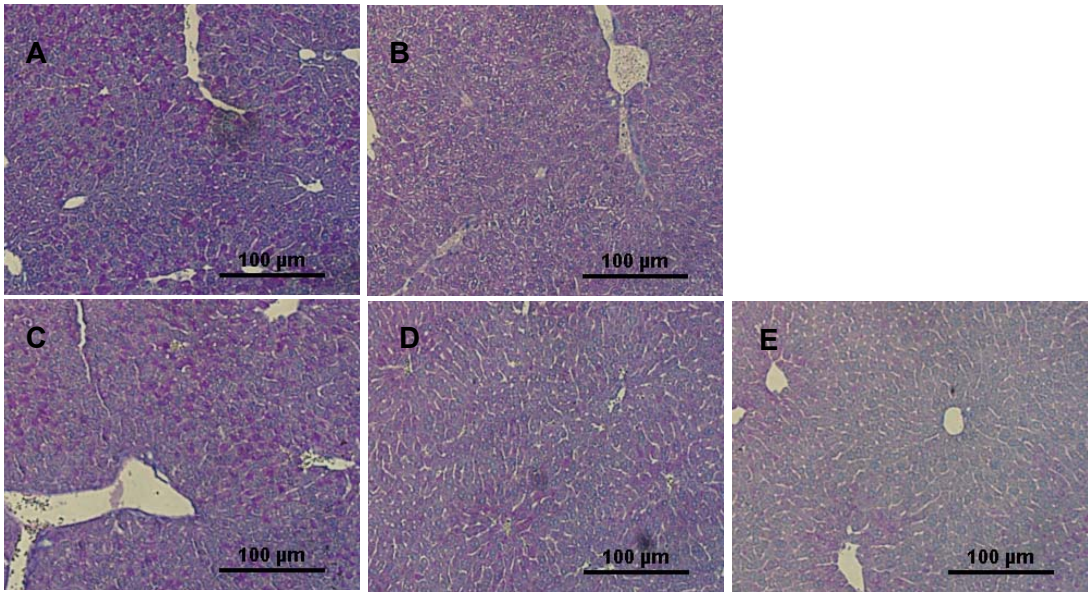


Figure 5.11 Liver glycogen storage of mice implanted with disk-shaped constructs. Sections, 5 µm thick, of formalin-fixed paraffin-embedded livers were stained by PAS and counterstained with hematoxylin. Magenta staining indicates stored glycogen. Liver sections were obtained from healthy mice (A), sham-treated diabetic mice (B), and diabetic mice treated with βTC-tet cells (C), GLUTag-INS cells (D), or GLUTag cells (E).

5.4 Discussion

The implantation of the GLUTag-INS cell-containing disk-shaped SIS constructs was not predicted to restore normoglycemia due to the subtherapeutic level of insulin secretion measured from these constructs, as discussed in CHAPTER 4. A significantly higher dose of insulin was measured from the disk-shaped SIS TEPS containing βTC-tet insulinomas in vitro, though it was only about 30% of a therapeutic dose. If that level of insulin secretion was maintained in vivo it may have affected the blood glucose levels of diabetic mice, though restoration of normoglycemia would be unlikely with only 30% of the therapeutic dose. Indeed, neither GLUTag-INS- nor βTC-tet-containing disk-shaped constructs lowered blood glucose levels to normoglycemic levels, though a small drop in blood glucose was noted for the βTC-tet constructs on day 2. TEPS of the microbead geometry containing βTC-tet cells, however, have been shown in several previous studies to correct hyperglycemia of diabetic mice (Black, Constantinidis et al. 2006), and it is no surprise that we saw a quick reversion to normoglycemia following implantation of βTC-tet microbeads and, in the absence of growth control by tetracycline, the subsequent decline of blood glucose to hypoglycemic levels. The volume of microbeads containing GLUTag-INS cells implanted in this study, however, was not sufficient to restore normoglycemia. Though more beads could have been implanted, the volume predicted to be necessary to treat diabetes in a mouse (12.4 ml), is quite large. To avoid this large implantation volume, an increase in cell density and/or further improvements in the insulin expression level of L-cells would be required.

Retrieved implants retained some insulin secretion capacity following the four day implantation, but the magnitude of the decline in insulin secretory function for the agarose/SIS constructs was surprising. GLUTag-INS and βTC-tet containing constructs retained just 25.9% and 2.8% respectively of their pre-implantation insulin secretion levels. There are several possible contributing factors for this rapid decline: (1) breakage

of the encapsulating agarose layer around the SIS, (2) immune response to the exposed cells and/or agarose, and (3) diffusion-limited supply of oxygen and nutrients.

The structural integrity of the SIS disk constructs following a relatively short time in vivo was significantly less than predicted by previous studies implanting agarose only disks of similar dimensions and composition (Stabler, Long et al. 2005). Though structural failure may have contributed to the rapid decline in insulin secretory function from the disk constructs, it cannot be solely blamed, as an SIS-free agarose construct containing GLUTag-INS cells, which did not experience any structural damage following four days of implantation, saw a similar drop in insulin secretion as the broken SIS-containing controls.

Leukocytes such as neutrophils and macrophages bind to implanted tissue engineered devices through surface-absorbed opsonins to result in attachment and activation, secretion of reactive oxygen intermediates, release of proteolytic enzymes and phagocytosis of the implant (Babensee, Anderson et al. 1998). Following any surgical procedure, it would be expected that some macrophages and neutrophils would be recruited to the site. The degree to which the excised agarose/SIS TEPS stained positive for macrophages and neutrophils four days after implantation, however was quite significant. Though CD68 is classically used as a macrophage marker, though there is evidence that other cells, such as fibroblasts may also express CD68 (Gottfried, Kunz-Schughart et al. 2008). Regardless of the specific origin of the CD68 staining, since it was not present in the non-implanted controls, it is still indicative of a host reaction. Though breakage of the agarose and exposure of the implanted cells directly to the host's immune cells probably exacerbated the immune reaction, it is likely that similar results would have been obtained regardless of breakage, as the endotoxin test of the agarose tested positive (>0.5 EU/ml). Indeed, the implanted cell-free constructs displayed an inflammation response. Neutrophils are the hallmark of acute inflammation, serving to remove foreign materials and bacteria from the injury site. They normally occur within

an hour of injury and with a short lifespan of about 24 hours give way to macrophages and eventually fibroblasts. If the inflammatory stimulus persists, however, chronic inflammation occurs (Babensee, Anderson et al. 1998). Given that the construct stained positive for neutrophils as far out as four days indicates a chronic inflammatory reaction. This inflammatory response certainly could have been detrimental to the cells contained within the TEPS, as non-specific lysis of encapsulated cells may be mediated by lysosomal enzymes or reactive oxygen or nitrogen intermediates released by activated leukocytes.

Other factors may have also had an impact on in vivo function, particularly a decrease in available oxygen and nutrients. Intraperitoneal dissolved oxygen concentration drops considerably to 0.06 mM compared to the level of 0.20 mM experienced under normal incubator conditions (Gross, Constantinidis et al. 2007). A drop to this level may certainly have an effect on insulin secretory function, as has been observed in insulinoma cells (Papas, Long et al. 1996).

Though normoglycemia was not achieved by implantation of the disk constructs, human insulin was measurable in GLUTag-INS-implanted mice, and mouse insulin levels were elevated for all cell-treated mice, relative to sham-treated diabetic mice. For the mice implanted with constructs containing either βTC-tet or GLUTag-INS cells, a straight-forward explanation is available for the presence of murine or human insulin, respectively, in serum samples, as these constructs demonstrated insulin secretion both before implantation and after retrieval. Interestingly, though the plasma insulin detected in diabetic mice implanted with βTC-tet-containing constructs was significantly higher than the plasma insulin of healthy controls, blood glucose concentrations were not returned to normal values. A possible explanation for this rests with the physiological changes that take place during extended periods of hyperglycemia, chiefly the increase in peripheral insulin resistance. It has been reported that in STZ-induced diabetes, hyprerglycemia leads to progressive insulin resistance of the peripheral tissue (Ordonez,

Moreno et al. 2007). Some of the changes that occur following hyperglycemia are an impairment of GLUT4 translocation in skeletal muscle (Dombrowski, Roy et al. 1998) and an inhibition in glycogen synthase in adipocytes (Parker, Taylor et al. 2004). Thus, glycemic regulation is not a state function. Whereas one level of insulin results in a certain blood glucose level prior to the onset of diabetes, a different amount of insulin may be required to achieve the same blood glucose level after the onset of diabetes. This means that curing diabetes by a cell substitute is further complicated by the path that glycemic regulation has taken. Therefore in the study with transgenic mice which produced insulin from intestinal K cells (Cheung, Dayanandan et al. 2000), the amount of insulin needed to avoid the onset of diabetes might have been significantly lower than the level that would have been needed if the same animals had experienced hyperglycemia and developed peripheral insulin resistance.

The reason for elevated serum murine insulin in the GLUTag cell-treated mice relative to sham-treated negative controls may be due to the delivery of GLP-1 from the construct which is known to potentiate the secretion of insulin from β-cells in the presence of glucose (Drucker 2001). The known actions of GLP-1 also include restoring glucose competence in glucose resistant β-cells, stimulating insulin gene expression and biosynthesis, and to acting as a growth factor by promoting cell proliferation, survival, and neogenesis (Buteau 2008). Since this is a model for IDD and the β-cells were specifically ablated by STZ, the precise action of GLP-1 is a little more complicated. It is possible that (1) some percentage of pancreatic β-cells evaded harm from STZ administration and GLP-1 served to potentiate insulin secretion, (2) damaged (but not destroyed) β-cells were able to recover some function following GLP-1 action, (3) GLP-1 worked to increase β-cell mass by regeneration, or (4) a combination of these mechanisms was at work in mice implanted with GLP-1-secreting cells. Indeed, GLP-1 has been shown to attenuate the destruction of β-cells by STZ (Li, Hansotia et al. 2003; Pospisilik, Martin et al. 2003).

Since the constructs were implanted intraperitoneally, and the insulin secreted by them would be picked up by the vasculature of the mesentery, which empties into the portal system, direct effects on the liver may have been observed even in the absence of correction of blood glucose levels. In healthy subjects, the liver is a major consumer of secreted insulin, with 50% of insulin (which empties directly from the pancreas into the portal vein of the liver) being used by the liver during the first pass (McPhee and Ganong 2006). Insulin's effect in the liver is primarily to promote fuel storage by way of stimulating glycogen synthesis and inhibiting gluconeogenesis and glycogenolysis.

Liver glycogen content for GLUTag-INS-treated mice and sham-treated diabetic controls was similar, but GLUTag-treated mice appeared to have less glycogen storage than the aforementioned groups. This was unexpected as we did see some elevation in plasma insulin in response to GLUTag-cell implantation, presumably due to GLP-1 action, but may be explained because GLUTag cells process proglucagon to release glucagon as well as GLP-1 (Drucker, Lee et al. 1992). Glucagon and insulin are antagonistic hormones, with glucagon promoting glycogenolysis and glucose production from the liver. We did observe in CHAPTER 3 that the level of GLP-1 secreted from GLUTag-INS cells is about 10-fold lower than that secreted from parental GLUTag cells. Since GLP-1 and glucagon are derived from the same prohormone, it is likely that glucagon would also be reduced in recombinant GLUTag-INS cells compared to the parental GLUTag cell line, though glucagon secretion from GLUTag-INS cells has not been characterized. Thus, the relative levels of glucagon secretion from GLUTag-INS cells compared to GLUTag cells, combined with the secretion of insulin by GLUTag-INS cells, may explain why a drop in glycogen storage was observed in GLUTag-implanted mice relative to GLUTag-INS-implanted mice.

Though not entirely comparable, βTC-tet-treated mice displayed liver glycogen staining much like healthy controls with localized deep staining present in many cells, a trait not evident in the other groups. This is to be expected given the relatively high level

of murine plasma insulin detected in βTC-tet-treated mice. Though the differences observed in liver glycogen storage among mice of various groups are interesting, the results would be much more convincing if a quantitative liver glycogen assay had been performed rather than the qualitative PAS staining and had the livers not been formalin-fixed, this sort of a quantitative assay would have been conducted. The repeatability of these results among all mice of each group also remains to be examined.

In summary, even though reversion of hyperglycemia was not expected or achieved from the agarose/SIS TEPS containing GLUTag-INS cells, a great deal about the in vivo performance of this type of construct was still obtained during this study. Though insulin secretory function of the constructs was diminished to a fraction of the pre-implantation value, insulin was secreted from constructs containing GLUTag-INS and βTC-tet cells in vivo and was detectible in the plasma samples taken four days after implantation. Intraperitoneal implantation of the agarose/SIS constructs exposed the predisposition towards rupture at the agarose-SIS interface. A significant inflammatory response was noted in the implanted constructs and was likely due to the presence of endotoxin from the agarose. Slight qualitative differences in the liver glycogen storage were noted for experimental groups of agarose/SIS TEPS treated mice, but these results should be further characterized with a quantitative assay for liver glycogen.

CHAPTER 6

CONCLUSIONS AND FUTURE DIRECTIONS

6.1 Conclusions

Though considered a treatable chronic disease, insulin-dependent diabetes (IDD) remains a serious public health issue. Because of patient compliance and the delay inherent to sampling blood glucose and administering insulin, exogenous insulin injections cannot provide the tight physiological control of blood glucose necessary to prevent the onset of secondary complications. Cell-based treatments which sense changes in surrounding blood glucose concentration and respond rapidly to secrete insulin in a physiological manner potentially represent a cure for IDD. The use of recombinant β-cell surrogates as a cell-based treatment for IDD has been investigated for several years, but many of the potentially autologous cell sources have significant drawbacks. Enteroendocrine cells have recently emerged as very promising potential surrogate β-cells. However, translating enteroendocrine cell therapy for IDD to a viable therapy for human use is not without its challenges. In this thesis, we have contributed to the field by studying the insulin secretory function of recombinant L-cells and evaluating their potential for use in an implantable tissue engineered pancreatic substitute both in vitro and in vivo.

To accomplish the goals set forth in this thesis, three aims were planned which progressed from the engineering L-cells at the cellular level, to then evaluating the performance of the cells within three-dimensional tissue engineered constructs in vitro, and finally determining their performance upon implantation into a diabetic mouse model.

The results of the first aim, discussed in CHAPTER 3 describe how we modified the murine GLUTag cell line by stable transfection with an expression plasmid which coded for human B10-mutated insulin driven from the CMV promoter and for neomycin resistance from the SV40 promoter. Following selection through the antibiotic resistance, we established a clonal cell line, called GLUTag-INS. This line was then characterized for insulin and GLP-1 secretion under normal culture conditions using both basal and inducing medium conditions. It was found that GLUTag-INS cells did co-secrete human insulin and murine GLP-1 in response to both putative and nutrient cues. Importantly, GLUTag-INS cells responded quickly to the step up in nutrient concentration by action of the regulated secretion pathway, without the delay found when relying on transcriptional regulation, as is necessary in non-endocrine cell types. Proinsulin was also efficiently converted to mature insulin through the action of the endogenous endoproteases PC1/3 and PC2 (Bara and Sambanis 2008).

The second aim of this thesis described in CHAPTER 4, evaluated three potential configurations of an implantable agarose disk construct in terms of their ability to support cellular growth and metabolic and secretory functions of the encapsulated GLUTag-INS cells. The three constructs evaluated in this work were agarose constructs which housed either a single cell suspension of GLUTag-INS cells, a suspension of pre-aggregated GLUTag-INS spheroids, or GLUTag-INS cells on SIS. It was learned that despite similarities in overall cellular growth and basal insulin secretion and glucose consumption rates, SIS-containing TEPS performed better than single cell or spheroid-containing TEPS in terms of induced insulin secretory function. This key aspect to regulated insulin secretion was not evident in the two constructs which did not contain the SIS substrate (Bara and Sambanis 2008, submitted to Biotechnology and Bioengineering).

Finally, in the third aim of this work, SIS-containing agarose constructs and alginate microbead constructs were implanted into the peritoneal cavity of STZ-diabetic

mice revealed interesting findings and uncovered several short-comings of the TEPS. As the developed TEPS based on agarose-encapsulated SIS, did not secrete a sufficient level of insulin to treat a diabetic mouse, blood glucose lowering and weight gain were not observed in GLUTag-INS treated mice. Likewise, GLUTag-INS-containing alginate beads did not display a blood glucose lowering effect. The volume of beads implanted in these preliminary experiments was only 1 ml whereas the volume predicted to restore normoglycemia is 12.4 ml. It was seen that the SIS-containing agarose was much more structurally unstable than predicted based on previous implantation of agarose only constructs of similar dimensions. Breakage of the encapsulating agarose layer, however was just one of the factors identified that may have played a role in the decline of insulin secretory function measured from the constructs post-explantation compared to the pre-implantation level. Other culprits may have included limitations in oxygen and other nutrients to cells housed in the constructs and an inflammatory response that included both macrophages and neutrophils. Importantly though, we were able to detect human insulin in the plasma samples of GLUTag-INS treated mice (which was not detected in sham-treated controls or healthy, non-diabetic mice) even after four days of implantation, when insulin secretory function of the TEPS was already quite low relative to initial levels. Qualitative liver glycogen storage images also suggested some benefit in metabolic health of the GLUTag-INS and especially the βTC-tet treated mice despite the subtherapeutic level of insulin delivered.

In summary, this thesis took the developed a tissue engineered pancreatic substitute based on recombinant intestinal L-cells from engineering and characterization of the L-cells themselves, to in vitro characterization, and carried it all the way through to in vivo testing.

6.2 Future Directions

6.2.1 Increasing Insulin Expression

In order to reach therapeutic levels of insulin secretion from a TEPS based on recombinant L-cells, the specific cellular insulin secretion rate and/or the total number of viable cells delivered in vivo would need to be improved. Increasing insulin expression from GLUTag-INS cells by additional gene delivery is one possibility (Clark, Quaade et al. 1997). As unforeseen changes may occur during additional rounds of stable clone selection, using another stable selection method is probably not the best option. This may have been why insulin-producing STC-1 K cells (Ramshur, Rull et al. 2002), which underwent two sequential rounds of stable clone selection, exhibited considerably different cell physiology than was expected based on the behavior of non-engineered cells in vivo. To avoid this complication, viral methods of gene delivery, such as lentiviral or retroviral vectors which provide stable gene expression, may be employed to facilitate additional gene expression in GLUTag-INS cells. Alternatively, another engineered L-cell source could be generated de novo starting with parental GLUTag cells, an L-cell enriched population of STC-1 cells, or even human NCI-H716 cells using the same viral vectors just mentioned.

6.2.2 Increasing Cell Loading of TEPS

Another option for increasing the level of insulin secretion from a TEPS based on recombinant L-cells is to deliver more of the GLUTag-INS cells and to improve the expected viability of the cells following implantation. With the tissue engineering approach, the number of cells that may be implanted is more limited due to space restrictions in vivo and the contribution of the biomaterial. Many preliminary studies in the area of cell-transplantation for IDD can bypass this limitation by implantation of cells alone, usually subrenally or subcutaneously, so as to localize the implanted cell mass.

Indeed, this was the approach used by Zhang et al. to evaluate recombinant K cells (STC-1-14) in vivo (Zhang, Yao et al. 2008). Though the initial amount of insulin secreted by the implanted cells, 78.6 μU/(10^6 cells · day), was not sufficient to reverse hypoglycemia, the cell mass expanded in vivo and 24 days after implantation, the mice were able to restore blood glucose levels to normal values. As the GLUTag-INS cells developed in this thesis secreted more insulin than STC-1-14 cells, it is reasonable to believe that a similar study using these cells would also demonstrate the ability to treat an IDD mouse model.

Many benefits may be seen, however, by incorporating the recombinant L-cells into a tissue engineered device, as such scaling up of the macroconstruct containing hydrated sheets of SIS described in this thesis is not a viable option, considering the limits imposed by what are considered to be implantable dimensions, especially given the breakage obtained at the current dimensions. TEPS consisting of alginate or agarose microbeads can easily be scaled up while retaining very good mechanical properties. It may also be possible to incorporate specific adhesive ligands or extracellular matrix proteins into the basic alginate or agarose microbeads. Given the work presented in this thesis, addition of solubilized SIS to the microbeads may be beneficial in enhancing insulin secretory function of GLUTag-INS cells (Lakey, Woods et al. 2001). Alternatively individual adhesive ligands or extracellular matrix components could be incorporated in the alginate or agarose gels (Rowley, Madlambayan et al. 1999; Connelly, Garcia et al. 2007). We have not compared the function of GLUTag-INS cells on the gel preparations of SIS, however, and cannot assume that there is any specific ligand in the hydrated sheets of SIS that supports the function of GLUTag-INS cells. It may well be that general adhesion to a semi-rigid surface is the mechanism by which induced insulin secretion was improved in GLUTag-INS cells on SIS compared to suspensions in agarose, as an even stronger induction response was observed on non-coated tissue culture plastic.

6.2.3 Genetic Engineering of Primary L-cells

A very important direction that this line of research should one day explore is the genetic engineering of intestinal stem cells which give rise to primary L-cells. Recently, the discovery of the intestinal stem cell marker, Lgr5, a transmembrane protein with a large extracellular domain for ligand binding, has opened doors to a number of important studies that were not possible just a couple of years ago (Barker, van Es et al. 2007). With the Lgr5 marker now available, it will be possible to visualize, isolate, and genetically modify stem cells of the adult intestine.

Obviously, a significant challenge to overcome remains the ability to establish primary cultures of murine L-cells, which are well defined and have good viability in vitro. As epithelial cells may require the presence of mesenchymal cells to survive and proliferate in vitro (Sanderson, Ezzell et al. 1996), isolation of stem cell/ mesenchymal cell aggregates, termed organoids rather than individual stem cells, may facilitate successful in vitro culture and successful transplantation (Choi and Vacanti 1997).

A likely implantation site for recombinant intestinal stem cells is back to the intestine itself and an interesting model has been studied as a site for heterotopic auxiliary liver is the isolated vascularized small intestinal segment (Berishvili, Liponava et al. 2003; Joseph, Berishvili et al. 2004). In this model, a segment of the small intestine along with its vascular supply is removed from the gastrointestinal tract, the ends of the intestine from which it was cut are anastomosed to restore flow of the intestine, and the isolated segment is denuded to remove the mucosa and the ends are ligated shut to create a sac. This model has recently been used by Avansino et al. in rats (Avansino, Chen et al. 2006) and mice (Avansino, Chen et al. 2006) to study the transplantation of intestinal organoids containing stem cells in vivo. It would be very interesting to see if this technique could be used to transplant recombinant intestinal stem cells, which drive the expression of insulin from the L-cell specific proglucagon promoter, as in vivo transplantation may facilitate longer study than in vitro culture.

In vivo engineering of the murine intestinal epithelium is another potential direction. A segment of the intestine with the mucosa intact, could be targeted by either the isolated vascularized segment just described or simply by temporary clamping of the segment (Sandberg, Lau et al. 1994). Then, a gene vector capable of chromosomal integration would be delivered to drive the expression of human insulin from the proglucagon promoter. Genetic modification of intestinal stem cells has also been proposed by use of an inducible Cre recombinase, integrated into the Lgr5 locus, such that Cre knock-ins can be directly inserted into intestinal stem cells (van der Flier and Clevers 2008) so long as the inserted gene did not interfere with the critical properties of the stem cells.

This is an exciting era in the advancement of intestinal stem cell gene therapy for the treatment of a number of disorders and diseases. Though much remains to be learned prior to human application, the elegance of using enteroendocrine cells for developing a potentially autologous cell therapy for IDD will certainly drive the progress in this area.

APPENDIX A

PRIMARY COLONIC CRYPT CULTURES

A.1 Introduction

Evaluating the efficiency of gene transfer to primary L-cells and more importantly to their progenitors—intestinal stem cells, would be a significant step toward the advancement of insulin gene therapy in somatic enteroendocrine cells. L-cells represent less than 1% of the population of intestinal epithelial cells; thus, obtaining a pure population of enteroendocrine L-cells would be very challenging. L-cells are primarily located at the base of structures called crypts of the distal small intestine (ileum) and the colon. The small intestine has numerous villi projecting into the lumen around the mouths of the crypts whereas the colon only has crypts. Colonic crypts are also larger than crypts found in the ileum and do not contain Paneth cells (Bach, Renehan et al. 2000; Kaeffer 2002); as such, only crypts from the colon were isolated. This Appendix describes the initial work toward a proof-of-concept experiment to isolate and transduce murine colonic crypts which are rich in intestinal stem cells. The studies described here evaluated the viability of various preparations of primary murine colonic crypts over the course of several days of in vitro culture.

A.2 Materials and Methods

A.2.1 Colonic Crypt Isolation

All procedures were conducted in accordance with the recommendations of the Georgia Tech Institutional Animals Care and Use Committee. Male 10-to-12-week-old BALB/c mice were obtained from Taconic Laboratories (Germantown, N.Y.) and sacrificed by CO_2 asphyxiation. Immediately after confirmation of death, a portion of the

colon was removed just distal to the illeal-cecal valve and a modification of the method described by Perreault and Beaulieu (Perreault and Beaulieu 1998) was initiated.

Complete culture medium for primary cultures was Dulbeco's Modified Eagle Medium supplemented with 2.5% FBS, 1% non-essential amino acids, 10 ng/ml Epithelial Growth Factor, 10 ng/ml Leukemia Inhibitor Factor, 100 U/ml penicillin, 100 µg/ml streptomycin, and 25 µg/ml gentamycin. The digestion solutions used were culture medium supplemented with (1) Matrisperse (a non-enzymatic solution used to depolymerize Matrigel Matrix) at a 50:50 ratio with complete medium or (2) 150 U/ml collagenase XIa and 40 µg/ml dispase A.

The intestine was flushed with cold Hanks' balanced salt solution (HBSS) to remove luminal contents followed by flushes containing 0.4 M N-acetyl-L-cysteine to remove mucous. The intestine was then cut open longitudinally and into 0.5-cm fragments. These were incubated with digestion solution at 22°C for solution (1) or 37°C for solution (2) on an orbital shaker platform for 3 hours or until crypts were seen to be released from the tissue. Upon completion of tissue digestion 5 ml additional culture medium was added and the centrifuge tubes were rapidly inverted 10 times to disperse the crypts. The suspension was allowed to settle for 1 minute and then 5 ml of the solution was collected by aspiration. This washing step was repeated five times for a total volume of crypt-containing media of 30 ml. This suspension was passed through a nylon mesh filter with a pore size of 180 µm to separate crypts from larger cell aggregates and then immediately plated on wells coated with either type I collagen or Matrigel. Crypts were separated from single cells by an additional sedimentation step in which the culture was incubated for 10 minutes and the supernatant was then replaced with fresh culture medium. A simplified schematic of the crypt isolation is shown in Figure A.1.

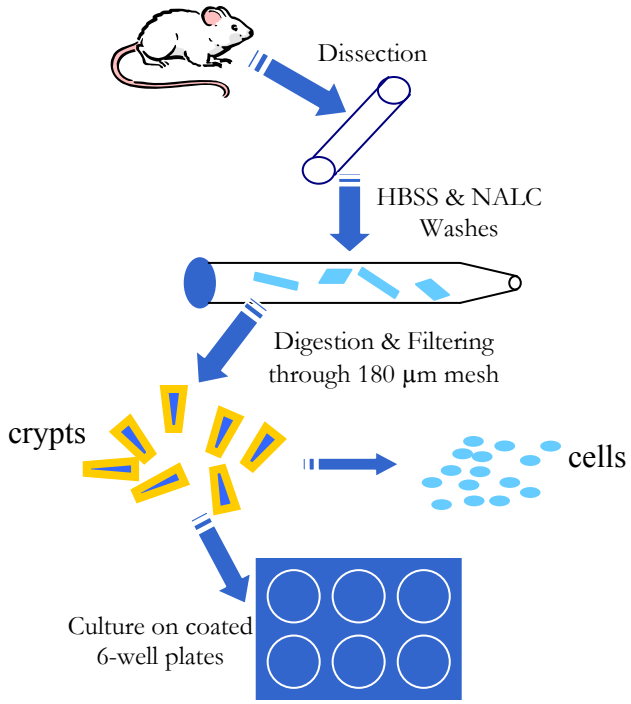


Figure A.1 Overall scheme of the isolation protocol used to obtain primary murine colonic crypts.

A.2.2 Viability Assays

Viability was assessed at various time points using the Trypan blue dye exclusion method and automated counting using the ViCell device or LIVE/DEAD staining as per the manufacturer's protocol and fluorescent confocal microscopy.

A.3 Results

Prior to digestion of the tissue, Live/Dead staining showed that the colonic tissue was highly viable without any appreciable red staining (Figure A.2). The dark areas of the images in the honeycomb-like lattice reveal where the crypts protrude or recede from the plane of view of the slice taken with confocal imaging.

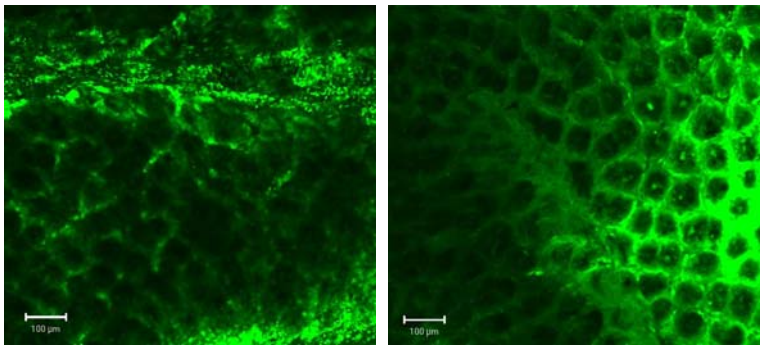


Figure A.2 Live/Dead stain of freshly isolated whole colonic tissue reveals highly viable intestinal tissue, with virtually no red staining detected using the multi-photon confocal microscope with dual channel settings for FITC and Rhodamine.

The protocol described for crypt isolation yielded cultures which were rich in cell clusters that had the structural characteristics of crypts, as seen in Figure A.3. The three dimensional cone shapes of the crypts are evident from the darker long edges of the clusters where many cells are stacked on one another to make the wall of the crypt perpendicular to the plane of the image.

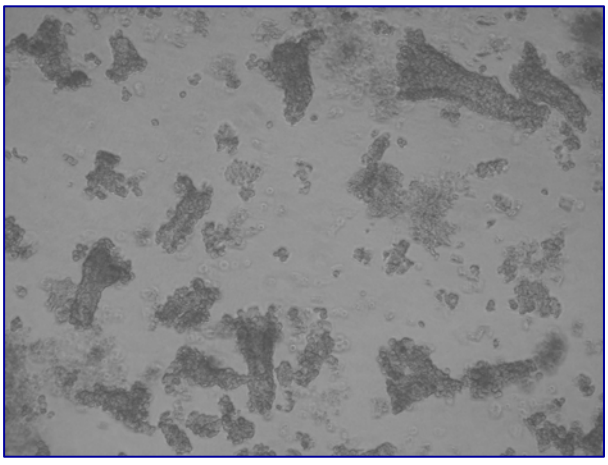


Figure A.3 Representative image of isolated murine colonic crypts isolated by Matrisperse digestion. The cone-shaped structure of the crypts is apparent in many of the cell clusters (10X).

Viability was also assessed on isolated crypt cultures by Live/Dead staining and is shown in Figure A.4. Although the Live/Dead staining performed just three hours after colonic crypt isolation revealed a mostly viable crypt structure (Figure A.4), by 24 hours after isolation, no viable staining was observed (not shown). Given this rapid decline, follow up studies were performed to compare the time course of viability of cultures isolated by the two different digestion solutions, or cultured under different conditions (surface coatings or medium compositions).

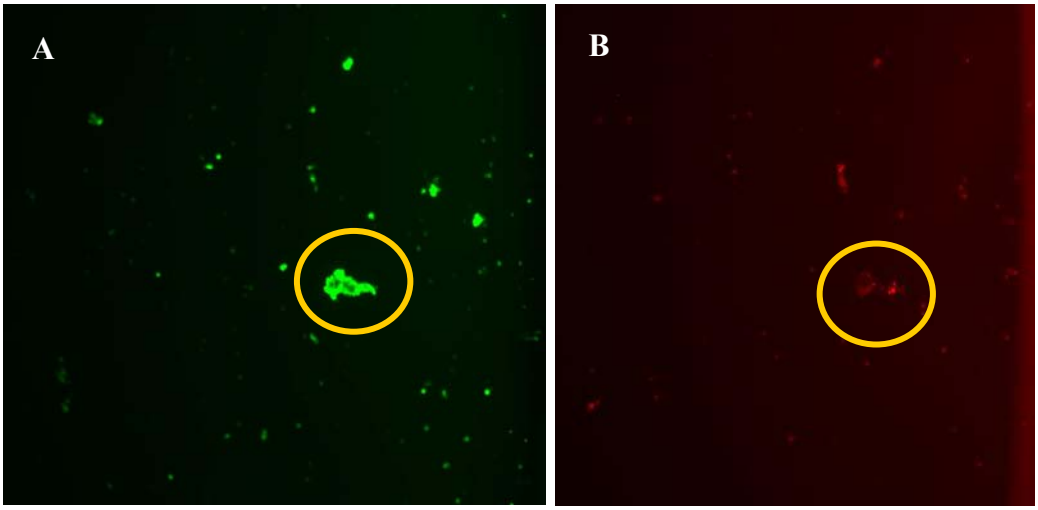


Figure A.4 Live/Dead staining of a colonic crypt cell preparation, plated on tissue culture plastic, three hours after the isolation (4X). Panels A and B were taken on the same area of interest, using different filter blocks of the fluorescent microscope for FITC and rhodamine respectively.

To quantify the difference in culture viabilities following digestion, dead cells were stained blue by Trypan blue and percent viability was determined using an automated counting device. Viabilities were assessed for two to three days, by which point all cultures were generally less than 30% viable. The effect of supplementing the culture medium with 2.5% FBS was also evaluated. Though the results presented here are very preliminary (n=1), some features are of note. With the exception of the isolation shown in Figure A.5, cultures generally retained good viability (greater than 60%) up to

24 hours after isolation. Beyond this point, however, the viability dropped steeply. In Figure A.5, crypts isolated by the non-enzymatic Matrisperse digestion solution and plated on Matrigel-coated wells, displayed very poor viabilities even at early time points. The presence or absence of FBS did not appear to influence the decline.

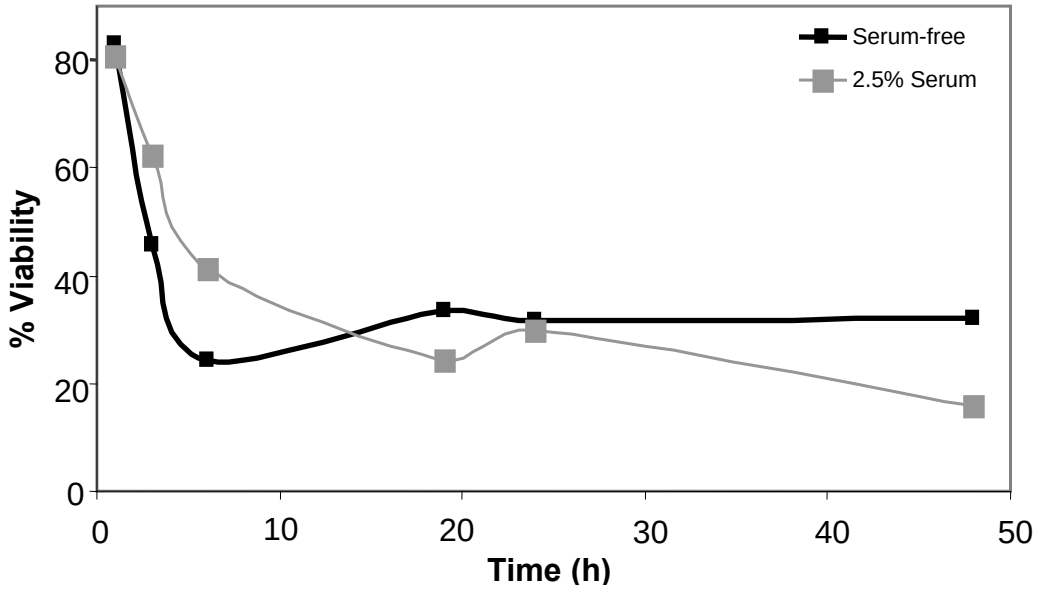


Figure A.5 Viability time course for crypts isoloated by the Matrisperse digestion solution and plated on Matrigel-coated wells. Cells were trypsinized and viability assessed by Trypan blue dye exclusion.

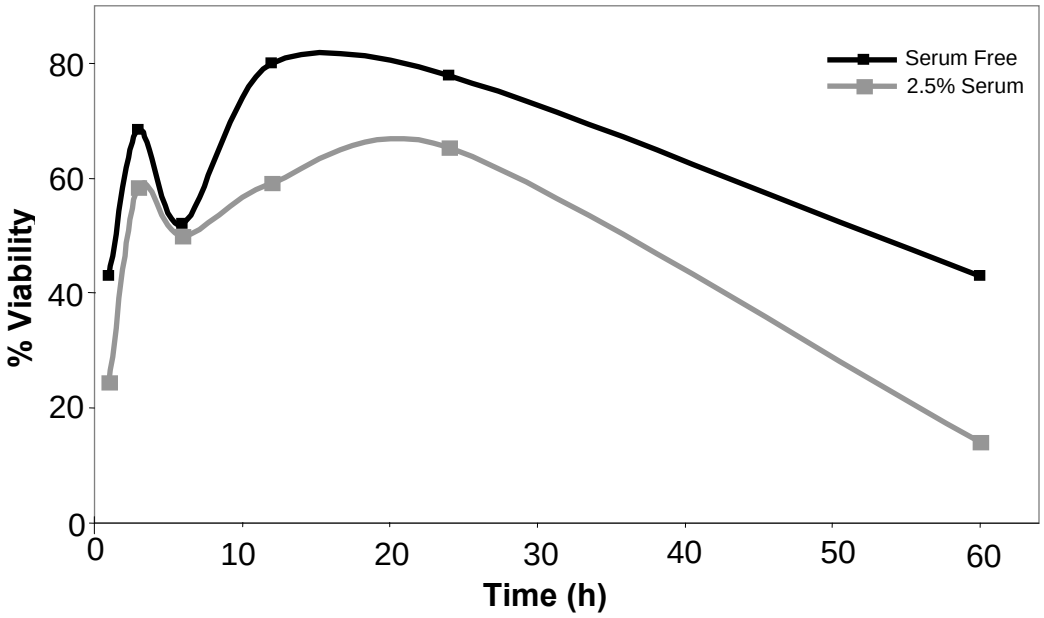


Figure A.6 Viability time course for crypts isolated by the collagenaese and dispase digestion solution and plated on Matrigel-coated wells. Cells were trypsinized and viability assessed by Trypan blue dye exclusion.

Significantly greater viabilities were seen for the cultures isolated by the enzymatic digestion solution containing collagenase and dispase (Figure A.6), though because this was a much more efficient solution at matrix disintegration than the Matrisperse solution, it is not clear if the viable cells are from the epithelium or from the underlying mucosa. Given this uncertainty, the more gentle Matrisperse digestion was the preferred method. In the case of the enzymatic digestion, however, it appeared that the presence of 2.5% serum was actually somewhat detrimental to the cells.

When comparing the viability of cultures obtained by Matrisperse digestion, crypt cells plated on collagen (Figure A.7) had appreciably higher viabilities than those plated on Matrigel (Figure A.5). The choice of extracellular matrix, however, does not lend any improvement to the viability beyond 24 hours.

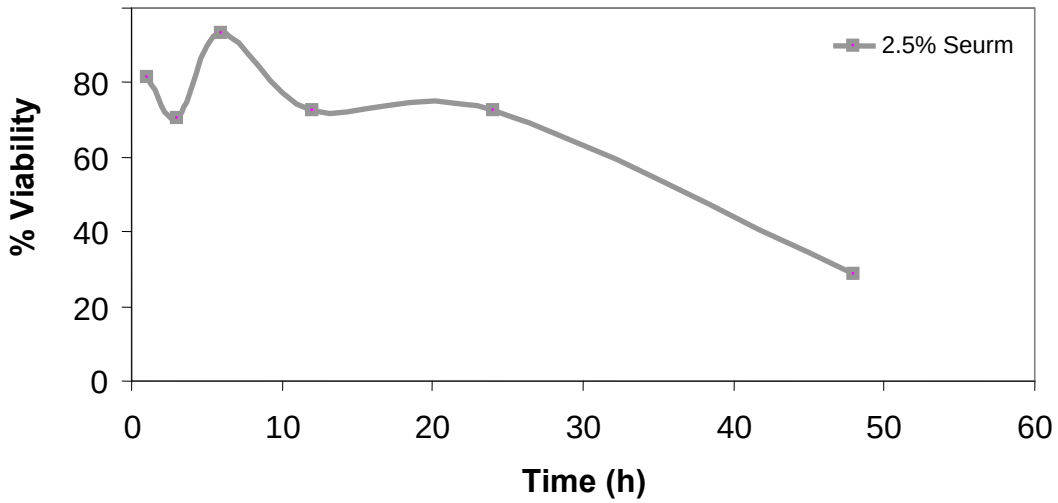


Figure A.7 Viability time course for crypts isolated by the Matrisperse digestion solution and plated on collagen-coated wells. Cells were trypsinized and viability assessed by Trypan blue dye exclusion.

A.4 Discussion

These preliminary experiments verified that organ cultures of adult intestinal tissue rapidly display necrosis and degeneration of the epithelium, with cultures in vitro being maintained for a maximum of twelve to twenty-four hours (Quaroni 1989; Kaeffer 2002).

The purity and/or viability of the cultures we obtained was not suitable for evaluating the possibility of gene transfer to the epithelium by viral transduction or chemical transfection, because of the long times necessary to visualize expression of the reporter protein. On a shorter time scale, however, it may be possible for one to evaluate binding of viruses to the cell surface of primary cultures, perhaps with co-localization of cell-specific antibodies to determine which cells are targeted by various viruses.

Factors that may have affected the viability of crypt cultures include (1) the length of procurement time; (2) the temperature at which isolation was performed; (3) the digestion solution used; (4) the coating used on the culture surface; and (5) culture medium. We attempted to improve upon a few of these factors, finding that the

Matrisperse digestion solution was better at obtaining cultures with greater content of crypts and that collagen-coated surfaces enhanced viability compared to Matrigel-coated surfaces. The results pertaining to culture medium supplementation with FBS, were unconvincing, and this supplement as well as a number of other possible supplements including growth factors should be further evaluated to determine an optimal maintenance medium. The isolation process could likely be improved by optimization of these factors.

Viability may also be superior when isolating fetal or neonatal tissue (Fukamachi 1992; Ramsanahie, Duxbury et al. 2003). Additionally leaving some of the surrounding mesenchymal tissue intact and isolating organoids may also significantly improve the longevity of the primary cultures (Sanderson, Ezzell et al. 1996).

APPENDIX B

GENE DELIVERY TO MURINE L-CELL LINES

Introduction

Previous work from our laboratory established methods to transiently transfect and preferentially transduce human NCI-H716 L-cells (Tang and Sambanis 2004), but in moving towards a murine L-cell line for developing a TEPS, initial studies needed to be performed on how to transfer genes of interest to the murine enteroendocrine GLUTag and STC-1 cell lines. As non-endocrine NIH-3T3 fibroblasts would be used as a negative control for the proglucagon promoter to be tested in APPENDIX C, these cells were also included in this study. In this study, the gene transferred to each cell type was the commonly used reporter gene, green fluorescent protein (GFP). Chemical transfections were performed using two commercially available reagents and transduction by an adenoviral vector was also performed.

Materials and Methods

Transfection of Cell Lines

GLUTag, STC-1, and NIH-3T3 cells were transfected with the chemical transfection reagents FugeneHD (Roche, Indianapolis, IN) and lipofectamine (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions, at varying concentrations of the transfection reagent in relation to amount of plasmid DNA. These protocols involved first the formation of a complex between the plasmid DNA and transfection reagent, then the dilution in serum-free medium, and delivery of the complex to cell monolayers that have been incubated with antibiotic-free medium overnight, to reduce the toxic effects of the antibiotic that may occur during the transfection procedure. The amount of plasmid DNA was held constant at 2 μg and the ratios of FugeneHD to DNA tested in this study

were: 3:2, 4:2, 5:2, 6:2, 7:2, and 8:2 (µl FugeneHD : µg DNA). For Lipofectamine the concentrations of reagent tested were 1X, 2X, 3X, and 4X.

Transduction of Cell Lines

Gene transfer for enteroendocrine cells was also evaluated by transduction with adenoviral vectors. The vectors used in this study, AdTrack and AdG3Fur/AdCMV-GFP were all generously supplied by the Thulé lab and contained expression cassettes for GFP. Transduction was performed simply by addition of the virus to the culture medium of the cell monolayers at a dose of viral particles to cells (multiplicity of infection, M.O.I.) of 1. Expression of the fluorescent protein was evaluated 24-48 hours after transduction by fluorescence microscopy using a GFP filter block.

Results and Discussion

Transfection of Cell Lines

As evident from the fluorescent micrographs (Figure B.1), FugeneHD was the better reagent for the transfection of the GLUTag cell line. The transfection efficiency was further characterized by flow cytometry and the 7:2 ratio yielded approximately 20% transfection efficiency. This was improved to 36% transfection by quadrupling the total amount of transfection complex used per well. STC-1 L-cells, however, were not transfected efficiently by either FugeneHD or Lipofectamine (Figure B.2). NIH-3T3 cells were transfected most efficiently with Lipofectamine with the 2x concentration of the complex (Figure B.3).

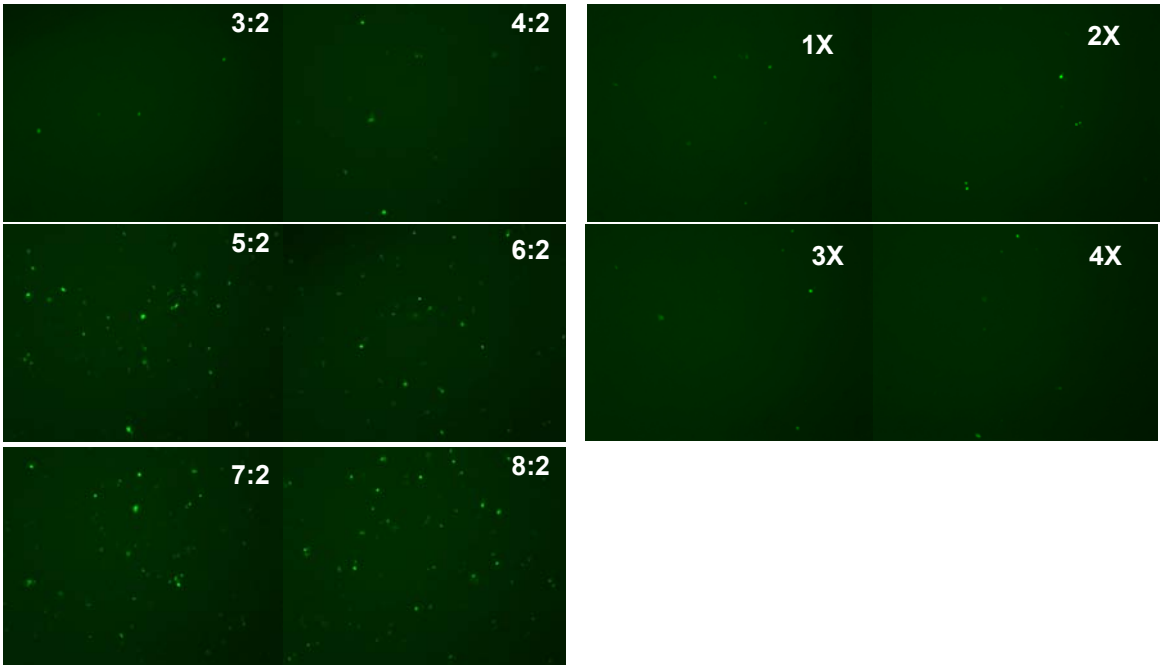


Figure B.1 GLUTag cells transfected with FugeneHD (left panel) or Lipofectamine (right panel). For FugeneHD, various ratios of µl transfection reagent to µg DNA were tested, while with Lipofectamine, various concentrations of the transfection reagent were tried.

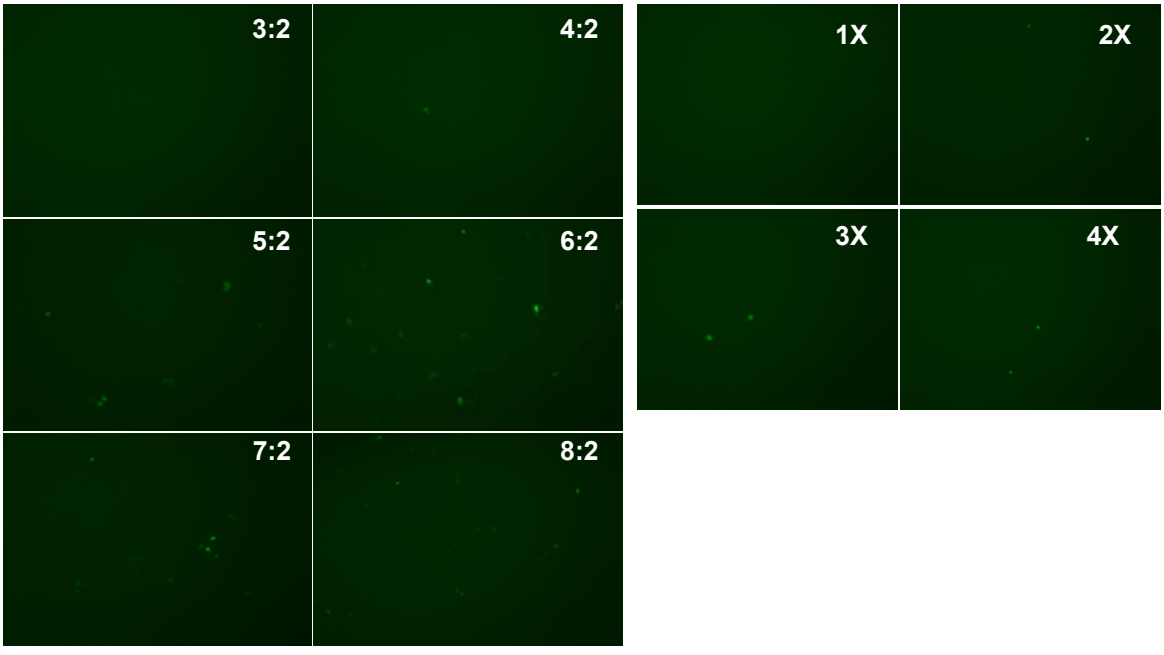


Figure B.2 STC-1 cells transfected with FugeneHD (left panel) or Lipofectamine (right panel). For FugeneHD, various ratios of µl transfection reagent to µg DNA were tested, while with Lipofectamine, various concentrations of the transfection reagent were tried.

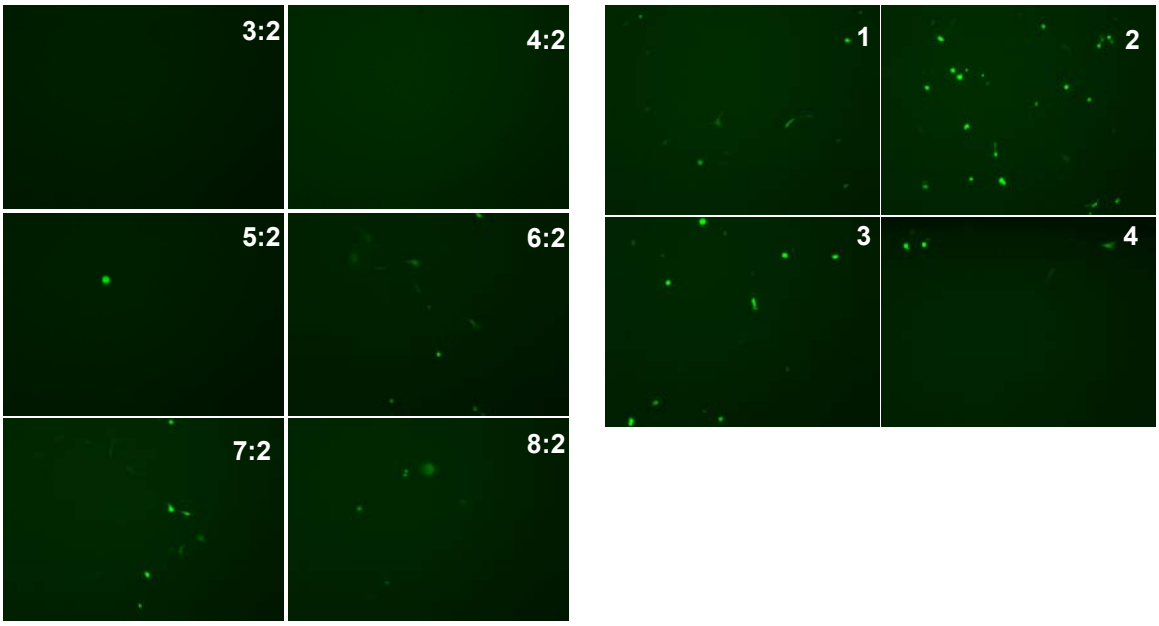


Figure B.3 NIH-3T3 cells transfected with FugeneHD (left panel) or Lipofectamine (right panel). For FugeneHD, various ratios of µl transfection reagent to µg DNA were tested, while with Lipofectamine, various concentrations of the transfection reagent were tried.

Transduction of Cell Lines

As expected based on previous studies with rat intestinal epithelial cells (Cheng, Kolls et al. 1997), adenoviral vectors strongly and efficiently transduced the enteroendocrine cell lines STC-1 (Figure B.4) and GLUTag (Figure B.5). This approach to gene delivery may be less useful when targeting L-cells in mixed populations such as primary cultures, as they would not show preferential gene transfer to enteroendocrine cells, as may an adeno-associated vector (Tang and Sambanis 2004).

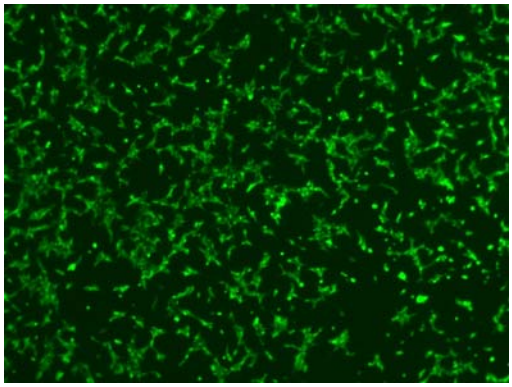


Figure B.4 STC-1 cells transduced with AdTrack (24 hours post transduction)

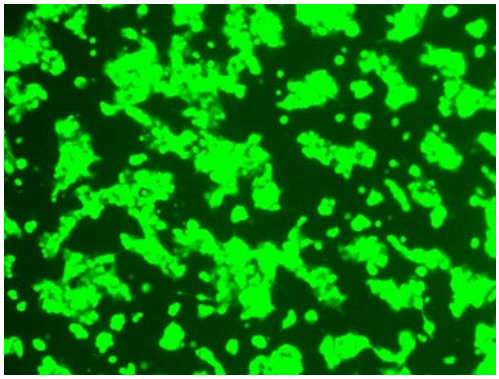


Figure B.5 GLUTag cells transduced with AdG3Fur/AdCMV-GFP (48 hours post transduction)

Though the methods of gene delivery investigated here typically yield only transient gene expression, as expression usually ceases after two to four days since episomal DNA is degraded by the cells. They are nonetheless very useful for conducting short-term studies. Transfection may also be adapted for stable expression by incorporation of an antibiotic resistance gene and delivery of the linearized plasmid to promote chromosomal integration. This approach to stable transfection was used in CHAPTER 3 to produce the GLUTag-INS cell line and the results obtained in the preliminary transfection studies described here, contributed to the success of that approach.

APPENDIX C

L-CELL SPECIFIC GENE EXPRESSION

C.1 Introduction

In moving to a more physiologically relevant system, as with transduction of primary intestinal L-cells either in vitro following isolation or in vivo, appropriate care must be taken in targeting L-cells for insulin expression. Given the complex nature of the intestinal epithelium and the multitude of surrounding non-L-cells, high specificity of transduction and/or expression is required to ensure that the insulin transgene is not expressed errantly by non-intended cells. Driving expression of insulin from an L-cell specific promoter would provide this specificity of gene therapy at the level of gene expression.

To obtain nutrient-regulated secretion of insulin in L-cells, reliance upon the cells' native regulated secretion pathway is crucial. It has been shown previously that insulin can be co-secreted with GLP-1, in genetically modified L-cells. Glucagon, GLP-1, GLP-2, and glicentin are all derived from preproglucagon and are expressed in a tissue-specific manner at the level of post-translational modification (Mojsov, Heinrich et al. 1986). GLP-1 is only produced by L-cells and is derived from the proglucagon gene. Therefore, we could hedge the expression of insulin off of the natural production of GLP-1 by inserting the insulin gene downstream of the proglucagon promoter. The goal of this study was to determine if the cloned murine proglucagon promoter would drive the cell-type specific expression of a transgene at a level that would be appropriate for insulin expression. The homogeneous GLUTag enteroendocrine cell line was used as a positive

control for expression while the non-enteroendocrine cell lines, NIH-3T3 (fibroblasts) and T84 (enterocytes) were used as negative controls for testing specificity of the vectors developed for this study.

C.2 Materials and Methods

C.2.1 Proglucagon Promoter Description and Primer Design

The mouse glucagon sequence was found by searching for "glucagon" on Entrez Gene (www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=gene), NCBI's database for gene-specific information. From this search, the record for Mus Musculus (mouse) was selected. The information about the glucagon (Gcg) gene was provided from Mouse Genome Informatics (MGI) and the full sequence (including flanking regions) was accessible from the MGI website. The "AF382209 Mus musculus glucagon gene promoter region 9/2002" was used for primer design and is shown in Figure C.1.

(-425bp) 5'—GAGATATAGCCAAATACCAAATCAAGGGATAAGACCCTCAAATGAGACTAGGCTCAT
TGACGTCAAAATTCACTTGAGAGAACTTTAGCAGTTTTTCGTGCCTGACTGAGACCGAA
GGGTGGATCTCCAAACTGCCCTTTCCATTCCCAAACAGAAAGGCACAAGAGTAAATAA
AATGTTTCCGGGCCTCTGCGGTCTCAACCCGGTATCAGCGTAAAAAG**CAGATG**AGCAA
AGTGAGTGGGCGAGTGAAAT**CATTTG**AACAAAACCCCATTATTT
A**CAGATG**AGAAATTTATATTGTCAGCGTAATATCTGCAAGGCTA
AACAGCCTGGAGAGCATATAAAAGCACACACCCTGGTGCAGAA
GGGCAGAGCTTGGGCCCAGGACACACTCAAAGTTCCCAAGGGGACTCCCT
CTGTCTAGAT—3'

Figure C.1 Sequence of the glucagon gene promoter region used for PCR cloning.

The section in larger size font matches the mouse proglucagon promoter sequence reported by Cordier-Bussat et al. (Cordier-Bussat, Morel et al. 1995). The sequences in bold are called E-boxes which have the consensus motif CANNTG and are known to interact with transcription factors characterized by a helix-loop-helix DNA-binding motif. These E-boxes are found in the promoter elements G1 and G4. In addition there are two other promoter elements G2 and G3 and a cAMP responsive element (CRE) also found in the proglucagon promoter. These are shown schematically in Figure C.2.

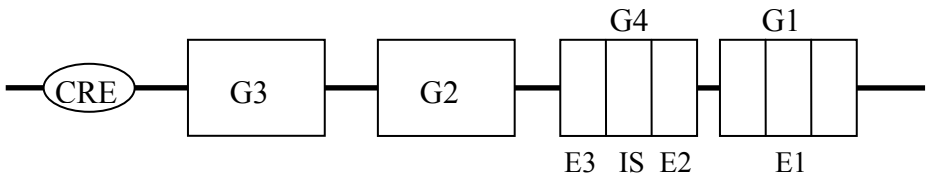


Figure C.2 Schematic of the 5' flanking enhancer and promoter elements of the proglucagon promoter from -50 bp to -350 bp (Cordier-Bussat, Morel et al. 1995).

In order not to diminish the strength or specificity of the promoter the full length of the proglucagon promoter including all of the elements just described was selected for cloning. As such, primers to initiate the PCR cloning were designed to match the upstream and downstream sequences of the proglucagon promoter shown above. Primers were selected based on the recommendations of Invitrogen's custom primer design application, Oligo Perfect^{TM}, and these included the insertion of appropriate restriction endonuclease recognition sequences on the 5' ends of both forward and reverse primers. In addition to the online tools, the primers were checked against the sequence using the Amplify program. The sequences and properties of the primers are described in Table C.1.

Table C.1 Primer Design for the PCR Cloning of the Mouse Proglucagon Promoter

	Sequence (5'→ 3')	5' Addition	Size (bp)	Primability of Match	Stability of Match
Fwd	cgcagatctgatatagccaaatac caaatcaagggataa	Bgl II site	39	93%	67%
Rev	gctagctagcgtagacagaggga gtcccttgggaa	Nhe I site	36	93%	68%

C.2.2 PCR Cloning of the Proglucagon Promoter

To obtain the full length mouse proglucagon promoter (mPGp), polymerase chain reaction (PCR) was employed and the mouse genomic DNA library served as template DNA. The PCR reaction was set up by the addition of 1 µl template mouse genomic DNA (Cat No. 69239-3, Novagen, San Diego, CA), 5 µl of 5 µM forward and 5 µl of 5 µM reverse primers (Invitrogen, Carlsbad, CA), 5 µl of 2 mM dNTP mix (Promega, Madison, WI), and 5 µl 10X Vent polymerase buffer (New England Biolabs, NEB, Ipswich, MA) to a PCR tube and adjusting the total volume to 50 µl by addition of DNase- and RNase-free water. The reaction mixture was covered with two drops of mineral oil to prevent evaporation and the tube was placed in the thermocycler set to the 80°C hot start setting to prevent nonspecific annealing. Then 1U Vent polymerase was added to the reaction and mixed with the pipette tip. The thermocycler was programmed with the following temperature steps, repeated for a total of 35 cycles: (1) 30 s at 94°C, (2) 30 s at 65°C, and (3) 90 s at 72°C. This PCR reaction was run in seven different 50 µl reactions, and the products were pooled and purified using the Wizard PCR prep kit (Promega). The final concentration of eluted mouse proglucagon promoter (mPGp) was

15.5 ng/µl as approximated by intensity comparison to the DNA standard following gel electrophoresis of a 1 µl sample.

C.2.3 Gene Construct Generation

The expression plasmid selected for initial testing of the proglucagon promoter was one containing the luciferase gene and it is depicted in Figure C.3. The luciferase system is very sensitive (less than 10^{-20} moles of luciferase can be detected under ideal conditions) and the half life of luciferase in mammalian cells is three hours, so the product does not accumulate, allowing changes in promoter activity to be detected. It was for these reasons the pRLnull vector was selected.

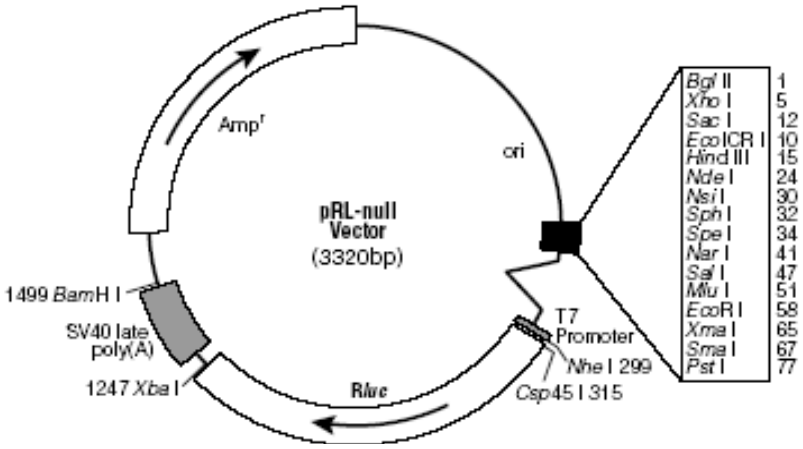


Figure C.3 Vector map of the pRLnull expression plasmid (Promega).

Erroneously the restriction sites chosen for cloning the proglucagon promoter into the pRLnull vector were Bgl II and Nhe I, this resulted in the release of the fragment containing the MCS, intron, and T7 promoter. This was done in an effort to ensure no contribution of expression due to the presence of the T7 promoter because of a misunderstanding about its function. The T7 promoter is included only for optional in

vitro transcription studies in E.-coli, yet as there is no ribosome binding site or Shine Delgarno sequence to initiate translation, the mRNA will not be translated into proteins.

Following PCR cloning of the promoter fragment, the murine proglucagon promoter (mPGp) was digested with the restriction endonucleases Bgl II and Nhe I (NEB) in NEB buffer 2 at 37°C for 12 to 16 hours to ensure digestion at the difficult to digest ends of the linear DNA. Similarly, the pRLnull vector backbone (Promega) was digested for 2 hours with Bgl II and Nhe I to release the fragment containing the MCS, intron, and T7 promoter and generate compatible ends with the promoter fragment. The vector backbone was also treated with Antarctic Phosphatase to remove the 5' phosphates and prevent self-annealing. Each component (vector backbone and promoter insert) was then purified by phenol/EtOH extraction to remove the restriction enzymes (Bgl II is not inactivated by heat). Ligation of these components was performed by the Quick Ligation Kit (NEB) and the ligation product (pRL-mPGp) was transformed into OneShot Stbl3 chemically competent cells (Invitrogen). Plasmid DNA from transformed cells was isolated using a midi-prep kit (Qiagen, Germantown, MD).

To correct for the error of removing the T7 promoter and intron by the cloning scheme described in the last paragraph, the scheme depicted in Figure C.4 was implemented in which the mPGp was excised from the pRL-mPGp vector and inserted into the pSE280 cloning vector to add additional restriction sites to the ends of the promoter and facilitate its transfer into the pRLnull vector at the appropriate site upstream of the T7 promoter and intron.

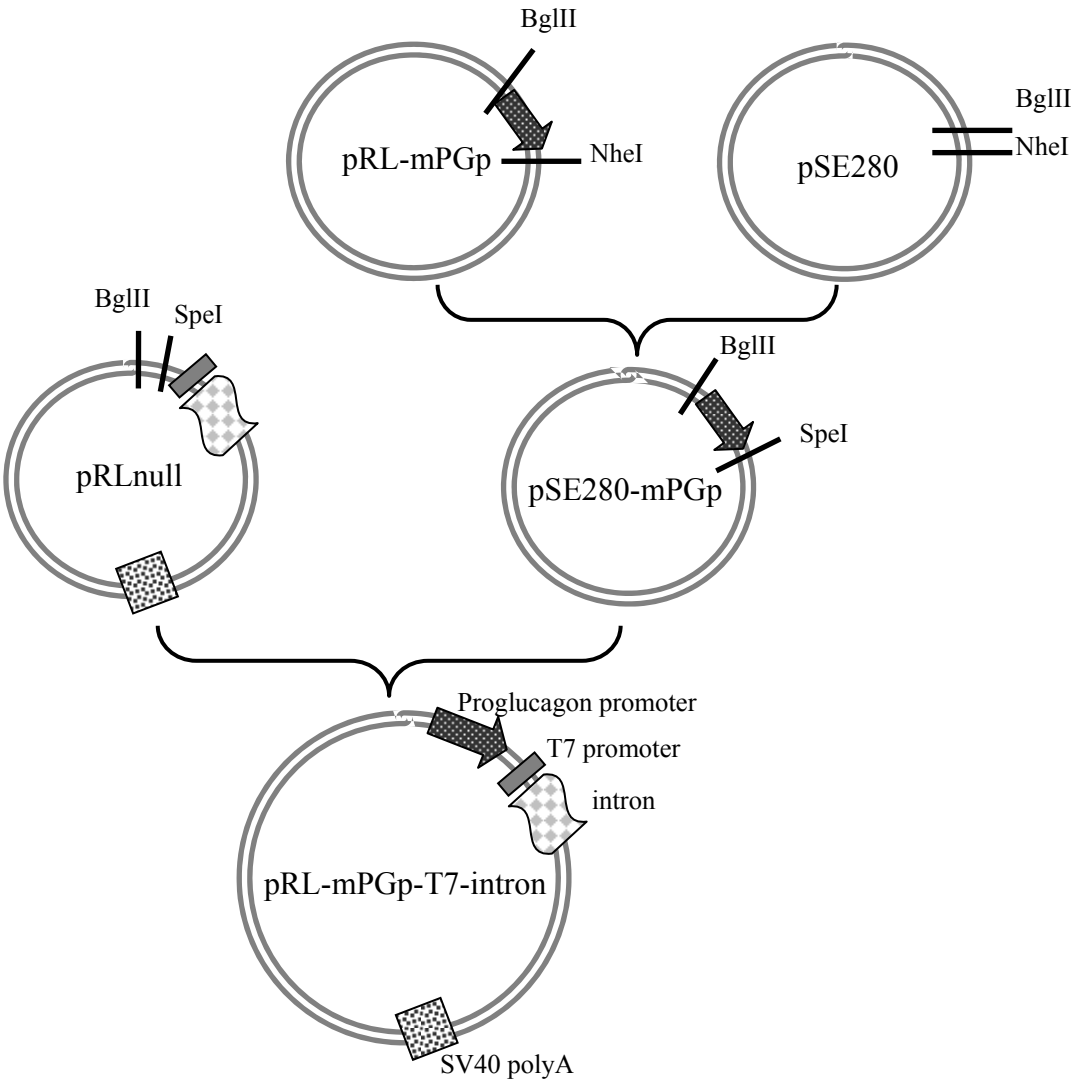


Figure C.4 Cloning scheme to correct the mPGp placement in the pRLnull vector. The mPGp was excised from the pRL-mPGp vector and inserted into the pSE280 cloning vector to facilitate its transfer into the pRLnull vector upstream of the T7 promoter and intron.

C.2.4 Transfection and Luciferase Expression Assay

Enteroendocrine GLUTag L-cells, T84 enterocytes, and NIH-3T3 fibroblasts were transfected via FugeneHD using the manufacturer's protocol at a ratio of 8 µl FugeneHD to 2 µg plasmid DNA. Three days after transfection, the supernatant was removed and cell monolayers were lysed and assayed for luciferase activity by the Renilla Luciferase Assay System (Promega) according to manufacturer's protocol.

C.3 Results and Discussion

Promoter insertion was confirmed by size comparison of bands after gel electrophoresis and sequencing performed by the Georgia Tech DNA Core Lab (Figure C.5).

TGTGNCTTTTGCTCCATGGCTCGAC*AGATCT*GATATAGCCAAATACCAAAT
CAAGGGATAAGACCCTCAAATGAGACTAGGCT**CATTTG**ACGTCAAAATTC
ACTTGAGAGAACTTTAGCAGTTTTTCGTGCCTGACTGAGACCGAAGGGTGG
ATCTCCAAACTGCCCTTTCCATTCCCAAACAGAAAGGCACAAGAGTAAAT
AAAATGTTTCCGGGCCTCTGCGGTCTCAACCCGGTATCAGCGTAAAAAGC
AGATGAGCAAAGTGAGTGGGCGAGTGAAAT**CATTTG**AACAAAACCCCAT
TATTTA**CAGATG**AGAAATTTATATTGTCAGCGTAATATCTGCAAGGCTAA
ACAGCCTGGAGAGCATATAAAAGCACAGCACCCTGGTGCAGAAGGGCAG
AGCTTGGGCCCAGGACACACTCAAAGTTCCCAAGGGGACTCCCTCTGTCTA
C*GCTAGC*CACCATGACTTCGAAAGTTTATGATCCAGAACAAAGGAAACGG
ATGATAACTGGTCCGCAGTGGTGGGCCAGATGTAAACAAATGAATGTTCT
TGATTCATTTATTAATTATTATGATTCAGAAAAACATGCAGAAAATGCTGT
TATTTTTTTACATGGTAACGCGGCCTCTTCTTATTTATGGCGACATGTTGTG
CCACATATTGAGCCAGTAGCGCGGTGTATTATACCAGACCTTATTGGTATG
GGCAAATCAGGCAAATCTGGTAATGGTTCTTATAGGGTTACTNGATCATTA
CAAATATCTTACTGCATGGTTTGAACTTCTTAATTTNCCNANAAANATCAT
TTTTNGTCGGCCATGNATTGGGGGNN

Figure C.5 Sequence of the cloned vector containing the proglucagon promoter. Underlined code matches with the target sequence identified in the materials and methods section. The italicized code is the added restriction sites, and bold text highlights the E-boxes.

Differences in expression level of the proglucagon promoter by various cell types was determined by normalizing the expression of the proglucagon promoter vector to expression from the promoterless vector (pRLnull, Figure C.6) or the CMV-containing vector (pRL-CMV, Figure C.7) to account for differences in transfection efficiencies among cell types.

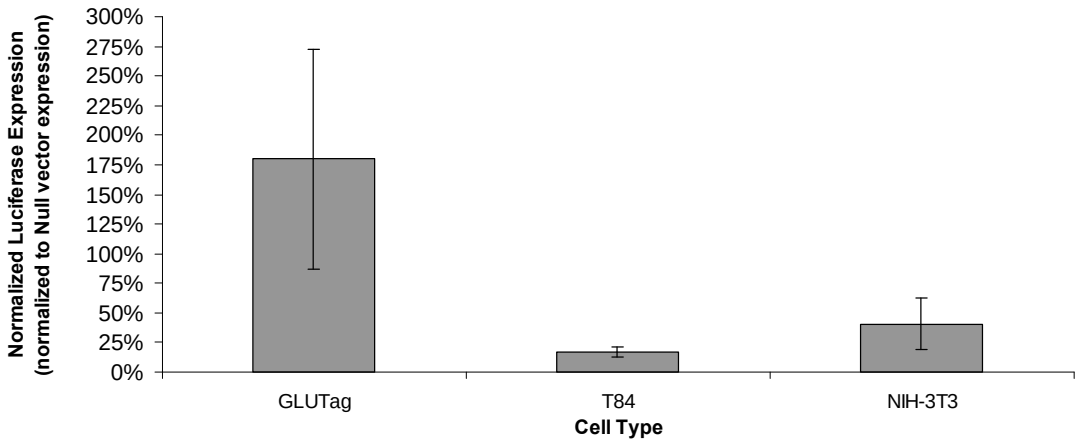


Figure C.6 Proglucagon promoter expression in various cell lines normalized to null vector expression. Parallel cultures of GLUTag L-cells, T84 enterocytes, and NIH-3T3 fibroblasts were transfected with three different plasmids: one containing no promoter (Null), one containing the CMV promoter (CMV) and finally one containing the murine proglucagon promoter. Luciferase expression from the proglucagon promoter in each of the cell types is expressed as a percentage of the Null promoter N=3.

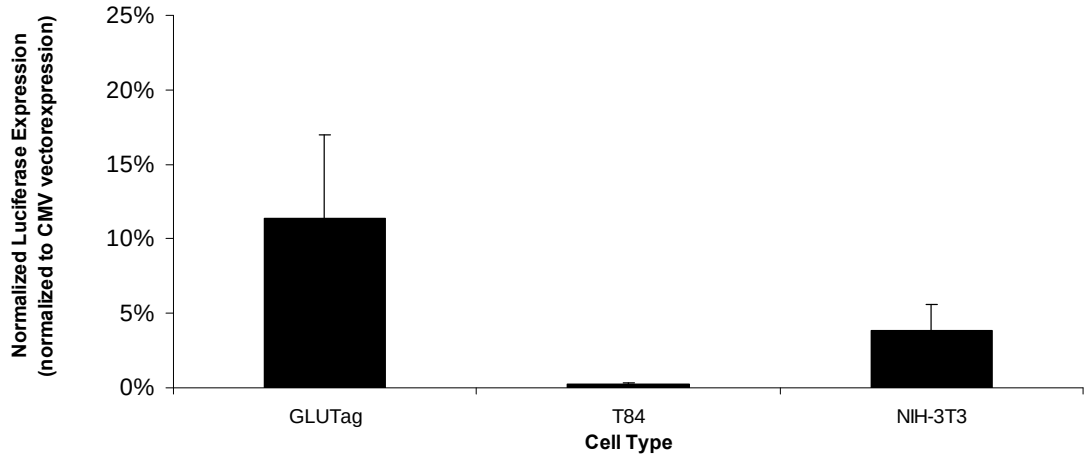


Figure C.7 Proglucagon promoter expression in various cell lines normalized to CMV vector expression. Parallel cultures of GLUTag L-cells, T84 enterocytes, and NIH-3T3 fibroblasts were transfected with three different plasmids: one containing no promoter (Null), one containing the CMV promoter (CMV) and finally one containing the murine proglucagon promoter. Luciferase expression from the proglucagon promoter in each of the cell types is expressed as a percentage of the CMV promoter N=3.

GLUTag cells displayed the highest level of expression from the proglucagon promoter vector, 179.8 ± 93.1% normalized to null vector expression compared to just 17.3 ± 4.3% for T84 and 40.7 ± 21.5% for NIH-3T3 cells (mean ± SD). Though L-cell specific expression was observed, the level of luciferase expression exhibited by the proglucagon promoter vector was only 11.4 ± 5.6% the level obtained from the CMV vector in GLUTag cells. Given however that the CMV promoter is one of the strongest known promoters, this comparison may not be entirely appropriate, and perhaps 11% of CMV-driven expression would be useful in certain studies.

The sequence cloned in this chapter included ~420 bp of the upstream flanking region, this should have been ample enough to include all of the promoter elements described in the proglucagon promoter (Cordier-Bussat, Morel et al. 1995), though the glucagon upstream enhancer (GUE) region is located between -1253 and -2292 bp (Jin and Drucker 1995) and was not included. It is possible that if a longer sequence had been cloned to include the GUE region, expression from the proglucagon promoter may have been improved. When this experiment was performed by Jin and Drucker, however, relative luciferase expression, compared to Null vector expression, of the vector containing 2058 bp of the 5' flanking sequence rose to only 290% compared to 200% from the vector containing only 476 bp of the 5' flanking sequence (Jin and Drucker 1995). Thus, though the contribution of the GUE region may be important for tissue specific processing, most of the expression level is determined by the proximal 5' flanking region.

It is important to note that the cell lysates were collected following three days of regular culture without any attempt to stimulate expression, for example by up-regulating transcription via the cAMP-promoting agent, forskolin (Brubaker, Schloos et al. 1998), to stimulate transcription through the CRE site. It would be interesting to see how expression levels change if the cells are stimulated prior to preparation of the lysates.

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