INSULIN SECRETION DYNAMICS OF RECOMBINANT HEPATIC AND INTESTINAL CELLS

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INSULIN SECRETION DYNAMICS OF RECOMBINANT HEPATIC AND INTESTINAL CELLS

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TO THOSE SUFFERING WITH INSULIN DEPENDENT DIABETES

ACKNOWLEDGEMENTS

I would like to thank everyone who supported and encouraged me throughout the years. I have developed an amazing network of friends and colleagues while at Georgia Tech whom I will truly miss as they were always nearby to share in laughs, sympathize with the stresses of research and provide positive outlets for frustration. We have made many memories together and hopefully we will continue to make many more together.

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LIST OF SYMBOLS AND ABBREVIATIONS

AAV(s)	adeno-associated virus(es)
Ad	adenovirus
β	beta
cAMP	cyclic adenosine monophosphate
CCK	cocktail
CDC	Center for Disease Control
CMV	cytomegalovirus
DEX	dexamethasone
DMEM	dulbecco's modified eagle's medium
EE	enteroendocrine
EGFP	enhanced green fluorescent protein
ES	embryonic stem
FBS	fetal bovine serum
GFP	green fluorescent protein
GIP	gastric inhibitory peptide
GLP-1	glucagon like peptide-1
GLUT2	glucose transporter type 2
hB10	human B10 modified
IDD	insulin-dependent diabetes
Kb	kilobases
MH	meat hydrolsate
MOI	multiplicity of infection
P	probability
PC	prohormone convertase
pen/strep	penicillin/streptomycin

PFU	plaque forming units
PPI	preproinsulin
rH4IIE	recombinant H4IIE
rHepG2	Recombinant HepG2
RIA	radioimmunoassay
R_{neo}	neomycin resistance
SE	standard error
STZ	streptozotocin

SUMMARY

Hepatic and intestinal endocrine cells are potentially helpful targets for recombinant insulin expression. As the two cell types exhibit different secretion kinetics, it has been hypothesized that a combination of the two would better approximate insulin secretion kinetics from normal, functioning beta-cells than either cell type alone. This hypothesis was tested using two hepatic cell lines transiently transduced with one of three adenoviruses for insulin expression along with a stably transfected recombinant intestinal L cell line.

The insulin secretion kinetics were analyzed for both the hepatic and intestinal cells to determine the potential of combining them to reproduce the insulin secretion kinetics of a normal, functioning beta-cell. It was observed that the two recombinant hepatic cell lines secreted insulin in a more sustained manner exhibiting slower release kinetics. They also exhibited an increase in insulin secretion when stimulated by the cocktail of nutrient secretagogues (glucose and meat hydrolysate) versus stimulating with only glucose. The cells transduced with the adenovirus containing an additional cytomegalovirus (CMV) promoter and green fluorescent protein (GFP) exhibited the highest insulin secretion after stimulation, whereas the cells transduced with an adenovirus encoding for destabilized preproinsulin mRNA exhibited the lowest secretion rates.

The recombinant intestinal cell line (GLUTag-INS) secreted insulin with rapid kinetics upon stimulation, apparently due to the presence of secretory granules containing pre-synthesized insulin. The experiments demonstrated that the cells stimulated with medium containing only meat hydrolysate exhibited a significantly higher insulin secretion relative to secretagogue-free controls. The insulin secretion was not further enhanced when meat hydrolysate was combined with glucose.

Overall, the results of this study indicate that a combination of these recombinant hepatic and intestinal cells would better approximate the biphasic kinetics of a normal, functioning beta cell.

CHAPTER 1

INTRODUCTION

Insulin-dependent diabetics lack the ability to regulate blood glucose levels. This may be due to 1) an autoimmune attack on pancreatic beta-cells (as seen in type 1 diabetes); or 2) an exhaustion of these insulin secreting cells (as seen in some late stages of type 2 diabetes). Currently, no cures are available for insulin-dependent diabetes (IDD) and treatments have limitations including: limited ability to achieve glucose homeostasis with insulin injections or infusions; limited availability of donor organs; and immune rejection issues with cell and tissue-based therapies.

A non-diabetic's pancreatic beta-cells respond to a glucose stimulus by secreting insulin in a biphasic manner. This method of control allows for rapidly lowering blood glucose during the first phase of insulin secretion followed by sustaining the lowered blood glucose over a period of hours during the second phase of insulin secretion.

A combination of hepatic and intestinal cells has the potential to reproduce the physiologic regulation of blood glucose levels. Our objective is to analyze insulin secretion kinetics of recombinant hepatic and intestinal cell lines and their ability to mimic those of beta-cells.

Hepatic cells possess glucose sensing proteins, which aid in glucose homeostasis in the body. This sensory mechanism may be utilized via genetic engineering to facilitate insulin secretion with regulated release at the transcription level. Recombinant hepatic cells have been shown to reproduce the sustained, second phase of insulin secretion after exposure to a stimulus of glucose.

Enteroendocrine (EE) L cells respond to the ingestion of a meal by releasing their incretin peptide, glucagon like peptide-1 (GLP-1). GLP-1 is released to potentiate glucose-induced insulin secretion, inhibit hepatic glucose production, and reduce appetite and food intake. This regulated secretion may be targeted through genetic engineering so that insulin accumulates in the same secretory vesicles and is therefore released with the same kinetics as endogenous GLP-1. Recombinant EE cells have demonstrated this ability to reproduce the acute and transient first phase of insulin secretion.

An intrinsic and closed loop method for achieving glucose homeostasis is the "holy grail" of blood glucose control. We will be analyzing the insulin secretion kinetics of hepatic and intestinal cell lines for their potential to provide this crucial regulation.

CHAPTER 2

BACKGROUND

2.1 Insulin-Dependent Diabetes

As a consequence of having no functional pancreatic beta-cells, insulin dependent diabetes (IDD) patients are unable to regulate blood glucose concentrations. IDD is an insulin deficiency caused by either 1) a genetically determined autoimmune disease, which destroys the insulin producing beta-cells of the pancreas (type 1 diabetes); or 2) insulin resistance and degeneration of the beta-cells due to chronically high insulin levels (type 2 diabetes).

The number of affected people is increasing worldwide. According to the Center for Disease Control (CDC), diabetes was the sixth leading cause of death in the United States in 2004. As of 2005, 20.8 million people in the United States have diabetes which is 7.0% of the population (<diabetes.org> 2006). Approximately 4 million of these people have IDD.

Many IDD patients must endure multiple daily injections of insulin to roughly maintain physiological levels of blood glucose. Even with insulin injections, the resulting fluctuations in blood glucose concentrations have severe medical implications such as retinopathy, heart disease and neuropathy. The tight control of these fluctuations can prevent or at least postpone dangerous long-term complications. (Chan, Fujimiya et al. 2003)

A treatment is needed to improve glycemic control, prevent severe hypoglycemia and improve quality of life.

2.2 Current Treatments

Currently no cures exist for diabetes, only limited treatment options. Current treatments for IDD include insulin injections, insulin pumps, whole pancreas transplantations or pancreatic islet transplantations (Bottino et al. 2003). These treatments have severe limitations including: limited availability of donor organs, immune rejection issues, and limited glucose regulation.

Insulin injections require IDD patients to manually monitor their blood glucose levels by sampling blood vi multiple daily finger sticks and administer multiple daily injections of precisely calculated amounts of insulin. This method has limited ability to prevent glycemic excursions and requires a high degree of patient compliance. It also does not reproduce natural insulin secretion dynamics of a normal, functioning pancreas seen in Figure 1 (Rorsman, Eliasson et al. 2000; Chan, Fujimiya et al. 2003).

In a healthy person, the pancreas releases insulin in two distinct phases (Figure 1). The first phase is a burst of insulin which begins within minutes of ingesting a meal and continues 30 to 60 minutes after rapidly lowering blood glucose levels in the patients. The second phase is a slower and sustained insulin release causing prolonged insulin secretion 1 to 3 hours after eating a meal; maintaining the blood glucose level in the patient (Reimann and Gribble 2002; Reimann, Ward et al. 2006).

Another current treatment is the insulin pump, which allows more frequent dosing and better controlled insulin administration. This device also has limited ability to prevent glycemic excursions as it neither automatically measures blood glucose levels nor calculates the appropriate amount of insulin to administer; therefore, this is not a closed loop system. The Medtronic Minimed real-time glucose monitoring system is one step closer to a closed loop system as it incorporates a continuous glucose monitor and an insulin pump, but it still requires finger sticks and user instructions for dispensing insulin (Medtronic.com). Insulin pumps are also limited by issues with infections at the

implantation site and their bulkiness of the devices (Schaepelynck-Belicar, Dufaitre-Patouraux et al. 2005). *No closed loop devices are currently available and no continuous blood glucose monitoring devices are approved to completely replace traditional monitoring methods.*

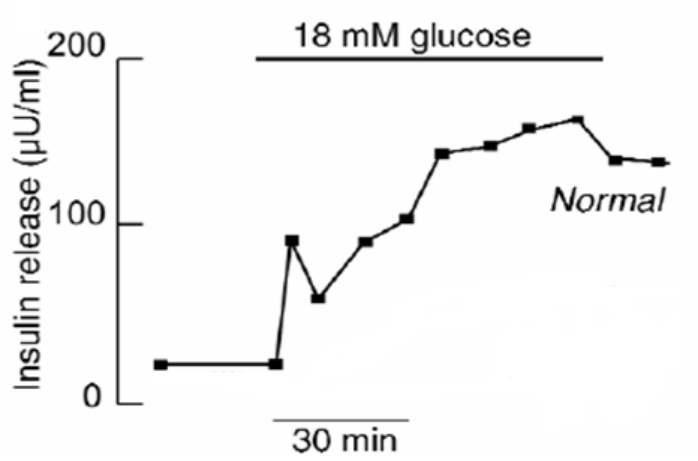

Figure 1: Insulin secretion from healthy patients. Insulin release in a healthy patient exhibits two phases of insulin secretion to regulate blood glucose levels and limit excursions. The first phase is a burst of insulin followed by the second phase containing more sustaining insulin release. Adapted from (Rorsman, Eliasson et al. 2000).

2.3 Sources of Beta-Cells

2.3.1 Islet Transplantation

The glucose-responsive and insulin secreting beta-cells are found in the islets of Langerhans in the pancreas. These spheroid structures contain specialized cells (alpha-, beta-, delta- and PP-cells) which produce and secrete hormones necessary for glycemic control (glucagon, insulin, somatostatin, and pancreatic polypeptide, respectively). Islets exist as “mini-organs” with highly organized cell clusters. Islets are

a potential treatment for IDD and may be transplanted into IDD patients as a whole-organ graft of the pancreas or as isolated islets.

One limitation with this islet transplantation is the number of donors. Approximately 6000 human pancreata are available each year in the United States with only half considered suitable quality for transplantation. Islets of 2-4 donors are required to treat an IDD patient with 2-4 implantations required as islets from each donor become available (Chan, Fujimiya et al. 2003; Xu, Li et al. 2003b; Wiseman and Gill 2005; O'Connell P, Hawthorne et al. 2006). Therefore, less than 1000 patients per year may be treated with this method— amounting to less than 0.1% of type 1 diabetics (Rother and Harlan 2004).

Shapiro et al studied IDD patients receiving islet transplantations in an international, multicenter trial of the Edmonton protocol, developed by physicians at the University of Alberta in Edmonton using donor pancreata transplants along with a steroid-free immunosuppression regimen (Shapiro, Ricordi et al. 2006). Islet transplantations exhibit varied results when treating IDD. Out of the 36 subjects in the study, 58% exhibited insulin independence (Figure 2). Out of the IDD patients gaining insulin independence, 76% of the subjects were reverted to insulin dependence 2 years after transplantation (Shapiro, Ricordi et al. 2006). Patients receiving the transplant also exhibited deficient first phase insulin secretion and delayed insulin secretion after an oral glucose load.

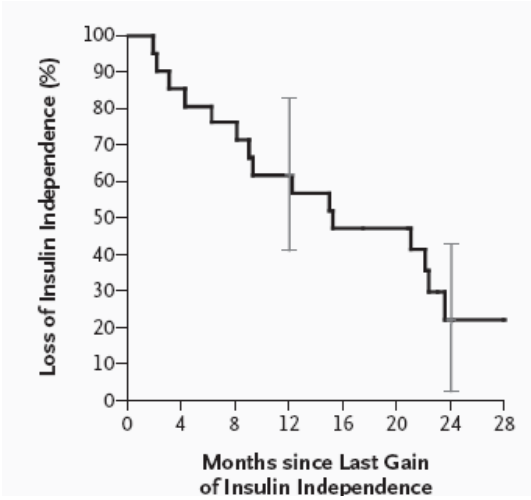

Figure 2: Results of international trial of the Edmonton protocol. 76% of subjects receiving islet transplantations reverted to insulin dependence within 2 years of transplantation. The bars represent a 95% confidence interval at these time points. Image adapted from (Shapiro, Ricordi et al. 2006).

Efforts to expand islet cellular mass and to locate an alternate source for islets are being pursued. The expansion of islet cellular mass has resulted in reduced insulin production after *in vitro* culturing of the cells. Xenogeneic sources, particularly porcine islets, may be a possible means to alleviate cell sourcing issues, as pigs expand into large litters and islets may be used from either neonatal or adult sources. They also produce an insulin form similar to humans. In fact, porcine insulin has been used to treat IDD patients for over 60 years (Rother and Harlan 2004). Concerns regarding a xenogeneic tissue source such as cross-species transmission of a pig retrovirus and need for immunosuppressive drugs limit the success of the technology.

Regardless of whether the source is an allo- or xenogeneic donor, chronic immunosuppression is necessary for islet transplantation. These immunosuppresive drugs result in side effects which some patients consider worse than living with IDD. The immune suppressing drugs also have a negative impact on the transplanted islets. They have been shown to destroy implanted islets, decrease beta-cell function, and to elicit an innate immune response. Other side effects include mouth ulcers, deteriorating

renal function, diarrhea, fatigue and hypertension (Shapiro, Ricordi et al. 2006). These side effects only exacerbate the secondary complications common to IDD. It is even possible that the use of these drugs actually increases patient mortality (Rother and Harlan 2004).

2.3.2 Continuous Beta-Cell Lines

An alternative cell source for treating IDD is transformed beta-cell lines. Cell lines have been developed by expressing oncoprotein transgenes in rodent beta-cells including βTC-tet and βTC3 (Efrat 2004). Cell lines offer the benefits of being well-defined, able to maintain differentiated properties for up to 50 passages, and are easily expanded into large cultures. Drawbacks of using cell lines as an IDD treatment include hypersensitivity to sub-physiologic glucose concentrations and the fear of adverse reactions from implanting a transformed cell line (Papas, Long et al. 1996; Efrat 2004). An engineered cell with the ability to maintain long-term glucose-responsiveness has yet to be developed (Sambanis 2000).

2.3.3 Differentiating Stem Cells into Pancreatic Beta-Cells

Another cell based therapy currently being studied is the use of stem cells differentiated into pancreatic beta-cells. Differentiation of embryonic stem (ES) cells remains a challenge. According to a review article by Rivas-Carrillo et al, successful derivations of ES cells into islet-cells is more difficult than initially expected (2007). More information on the mechanisms driving the development of the pancreas is needed to successfully produce these cells (Rivas-Carrillo, Okitsu et al. 2007). ES cells are typically allogeneic or xenogeneic; therefore, they are also expected to be immunogenic. The current ES cell lines are not a feasible treatment for diabetes. These cells were

determined to be contaminated with a mouse protein from mouse feeder layers used for culturing the ES cell lines (Kuleshova, Gouk et al. 2007).

Adult progenitor cells have also been studied to determine their potential to differentiate into insulin-secreting cells. Progenitor cells have been investigated from a variety of sources including the pancreatic ducts, exocrine pancreas, pancreatic islets, liver, spleen, and bone marrow. At this time, some of these cell types have demonstrated the ability to maintain normoglycemia in animals (Lock and Tzanakakis 2007). Though, an additional concern is that the immune system will continue to recognize and destroy these cells after they differentiate into beta-cells due to the presence of the genetic disease in type 1 diabetes (Xu, Li et al. 2003b; Fujimoto, Sasaki et al. 2005; Han, Lee et al. 2007).

2.3.4 Engineered Non-Beta-Cells

Non-beta-cells cells offer a promising cell source for the development of personalized medicine to treat diabetes. These are potentially autologous cells which could relieve the cell sourcing and immune acceptance issues. They offer an alternate blood glucose management system creating a closed loop system to monitor blood glucose and secrete appropriate amounts of insulin. They do not express beta-cell antigens allowing these cells to avoid an attack by the immune system, as seen in type 1 diabetes (Han, Lee et al. 2007).

Various non-beta cells are being researched including enteroendocrine (EE), hepatic, skeletal, neuroendocrine, and adipocytes. Preadipocytes have been engineered to secrete furin compatible insulin, but the lack of glucose sensitivity prevents normalization and stabilization of glucose (Fujimoto, Sasaki et al. 2005; Ito, Bujo et al. 2005). Ito et al expressed furin-cleavable insulin in primary adipocytes and transplanted the cells into diabetic mice (Ito, Bujo et al. 2005). The cells normalized the

blood glucose levels, but the researchers failed to mention the effect of cell number on hypoglycemia and the kinetics of insulin release (Ito, Bujo et al. 2005; Wideman, Fujita et al. 2007).

Another cell source, skeletal muscle was injected with two adeno-associated viruses (AAVs)—one with furin-cleavable proinsulin and one with glucokinase. IDD was reversed in streptozotocin (STZ) mice injected with these AAVs, but normoglycemia was not maintained. This demonstrated the need for glucose-responsive insulin secretion (Wideman, fujita et al. 2007).

2.3.4.1 Hepatic Cells

One promising cell source for autologous cell therapy are hepatic cells which express glucose sensing components similar to pancreatic beta-cells (Xu, Li et al. 2003b). An analogous glucose-sensing component is present in liver and beta-cells—glucose transporter type 2 (GLUT2). Auricchio et al consider, GLUT2 and glucokinase "…essential factors for glucose metabolism" (Auricchio, Gao et al. 2002). Once glucose enters the cells via the GLUT2 transporter it is phosphorylated by glucokinase. This is considered the rate limiting step enabling the sensing of extracellular glucose concentration in liver cells; making these cells a potential source of non-beta cells for treating IDD (Reimann and Gribble 2002; Efrat 2004; Klover and Mooney 2004).

2.3.4.1.1 Recombinant Hepatic Cell Lines

Primary hepatic cells are candidates for genetic engineering due to their low cell turn over rate, high regenerative capacity, and ability to sense glucose concentration fluctuations. This response to glucose may be targeted via genetic engineering to develop insulin secreting and glucose-responsive cells. These cells offer a potential *in vivo* therapy for treating diabetes.

To aid in studying the potential of the hepatic cells to treat IDD, recombinant hepatic cell lines have been developed by modifying rat and human hepatoma cell lines, (H4IIE and HepG2 cells respectively). Although, hepatic cells do not possess the capacity for regulated secretion, transcriptional regulation of insulin biosynthesis is incorporated into these cell lines using promoters which are responsive to glucose concentrations. This transcriptional regulation is slower and more sustained simulating the second phase of insulin secretion from a beta-cell as seen previously in Figure 1. *Recombinant* h*epatic cells are unable to provide the acute first phase of insulin secretion as seen in beta-cells (Ito, Bujo et al. 2005; Wideman, fujita et al. 2007); therefore, requiring the combination with an additional cell type to reproduce the biphasic kinetics of the beta-cells.*

2.3.4.1.2 Furin Compatible Insulin for Hepatic Cell Lines

Hepatic cells do not contain the endoproteases required to process proinsulin into insulin—prohormone convertases PC1/3 and PC2— as seen in beta-cells. To alleviate this problem, a mutated proinsulin that is cleavable by furin, a ubiquitous protease, can be incorporated into the transgene (Wideman, fujita et al. 2007). The furin-compatible transgene allows cleavage of C-peptide from the A and B chains of insulin to activate the insulin. Furin modified human insulin cDNA has been shown to lower blood glucose levels in diabetic animals (Short, Okada et al. 1998; Thule and Liu 2000; Shifrin, Auricchio et al. 2001; Auricchio, Gao et al. 2002). Release of C-peptide also has an added benefit in treating diabetes. When C-peptide was injected into patients demonstrating early signs of diabetic neuropathy, it improved blood vessels and nerves in diabetic patients (Sadrzadeh, Glembourtt et al. 2007).

Transgenes with glucose-responsiveness and furin compatibility have been created by Thulé et al (Veterans Administration Medical Center, Atlanta, GA) (Figure 3).

These transgenes respond to glucose stimulations through transcriptional regulation of the promoter (Thule, Liu et al. 2000). They have three promoter elements from hepatic cells (referred to as G3) which exploit the cells' glucose sensitivity and responsiveness. The transgenes also include a furin compatible human insulin cDNA (2xfur). Diabetic rats injected with an adenovirus containing the G3-2xfur insulin transgene (Figure 3A) maintained normoglycemia (Thule and Liu 2000; Thule, Campbell et al. 2006). According to Thulé and Liu, they "demonstrated the feasibility of utilizing transcription to control transgenic insulin production in a rodent model of diabetes mellitus" (2000). This transgene exhibited many of the necessary characteristics for lowering blood glucose and has also been combined with a cytomegalovirus (CMV) promoter for green fluorescent protein (GFP) allowing visualization of the transduction. *The genetic engineering of hepatic cells requires 1) a vector for genetically engineering the cells such as adenovirus transduction and 2) modifications to the insulin enabling processing of the prohormone.*

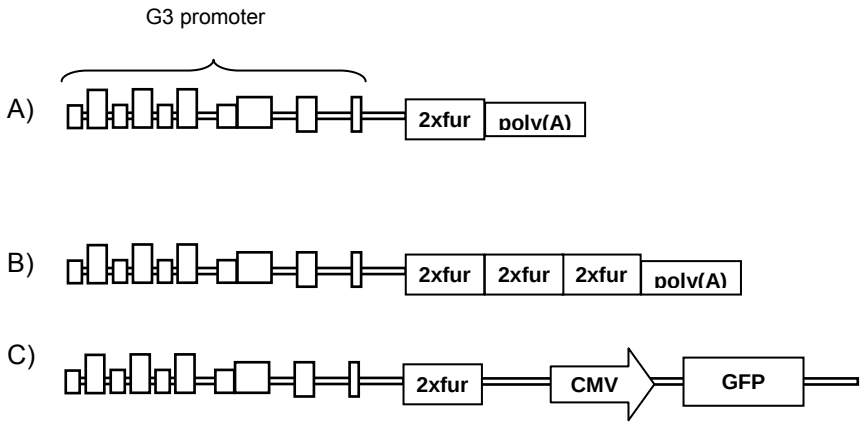

Figure 3: Transgenes for adenovirus transduction. These vectors were developed by Thulé et al: A) G3-2xfur containing the G3 promoter, engineered PPI mRNA and a poly(A) tail B) G3-InsTail containing the G3 promoter, three copies of the 2xfur PPI and a poly(A) tail C) G3-Track containing the G3-2xfur and the cytomegalovirus (CMV) promoter for green fluorescent protein (GFP).

2.3.4.1.3 Nonsense-Mediated mRNA Decay (NMD)

The transcriptional expression of insulin in recombinant hepatic cells may be further regulated by incorporating engineered PPI mRNA with enhanced down-regulation secretion of insulin (Figure 3C). Tang and Sambanis demonstrated that "the prolonged stability of PPI (PPI) mRNA causes the sluggishness of secretion down-regulation…resulting in the secretion of insulin long after the stimulus was removed" (Tang and Sambanis 2003b). The inability of the recombinant cells to rapidly down-regulate insulin secretion may result in hypoglycemic episodes. This was demonstrated *in vitro* by Tang and Sambanis who compared the engineered PPI mRNA (InsTail) with a control PPI mRNA *in vitro* (2003b). The expedited decay of the engineered PPI mRNA allows for faster elimination of the mRNA, which may prevent hypoglycemia in animal models. In the experiment by Tang and Sambanis (2003b), the control and InsTail vectors were responsive to tetracycline (tet) due to presence of the tet regulated promoter. When the cells were exposed to doxycycline (DOX), a tet isomer, the process of transcription was stimulated; resolving the up- and down-regulation kinetics for the two vectors. The InsTail vector exhibited faster kinetics in down regulation of engineered PPI mRNA and consequently insulin secretion after exposure to DOX (Figure 4). As predicted, the control vector did not experience a significant down-or up-regulation of insulin expression.

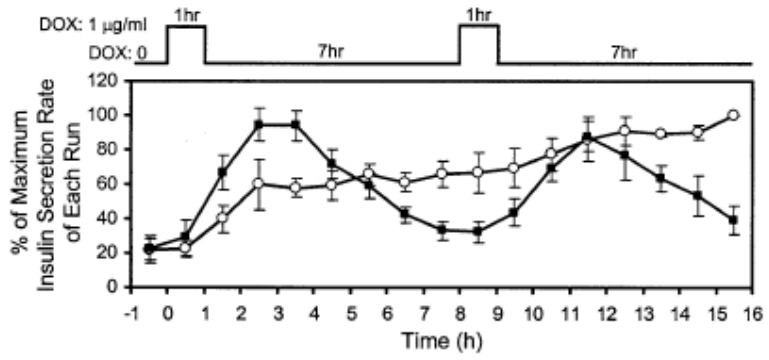

Figure 4: Down-regulation of destabilized insulin. The transcription of engineered PPI mRNA (InsTail) (filled squares) and control PPI mRNA (open circles) were stimulated by a doxycycline (DOX) square wave (Tang and Sambanis 2003b).

Thulé et al have exploited this enhanced down-regulation kinetics by modifying the G3-2xfur transgene to express the engineered PPI mRNA (G3-InsTail) (Figure 3B). The G3-2xfur was also modified in a separate vector by adding a cytomegalovirus promoter (CMV promoter) controlling expression of green fluorescent protein (GFP) (G3-Track) (Figure 3C). These three vectors allow for analysis and comparison of insulin secretion kinetics. Additionally, G3-Track allows an assessment of the transduction efficiency through examining cells with fluorescent microscopy.

2.3.4.1.4 Adenovirus Transduction

An adenovirus transduction may be used to genetically engineer the hepatic cells for glucose responsive insulin secretion. Recombinant adenoviruses are non-enveloped DNA viruses which are replication deficient due to the deletion of the E1 and E4 viral genes. Adenoviruses are able to efficiently transduce both proliferating and quiescent cells. High titres may be generated with this virus on the order of 10^{10} to 10^{11} PFU/mL. They package large genes up to 8kb and have a broad tropism. They do not integrate into the genome, therefore, preventing insertion into an oncogene as seen in

retroviruses (Ritter, Lehmann et al. 2002) Adenoviruses have some limitations *in vivo* including the elicitation of an immune response which may require immunosuppressive drug treatment and result in loss of expression over time due to the transient expression of the virus. Strategies for circumventing the immune response issues including monoclonal antibody therapy and neutralization of proinflammatory cytokines. *Overall, adenoviruses are a good candidate for in vitro gene therapy studies especially the transduction of hepatic cells, but are limited for in vivo applications.*

2.3.4.2 Enteroendocrine (EE) Cells

Another promising cell source for autologous cell therapy are the EE cells. EE cells are responsible for 60% of the glucose dependent insulin secretion following an oral glucose load (Theodorakis, Carlson et al. 2006). L and K cells, two types of EE cells, have been confirmed to express the same glucose sensor, glucokinase, as found in beta cells (Theodorakis, Carlson et al. 2006). Gastric inhibitory polypeptide (GIP) and glucagon-like peptide-1 (GLP-1) are two glucose-dependent incretins secreted by the K and L cells, respectively, in response to food intake.

L cells and K cells are located in the gut, enabling direct nutrient contact. K cells are located in the duodenum. The exact location of L cells is under debate, but most are limited to the region between the ileum and colon (Reimann, Maziarz et al. 2005). The location of both of these EE cells in the gut supports the observation that GLP-1 secretion exhibits similar kinetics as GIP as shown in Figure 5 (Schirra, Katschinski et al. 1996; Theodorakis, Carlson et al. 2006). In healthy subjects, GLP-1 aids in maintaining blood glucose homeostasis by enhancing insulin secretion of beta-cells in a glucose dependent manner. GLP-1 secretion is stimulated by an oral administration of carbohydrates, lipids and amino acids (Reimer, Darimont et al. 2001). In contrast, GIP is not effective in stimulating insulin secretion (D'Alessio and Vahl 2004). GLP-1 has been

demonstrated to increase insulin release and promote glucose homeostasis; even correcting hyperglycemia in type 2 diabetic subjects (D'Alessio and Vahl 2004). It is also known to 1) stimulate the proliferation and neogenesis of beta-cells; 2) decrease appetite; 3) decrease gastric emptying and acid secretion in the stomach; and 4) decrease glucagon production in patients (Wideman, Yu et al. 2006). GLP-1 is being studied (Reimann and Gribble 2002) and mimetic drugs (Byetta) are being marketed to treat type 2 diabetics. Though, its major limitation is the 1-2 minute half-life of GLP-1 (D'Alessio and Vahl 2004), which Byetta circumvents by implementing a mimetic drug (approved for type 2 diabetics only).

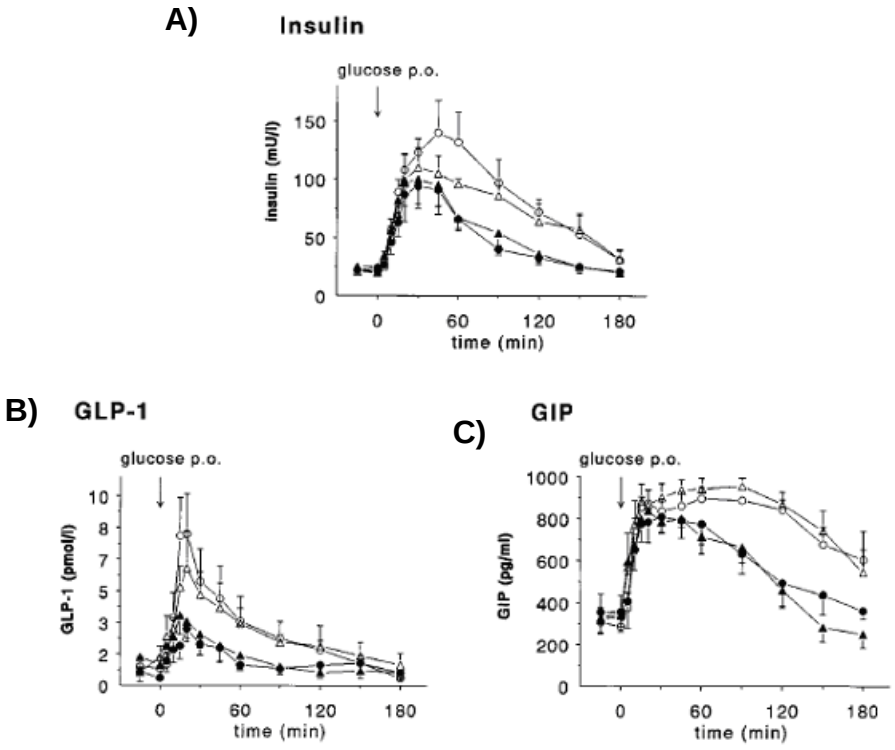

Figure 5: Response ton oral glucose ingestion. The release of A) insulin B) glucagon like peptide-1 (GLP-1) and C) gastric inhibitory peptide (GIP) are stimulated by an increase in the plasma glucose concentration. The oral ingestion of glucose ranged from 50 to 100 grams. Image adapted from (Schirra, Katschinski et al. 1996).

L cells naturally release GLP-1 from secretory vesicles in response to nutrient stimulation. The acute response exhibited by GLP-1 is due to its release from secretory vesicles in response to nutrient ingestion. The release of GLP-1 mimics the acute and transient first phase of beta-cell insulin secretion (Schirra, Katschinski et al. 1996)—the phase not reproduced by recombinant hepatic cells. This innate glucose-responsiveness can be targeted through genetic engineering to express insulin. *EE cells offer a promising ancillary system for treating diabetes when combined with a cell type capable of producing a second phase release of insulin such as hepatic cells.*

A pure population of primary L cells would be the ideal culture to study the response of GLP-1 to a secretagogue. Unfortunately, cells isolated from the intestinal epithelium constitute a mixed population which, currently, cannot be separated into a single-cell type. The isolated cells typically contain only 10-30% L cells (Reimann and Gribble 2002; Reimann, Ward et al. 2006). Therefore, the purification of a primary cell population to a single-cell type is not possible at this time. A solution to this problem is EE cell lines, which provide a homogeneous population for these studies.

2.3.4.2.1 L cell line: NCI-H716

After transducing an L cell line (human NCI-H716 cells) with an adeno-associated virus (AAV) containing an insulin gene, the recombinant cells (rNCI-H716) responded to secretagogue stimulations by secreting insulin with the same kinetics as their release of GLP-1 (Figure 6). The engineered EE cell line responded acutely simulating the first phase of insulin secretion from a beta cell. This system could potentially eliminate the glycemic excursions in the blood when combined with a cell releasing insulin in a sustained manner; providing a more physiological condition for the patient.

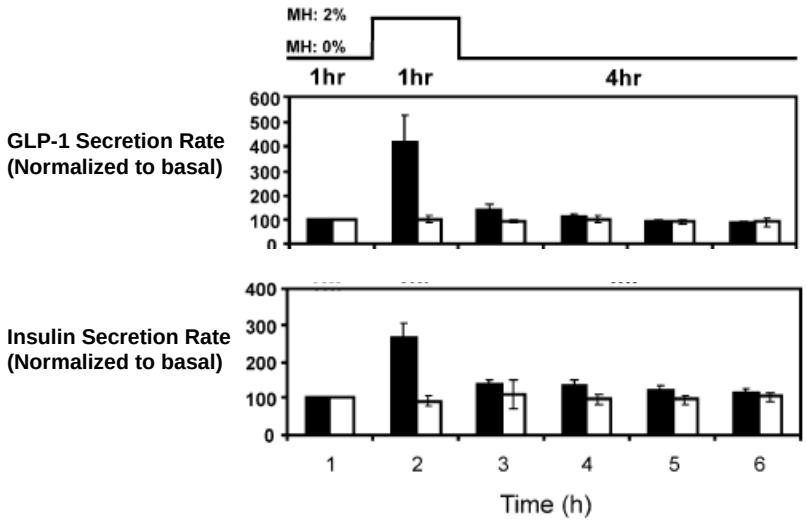

Figure 6: Response of a recombinant L cell line to a nutrient stimulation. NCI-H716 cells were transduced with an adeno-associated virus (AAV) containing insulin and enhanced green fluorescent protein (EGFP). The rNCI-H716 cells experienced a 2.7 fold increase in insulin secretion after stimulation with meat hydrolysate (a nutrient secretagogue). The secretion dynamics mimicked the 4.2 fold increase in glucagon like peptide-1 (GLP-1) after the same stimulation with meat hydrolysate.
The graphs are adapted from (Tang and Sambanis 2003a).

It has been demonstrated that NCI-H716 genetically engineered to express insulin will accumulate insulin in the same secretory granules used to store GLP-1 (Tang and Sambanis 2003a). The cells are co-localize insulin and GLP-1 in the same secretory granules and the contents of the granules are released upon stimulation with a nutrient secretagogue; therefore, experiencing similar secretion kinetics.

This cell line was not used in the experiments included in this thesis, but it does exemplify the insulin secretion kinetics of recombinant L cells.

2.3.4.2.2 L cell line: GLUTag

A transgenic mouse L cell line (GLUTag) was developed to study the signaling pathways responsible for the release of GLP-1 in response to physiologic stimuli. This cell line is a homogenous population of L cells and contains the proglucagon promoter

controlling the SV40 large T antigen (Reimann, Maziarz et al. 2005). As seen with NCI-H716 cells, GLUTag cells should also express the prohormone convertases, PC 1/3 and PC 2, required to process proglucagon into glucagon releasing the GLP-1 molecule. These are also the same endoproteases required to process proinsulin into insulin. Therefore, the GLUTag cells do not require the furin mutated PPI cDNA to allow for proteolytic processing.

To analyze insulin secretion from this glucose-responsive cell line, recombinant GLUTag cells were developed in the Sambanis lab by Heather Bara. The human B10 mutated insulin gene was stably transfected into GLUTag cells. The gene was expressed along with neomycin resistance allowing for selection of a stable clone (Figure 7). The most robust insulin secreting colony was selected for this study and named GLUTag-INS (Bara 2007). These cells were demonstrated to express both PC 1/3 and PC 2 prohormone convertases (Bara and Sambanis, in preparation).

Figure 7: Plasmid used for generation of the stable GLUTag clone (GLUTag-INS). The plasmid contains the cytomegalovirus promoter (CMV), human B10 modified PPI (hB10 Insulin), SV40 promoter (SV40) and neomycin resistance (R_{neo}). Diagram obtained from Heather Bara.

2.3.4.2.3 K cell line

Another EE cell type, K cells, has been targeted by Cheung et al and Han et al for glucose-responsive insulin secretion (Cheung, Dayanandan et al. 2000; Han, Lee et al. 2007). Han et al created a stable insulin secreting clone from STC-1 cells (a mixed population of mouse EE cells). These cells were implanted into diabetic mice and restored normoglycemia within 2 weeks of implantation. Normoglycemia was

maintained for 2 to 3 weeks post-implantation after which mice became hypoglycemic (Han, Lee et al. 2007).

2.4 Combination Approach

Recombinant hepatic and recombinant EE cells have been demonstrated to have differing insulin secretion dynamics. Recombinant hepatic cells better approximate the slower and more sustained second phase of insulin secretion from a beta-cell while the recombinant EE cells approximate the acute first phase of insulin secretion. Neither cell type alone could reproduce the secretory dynamics of a normal, functioning beta-cell. *A combination of hepatic and EE cells is proposed to mimic the biphasic response of a functioning beta cell. It should more accurately reproduce beta-cell kinetics than with either cell type alone.*

CHAPTER 3

MATERIALS AND METHODS

3.1: Cell Culture and Transduction

3.1.1 Recombinant Intestinal Cell Line: GLUTag-INS

GLUTag-INS were developed by Heather Bara in the Sambanis lab (Georgia Institute of Technology, Atlanta, GA) by stable transfection of GLUTag cells obtained from Dr. Brubaker (University of Toronto, Toronto, Canada) with the permission of Dr. Drucker (University of Toronto, Toronto, Canada). The transgene for stable insulin expression was constructed by Heather Bara who inserted the human B10 mutated insulin gene (Genentech, San Francisco, CA) into the EcoRI site of the pcDNA3.1(+) vector (Invitrogen, Carlsbad, CA) (Figure 7). Selection of a stable clone was facilitated by the Geneticin resistance of the vector.

The GLUTag-INS cells were grown in culture medium (Dulbecco's Modified Eagle's Medium (DMEM, Sigma) supplemented with 10% Fetal Bovine Serum (FBS, Sigma), and 1% penicillin/streptomycin (pen/strep, Sigma) in a humidified incubator at 37^{o}C and 5% CO_2. They were split at a ratio of 1:5 at approximately 80% confluency.

3.1.2 Recombinant Hepatic Cell Lines: rHepG2 and rH4IIE

HepG2 cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA) and were propagated in culture medium (Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FBS, and 1% pen/strep) in a humidified incubator at 37^{o}C and 5% CO_2. Culture flasks were coated with 0.4mg/mL PureCol

collagen (Inamed Biomaterials, Fremont, CA). The cells were split at a ratio of 1:6 at approximately 80% confluency.

H4IIE cells were obtained from the ATCC (Manassas, VA). The cells were propagated in culture medium (DMEM supplemented with 10% FBS, and 1% pen/strep) in a humidified incubator at 37^{o}C and 5% CO_2. They were split at a ratio of 1:10 at approximately 80% confluency

To produce recombinant HepG2 (rHepG2) and recombinant H4IIE (rH4IIE) cells, cells were transduced with either AdG3-InsTail, AdG3-Track or AdG3-2xfur (described in further detail in section 3.2.2), obtained from the laboratory of Dr. Peter Thulé (VA Hospital, Atlanta, GA). The adenoviruses incorporated the vectors described in Figure 3. The AdG3-InsTail virus contained three copies of the glucose sensitive promoter GlRE (G3) and furin compatible human insulin (2xfur) which was modified to enhance destabilization. The AdG3-2xfur virus contained the G3 promoter and furin compatible human insulin (2xfur). The AdG3-Track virus contained the G3-2xfur vector with an additional CMV promoter for GFP (Figure 3).

3.2 Secretion Studies

3.2.1 Intestinal Cell Line

Cells were plated on day -1 at a concentration of 1x10^{6} cells per well of a 6 well plate containing 2mL culture medium. The numbering convention for experimental days was based on the hepatic cells. The intestinal cells were not altered on day 0. Medium was changed on day 1 to BASAL medium (DMEM containing 5mM glucose (Sigma), 1x10^{-7}M Dexamethosone (DEX, Sigma), 2% FBS, 1% pen/strep. Samples were taken at 0, 12 and 24 hours after medium change on day 1.

On day 2 (24 hours after 0 hour sample), the medium was changed to either MH only stimulation (MH) medium [DMEM containing, 2% protein hydrolysate from meat (MH/peptone, Sigma), 10^{-7}M DEX, 2% FBS, 1% pen/strep], cocktail medium (cocktail) (DMEM containing 20mM glucose, 2% MH, 10^{-7}M DEX, 2% FBS, 1% pen/strep) or basal medium. Samples were taken at 24, 26, 28, 36 and 48 hours.

Insulin accumulation was calculated for each of the time points. It was assumed that the wells contained 0mM of insulin immediately after a change in medium. A total of four independent experiments (each containing 2 sampled wells) were averaged and the standard deviation and standard error between the experiments were calculated. A Grubb's T test was performed to determine the existence of outliers. The calculations showed that the experiment from 12/08/07 was an outlier for almost every well. Because of this statistical determination, the entire experiment was removed from the calculations. Hence, reported results are the averages of three independent experiments each containing 2 sampled wells. A complete listing of the data points may be found in Appendix A.

Insulin secretion rate (ISR) was calculated by determining the differential secreted insulin secretion between two time points and dividing this insulin amount by the time difference between the two points. The average, standard deviation and standard error were calculated for each ISR time point.

3.2.2 Hepatic Cell Lines

3.2.2.1 rHepG2 Cells

Cells were plated on day -1 at a concentration of $1x10^6$ cells per well of a 6 well plate containing 2mL culture medium. Cells were transduced in culture medium on day 0 with either AdG3-2xfur (Multiplicity of infection (MOI) =1), AdG3-Track (MOI=0.17) or

AdG3-InsTail (MOI=0.25). The MOI was calculated by dividing the plaque forming units (PFU) of each virus by the average number of cells per well. The PFU value for each virus was experimentally determined by the laboratory of Dr. Peter Thulé. Medium was changed on day 1 to basal medium. Samples were taken at 0, 12 and 24 hours after medium change on day 1.

On day 2 (24 hours after 0 hour sample), the medium was changed to either glucose only medium (glucose), cocktail, or basal medium (for control wells). Samples were taken at 24, 26, 28, 36 and 48 hours.

Insulin accumulation was calculated for each of the time points. It was assumed that the wells contained 0mM of insulin immediately after a change in medium. Four independent experiments (each containing 2 sampled wells) were averaged and the standard deviation and standard error between the experiments were evaluated. A Grubb's T test was performed to determine the existence of outliers. The 12/08/07 experiment was not performed on the HepG2 cells, therefore it was not necessary to remove this test from the calculations and only analyze three independent experiments. A complete listing of data points may be found in Appendix A.

Insulin secretion rate (ISR) was calculated as seen with the GLUTag-INS cells on the previous page.

3.2.2.2 rH4IIE Cells

Cells were plated on day -1 at a concentration of $0.5x10^6$ cells per well of a 6 well plate containing 2mL culture medium. Cells were transduced in culture medium on day 0 with either AdG3-2xfur (MOI=4), AdG3-Track (MOI=1) or AdG3-InsTail (MOI=4). The MOI was calculated as described in section 3.2.2.1 for rHepG2 cells. Media in all wells were changed on day 1 to BASAL medium. Samples were taken at 0, 12 and 24 hours after medium change on day 1.

On day 2, the medium was changed to glucose, cocktail or basal (for control wells) medium. Samples were taken at 24, 26, 28, 36 and 48 hours (after medium change on day 1).

Insulin accumulation was calculated for each of the time points as seen with the rHepG2 and GLUTag-INS cell lines. It was assumed that the wells contained 0mM of insulin immediately after a change in medium. The four independent experiments (each containing 2 sampled wells) were averaged and the standard deviation and standard error between the experiments were evaluated. A Grubb's T test was performed to determine the existence of outliers. The calculations showed that the experiment from 12/08/07 was an outlier for almost every well. Because of this statistical determination, the entire experiment was removed from the calculations and only three independent experiments were analyzed. A complete listing of the data points may be found in Appendix A.

Insulin secretion rate (ISR) was calculated as seen with the GLUTag-INS cells and rHepG2 cells on the previous pages.

3.3 Analytical Techniques

Insulin concentrations were measured using either a human insulin specific radioimmunoassay (RIA) kit (Linco Research, St. Charles, MO) or ultra sensitive human insulin RIA kit (Linco Research) depending on the insulin concentration in the sample. Radioactivity was determined using an Auto-Gamma Counting System, Cobra II (Packard, Meriden, CT).

Light and fluorescent microscopy pictures were captured using Olympus IX71 microscope and DP software (Olympus, Japan).

Cell number was measured daily using parallel wells for each vector type. The counted wells were exposed to the glucose only stimulation protocol. The viable cell

number was measured using 0.4% trypan blue (Sigma) at a ratio of 100μL cell suspension to 100 μL trypan blue.

Several two-way ANOVAs followed by the Tukey's post-hoc analysis were performed to determine the statistical significance of the data. A Grubb's T test was performed to determine if outliers could be removed from the calculations.

CHAPTER 4

RESULTS

This chapter describes results on the insulin secretion kinetics of recombinant hepatic and intestinal cell lines and the potential for combining these two cell types to reproduce the glucose responsive insulin secretion kinetics of a normal, functioning beta-cell. The adenovirus vectors containing glucose responsive promoters and furin compatible insulin were obtained from the laboratory of Dr. Peter Thulé. The stably transfected GLUTag-INS cells were developed in the Sambanis lab by Heather Bara.

For the data in this chapter, two hepatic cell lines were transduced with these viruses and subjected to secretion studies to analyze the insulin secretion kinetics in response to glucose only or a cocktail of nutrient secretagogues [meat hydrolysate (MH) and glucose]. With GLUTag-INS cells, the secretion studies were performed to analyze the insulin secretion kinetics of these cells in response to a stimulus of MH only or a cocktail of MH and glucose.

4.1 Stimulation Response of Recombinant H4IIE Cells

The rH4IIE cells were transduced with either AdG3-2xfur (MOI = 4), AdG3-Track (MOI = 1) or AdG3-InsTail (MOI = 4). Following transduction, cells were maintained in basal medium for 24 hours and then changed to either stimulation medium or fresh basal medium for control cells for an additional 24 hours (48 hours total). Stimulation media consisted of either 20mM glucose or a cocktail of 20mM glucose and 2% MH.

4.1.1 Stimulation Response of H4IIE Cells Transduced with AdG3-2xfur

At the 48 hour time point (24 hours of insulin accumulation), the H4IIE cells transduced with AdG3-2xfur and stimulated by the cocktail medium exhibited a significant increase in accumulated insulin over the cells stimulated by only glucose medium ($p \leq 0.001$) ($n = 3$) and the control cells ($p \leq 0.001$) ($n = 3$) (Figure 8). The cocktail stimulated cells exhibited a significant increase in accumulated insulin over time ($p < 0.05$). The glucose only and control cells did not experience a statistically significant increase in insulin secretion over time ($p > 0.05$).

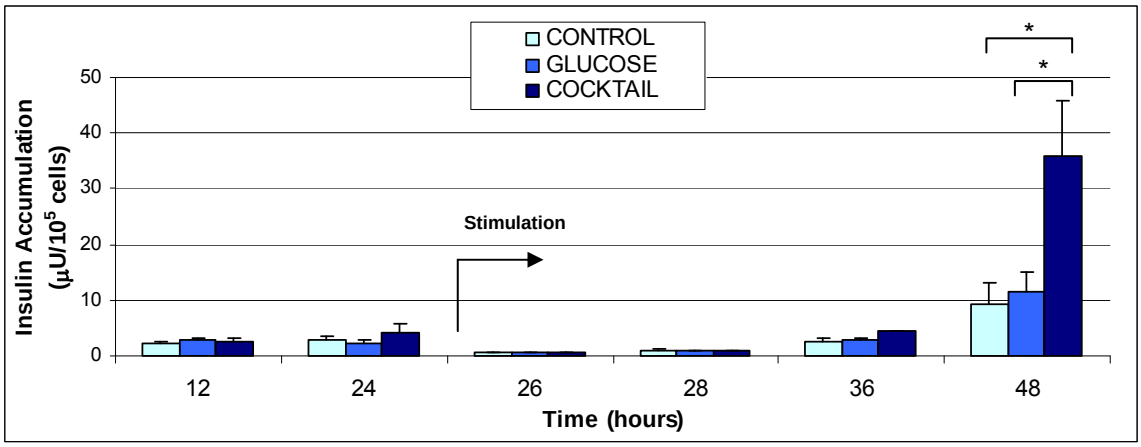

Figure 8: Insulin accumulation of H4IIE cells transduced with AdG3-2xfur. The H4IIE cells were plated in 6 well plates, transduced with AdG3-2xfur (MOI=4), and were exposed to basal medium for 24 hours then stimulated with either basal medium (for control cells), glucose only medium, or a cocktail of glucose and meat hydrolysate medium. Insulin accumulation was measured over time. The cocktail stimulated cells also exhibited a significant increase in insulin accumulation over time ($p < 0.05$) when comparing 48 to 28 and 48 to 36 hours, but not when comparing 36 to 28 hours. The cells stimulated by only glucose and the control cells did not exhibit a significant increase in insulin accumulation over time ($p > 0.05$) when comparing 48 to 36, 48 to 28 or 36 to 28. *$p \leq 0.001$; bars are ± SE; $n = 3$ for each data point; arrow indicates change in medium to stimulation medium.

4.1.2 Stimulation Response of H4IIE Cells Transduced with AdG3-Track

At the 48 hour time point (24 hours of insulin accumulation), H4IIE cells transduced with AdG3-Track and stimulated by the cocktail medium did not exhibit a significant increase in accumulated insulin over the cells stimulated by only glucose medium (n = 3) and the control cells (n = 3) (Figure 9). Also, the cells stimulated by glucose only medium did not exhibit a significant increase in accumulated insulin secretion over the control cells ($p>0.05$). The cocktail stimulated cells exhibited a significant increase in accumulated insulin over time ($p < 0.05$). The cells stimulated by glucose only medium did not experience a significant increase in accumulated insulin over time ($p > 0.05$). The control cells did not exhibit a significant increase in insulin secretion over time ($p > 0.05$)

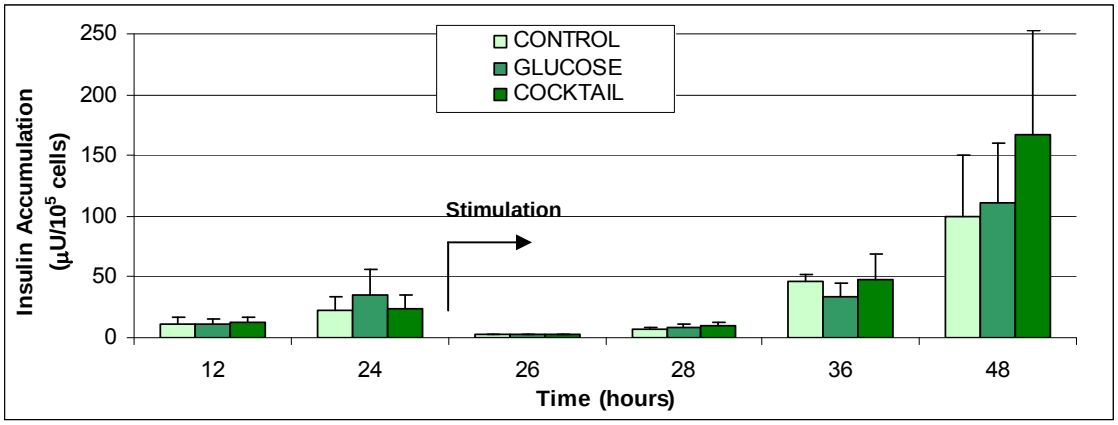

Figure 9: Insulin accumulation of H4IIE cells transduced with AdG3-Track. The H4IIE cells were transduced with AdG3-Track (MOI = 1) and were exposed to basal medium for 24 hours then stimulated with either basal medium (for control cells), glucose only medium, or a cocktail of glucose and meat hydrolysate medium. The cocktail stimulated cells exhibited a significant increase in insulin accumulation over time ($p < 0.05$) when comparing 48 to 28 and 48 to 36 hours, but not when comparing 36 to 28. The glucose only stimulated cells exhibited a significant increase in insulin secretion over time ($p < 0.05$) when comparing 48 to 28 hours, but not when comparing 48 to 36 and 36 to 28 ($p > 0.05$). The control cells did not exhibit a significant increase in insulin secretion over time ($p > 0.05$). *$p \leq 0.001$; bars are ± SE; n = 3 for each data point; arrow indicates change in medium to stimulation medium.

Fluorescent imaging of the transduced cells showed the high transduction efficiency of the AdG3-Track virus. It appears that almost 100% of the cells were effectively transduced.

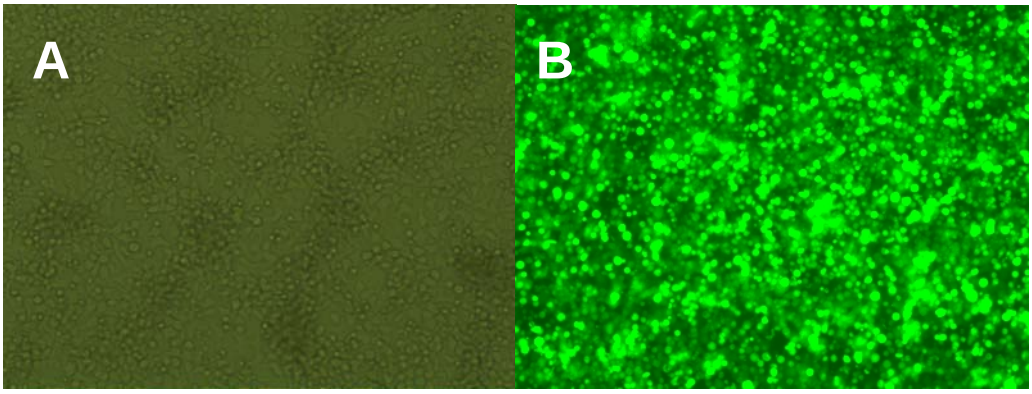

Figure 10: Microscope images of H4IIE transduced with AdG3-Track. The H4IIE were transduced in 6 well plates at MOI of 1. The wells were imaged using a fluorescent microscope to compare the same section of the plate. A) light image of rH4IIE cells B) fluorescent image of rH4IIE cells (mag x10)

4.1.3 Stimulation Response of H4IIE Cells Transduced with AdG3-InsTail

At the 48 hour time point (24 hours of insulin accumulation), H4IIE cells transduced with AdG3-InsTail and stimulated by the cocktail medium exhibited a significant increase in accumulated insulin over the cells stimulated by only glucose medium ($p \leq 0.001$) ($n = 3$) and the control cells ($p \leq 0.001$) ($n = 3$) (Figure 11). The cocktail stimulated cells exhibited a significant increase in accumulated insulin over time ($p \leq 0.001$). The cells stimulated by glucose only medium also experienced a significant increase in insulin expression over time ($p \leq 0.05$). The AdG3-Ins Tail cells also exhibited significantly less insulin secretion after 24 hours of stimulation with cocktail medium (1.61 ± standard deviation of 0.15 $\mu U/10^5$ cells) compared to the AdG3-Track

(167.14 ± standard deviation of 85.03 μU/10^5 cells) and AdG3-2xfur cells (36.04 ± standard deviation of 9.90 μU/10^5 cells).

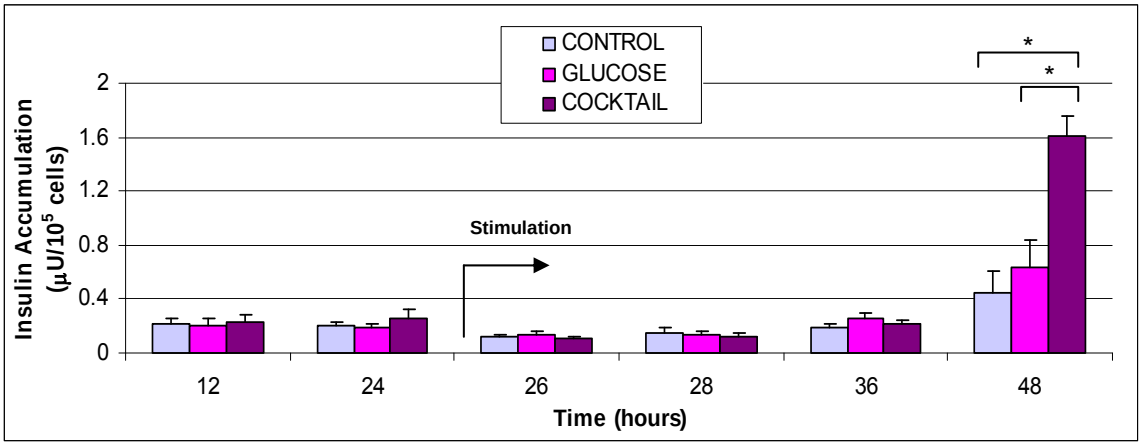

Figure 11: Insulin accumulation of H4IIE cells transduced with AdG3-InsTail. The H4IIE cells were plated in 6 well plates, transduced with AdG3-InsTail (MOI = 4), and were exposed to basal medium for 24 hours then stimulated with either basal medium (for control cells), glucose only medium, or a cocktail of glucose and meat hydrolysate medium. The cocktail stimulated cells exhibited a significant increase in insulin accumulation over time ($p \leq 0.001$) when comparing 48 to 28 and 48 to 36 hours, but not when comparing 36 to 28 The cells stimulated by glucose only medium exhibited a significant increase in insulin accumulation over time ($p \leq 0.05$) when comparing 48 to 28 and 48 to 36 hours. The control cells did not experience a significant increase in insulin over time when comparing 48 to 28, 48 to 36 or 36 to 28 ($p > 0.05$). *$p \leq 0.001$; bars are ± SE; n = 3 for each data point; arrow indicates change in medium to stimulation medium.

4.2 Stimulation Response of Recombinant HepG2 Cells

The HepG2 cells were transduced with either AdG3-2xfur (MOI = 1), AdG3-Track (MOI = 0.17) or AdG3-InsTail (MOI = 0.25). The cells were maintained in basal medium for 24 hours and then changed to stimulation medium for an additional 24 hours (48 hours total). Stimulation was either 20mM glucose or a cocktail of 20mM glucose and 2% MH.

4.2.1 Response of HepG2 Cells Transduced with AdG3-2xfur

At the 48 hour time point (24 hours of insulin accumulation), the HepG2 cells transduced with AdG3-2xfur and stimulated by the cocktail medium exhibited a significant increase in accumulated insulin over the cells stimulated by only glucose medium ($p \leq 0.001$) ($n = 3$) and the control cells ($p \leq 0.001$) ($n = 3$) (Figure 12). The cocktail stimulated cells exhibited a significant increase in accumulated insulin over time ($p \leq 0.001$).

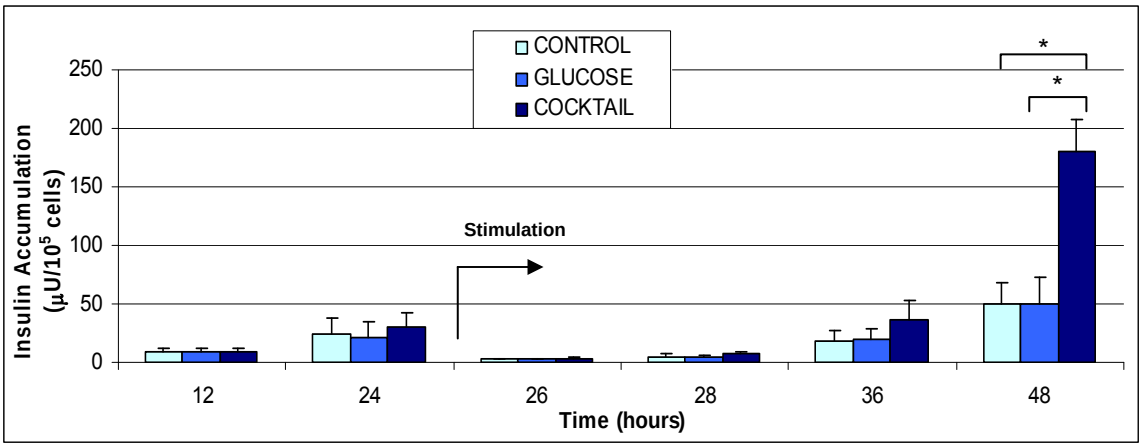

Figure 12: Insulin accumulation in HepG2 cells transduced with AdG3-2xfur. The HepG2 cells were transduced with AdG3-2xfur (MOI = 1) and exposed to basal medium for 24 hours then stimulated with either basal medium (for control cells), glucose only medium, or a cocktail of glucose and meat hydrolysate medium. The cocktail stimulated cells exhibited a significant increase in insulin accumulation over time ($p \leq 0.001$) when comparing 48 to 28 and 48 to 36 hours, but not when comparing 36 to 28 ($p > 0.05$). The cells stimulated by only glucose medium did not experience a significant increase in insulin over time when comparing 48 to 28, 48 to 36 and 36 to 28 ($p > 0.05$). The control cells did not experience a significant increase in insulin over time when comparing 48 to 28, 48 to 36 and 36 to 28 ($p > 0.05$). *$p \leq 0.001$; bars are ± SE; n = 3 for each data point; arrow indicates change in medium to stimulation medium.

4.2.2 Response of HepG2 Cells Transduced with AdG3-Track

At the 48 hour time point (24 hours of insulin accumulation), HepG2 cells transduced with AdG3-Track and stimulated by the cocktail medium exhibited a significant increase in accumulated insulin over the cells stimulated by only glucose medium ($p \leq 0.001$) ($n = 3$) and the control cells ($p \leq 0.001$) ($n = 3$) (Figure 13). The cocktail and glucose only stimulated cells exhibited a significant increase in accumulated insulin over time ($p \leq 0.05$).

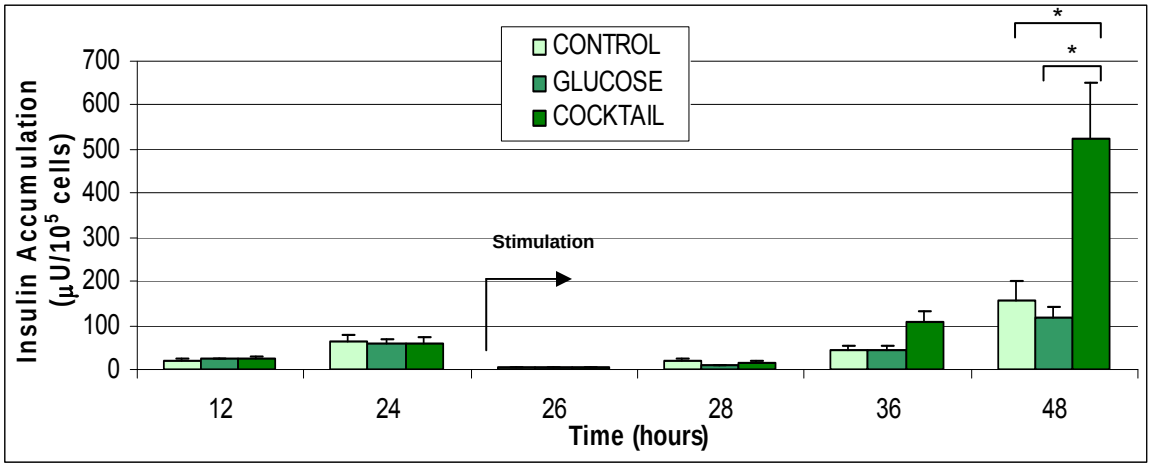

Figure 13: Insulin accumulation in HepG2 cells transduced with AdG3-Track. The HepG2 cells were transduced with AdG3-Track (MOI = 0.17) and exposed to basal medium for 24 hours then stimulated with either basal medium (for control cells), glucose only medium, or a cocktail of glucose and meat hydrolysate medium. Insulin accumulation was measured over time. The cocktail stimulated cells exhibited a significant increase in insulin accumulation over time ($p \leq 0.001$) when comparing 48 to 26, 48 to 28, and 48 to 36 hours, but not when comparing 36 to 28 did not experience a significant increase in insulin over time when comparing 48 to 28, 48 to 36 and 36 to 28 ($p > 0.05$). The cells stimulated by only glucose medium experienced a significant increase in insulin over time ($p \leq 0.05$) when comparing 48 to 28 and 48 to 36 hours, but not when comparing 36 to 28 ($p > 0.05$). The control cells experienced a significant increase in insulin secretion over time ($p \leq 0.05$) when comparing 48 to 28, and 48 to 36 hours, but not when comparing 36 to 28 ($p > 0.05$). *$p \leq 0.001$; bars are ± SE; $n = 3$ for each data point; arrow indicates change in medium to stimulation medium.

Fluorescent imaging of the HepG2 cells transduced with AdG3-Track demonstrated the high transduction efficiency of the virus. It appears that almost 100% of the cells were transduced by the virus (Figure 14).

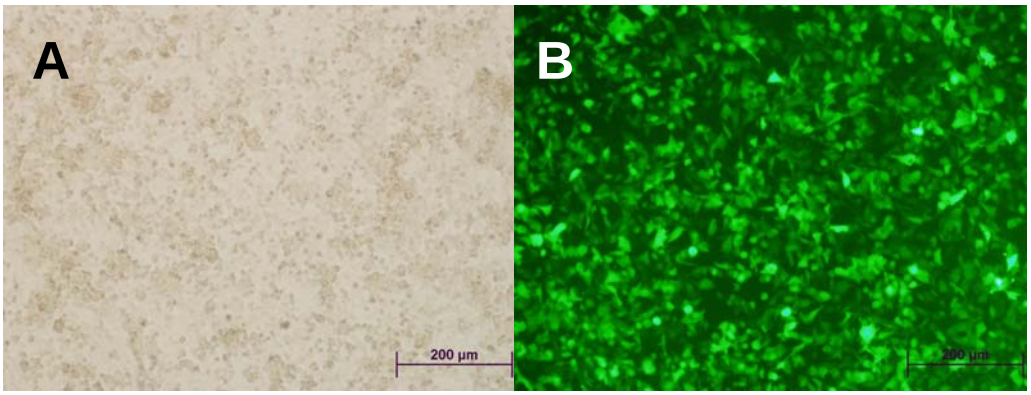

Figure 14: Microscope images of HepG2 Cells transduced with AdG3-Track. The HepG2 cells were transduced with AdG3-Track in 6 well plates at MOI of 0.17. The wells were imaged using a fluorescent microscope to compare the same section of the plate. A) light image of rHepG2 cells B) fluorescent image of rHepG2 cells (mag x10)

4.2.3 Response of HepG2 Cells Transduced with AdG3-InsTail

At the 48 hour time point (24 hours of insulin accumulation), HepG2 cells transduced with AdG3-InsTail and stimulated by the cocktail medium exhibited a significant increase in accumulated insulin over the cells stimulated by only glucose medium ($p \leq 0.001$) ($n = 3$) and the control cells ($p \leq 0.001$) ($n = 3$) (Figure 15). The cocktail stimulated cells exhibited a significant increase in accumulated insulin over time ($p \leq 0.001$). The glucose only stimulated cells and control cells exhibited an increase in insulin secretion with time which was less pronounced than the cocktail stimulated cells. The AdG3-Ins Tail cells also exhibited significantly less insulin secretion after 24 hours of stimulation with cocktail medium (2.88 ± standard deviation of 0.52 $\mu U/10^5$ cells)

compared to the AdG3-Track (119.36 ± standard deviation of 24.88 μU/10^5 cells) and AdG3-2xfur cells (180.33 ± standard deviation of 26.84 μU/10^5 cells).

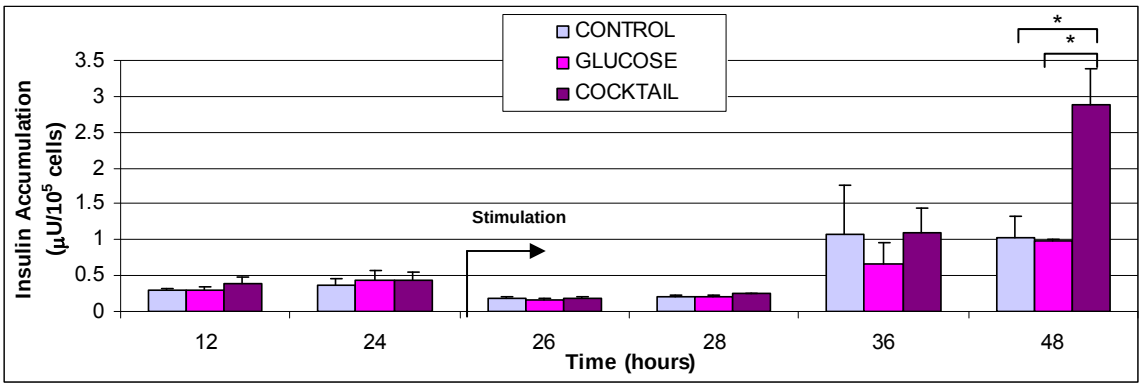

Figure 15: Insulin accumulation in HepG2 cells transduced with AdG3-InsTail. The HepG2 cells were transduced with AdG3-InsTail (MOI = 0.25) and exposed to basal medium for 24 hours then stimulated with either basal medium (for control cells), glucose only medium, or a cocktail of glucose and meat hydrolysate medium. Insulin accumulation was measured over time. The cocktail stimulated cells exhibited a significant increase in insulin accumulation over time ($p \leq 0.001$) when comparing 48 to 26, 48 to 28, and 48 to 36 hours, but not when comparing 36 to 28 ($p > 0.05$). The cells stimulated by only glucose medium experienced a significant increase in insulin over time ($p \leq 0.05$) when comparing 48 to 28 hours, but not when comparing 36 to 28 ($p > 0.05$). The control cells experienced a significant increase in insulin secretion when comparing 48 to 28, but not when comparing 48 to 36 and 36 to 28 ($p > 0.05$). *$p \leq 0.001$; bars are ± SE; n = 3 for each data point; arrow indicates change in medium to stimulation medium.

4.3 Stimulation Response of GLUTag-INS Cells

GLUTag-INS cells were stably transfected with the vector shown in Figure 7 and stimulated with either 2% MH alone (MH) or a cocktail of 20mM glucose and 2% MH. The cells were maintained in basal medium for 24 hours and then changed to stimulation medium for an additional 24 hours (48 hours total).

At the 48 hour time point (24 hours of insulin accumulation), GLUTag-INS stimulated by the cocktail medium exhibited a significant increase in accumulated insulin over the control cells ($p \leq 0.05$) (n = 3) (Figure 16). The GLUTag-INS cells stimulated by MH only (MH) also exhibited a significant increase in accumulated insulin over the

control cells ($p \leq 0.05$). There was no difference between MH only stimulated cells and cocktail stimulated at 48 hours.

The cocktail stimulated GLUTag-INS cells exhibited a significant increase in accumulated insulin over time ($p \leq 0.001$). The MH stimulated GLUTag-INS cells also exhibited a significant increase in accumulated insulin over time ($p \leq 0.001$)

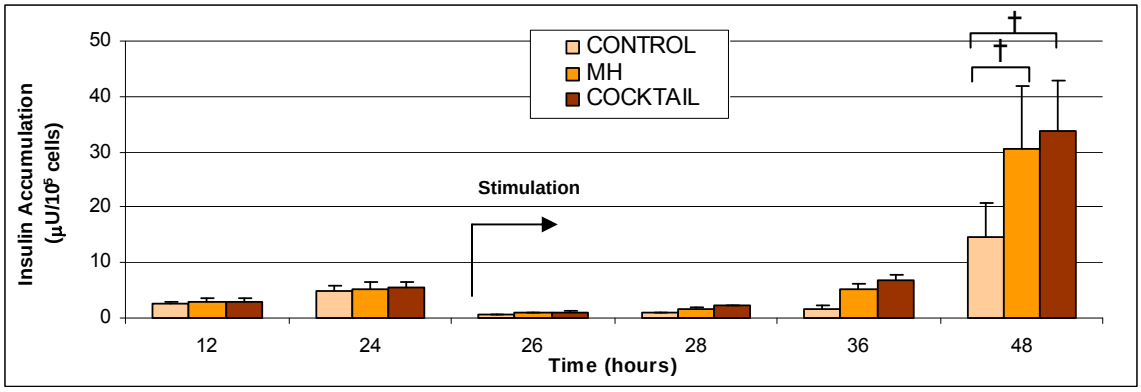

Figure 16: Insulin accumulation in GLUTag-INS cells. GLUTag-INS cells were stably transfected and exposed to basal medium for 24 hours then stimulated with either basal medium (for control cells), meat hydrolysate (MH) only medium, or a cocktail of glucose and meat hydrolysate medium. Insulin accumulation was measured over time. The cocktail stimulated cells exhibited a significant increase in insulin accumulation over time ($p \leq 0.05$) when comparing 48 to 28, 48 to 36 and 36 to 26 hours. The cells stimulated by only MH medium exhibited a significant increase in insulin accumulation over time ($p \leq 0.05$) when comparing 48 to 26, 48 to 28 and 48 to 36 hours, but not when comparing 36 to 28 ($p > 0.05$). The control cells exhibited a significant increase in insulin accumulation over time ($p \leq 0.05$) when comparing 48 to 26 hours and 48 to 36, but not when comparing 36 to 28 ($p > 0.05$). † $p \leq 0.05$; bars are ± SE; n = 3 for each data point; arrow indicates change to stimulation medium.

CHAPTER 5

DISCUSSION

5.1 Comparison of Adenovirus Vectors within H4IIE and HepG2 Cells

Cell sourcing is a critical issue in creating a cell-based therapy for IDD. Non-beta cells such as hepatic and intestinal cells offer potentially autologous cell sources. It has been shown in this paper that recombinant hepatic cells will respond to a glucose stimulation, but exhibit sluggish kinetics. These cells must be combined with a complimentary cell type, such as recombinant EE cells, which provide the initial burst of insulin from secretory granules.

Three adenoviral vectors were analyzed in HepG2 and H4IIE hepatic cell lines. The three vectors exhibited various responses to stimulation. This was demonstrated when recombinant hepatic cells transduced with AdG3-2xfur, AdG3-Track, and AdG3-InsTail were stimulated with the cocktail medium. The data reported in Figures 8, 9, 11, 12, 13, and 15 have been re-graphed to compare the three vectors in H4IIE (Figure 17A) and HepG2 (Figure 17B) cells under the same stimulation condition.

At the 48 hour time point (24 hours of induced insulin accumulation), the AdG3-Track vector secreted significantly more insulin compared to the AdG3-2xfur ($p \leq 0.001$) and AdG3-InsTail ($p \leq 0.001$) vectors in both H4IIE and HepG2 cells (Figure 17). The H4IIE and HepG2 cells transduced with AdG3-2xfur also secreted significantly more insulin than AdG3-InsTail ($p \leq 0.001$).

A

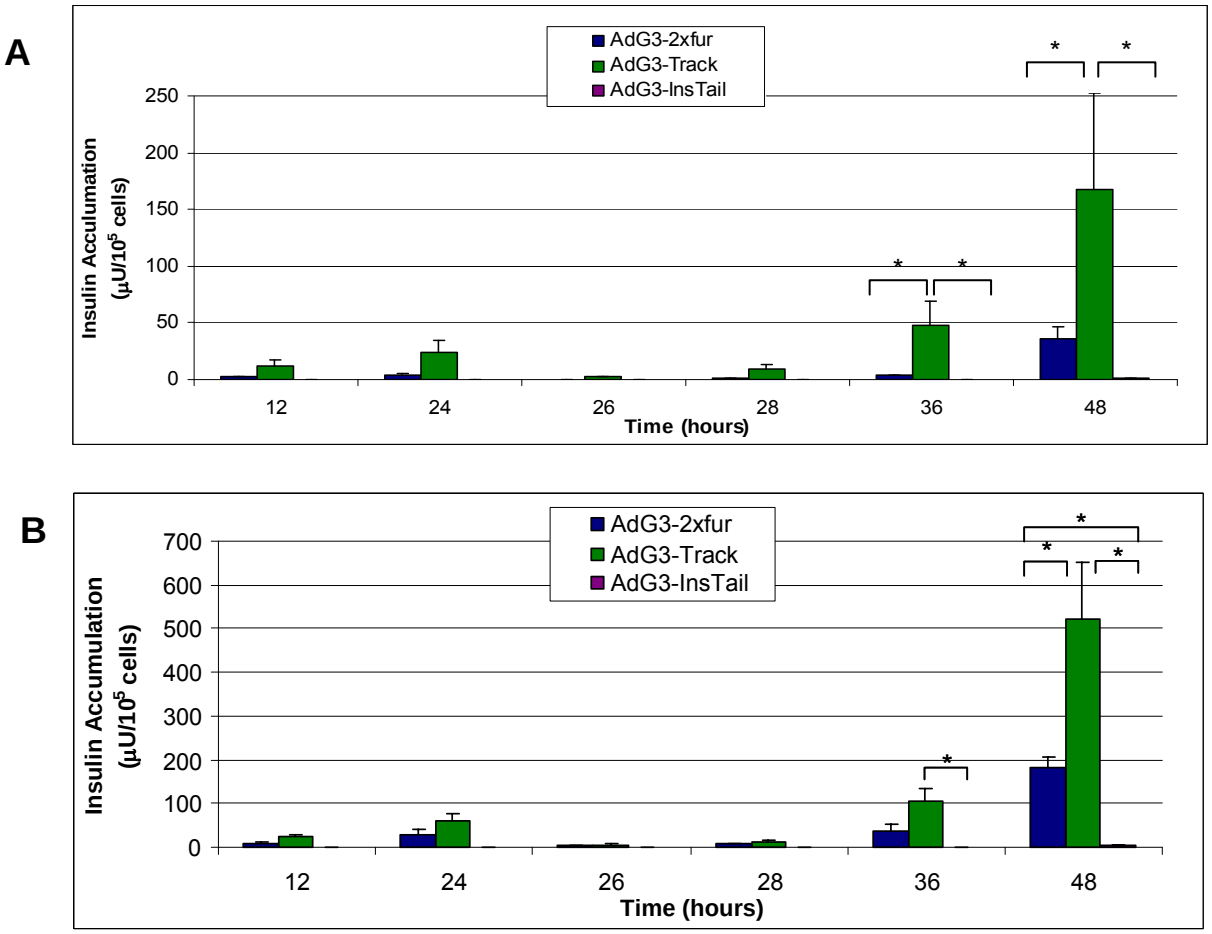

Figure 17: Comparison of vectors stimulated with cocktail medium. A) H4IIE Cells transduced with either AdG3-2xfur, AdG3-Track or AdG3-InsTail. B) HepG2 Cells transduced with either AdG3-2xfur, AdG3-Track or AdG3-InsTail. The HepG2 and H4IIE cells transduced with AdG3-Track secreted significantly more insulin than AdG3-2xfur and AdG3-InsTail. Also AdG3-2xfur secreted significantly more insulin than AdG3-InsTail. *p ≤ 0.001; values are ± SE; n = 3 for each data point.

After stimulation with cocktail medium, AdG3-InsTail secreted less insulin likely due to the expedited decay of the engineered PPI mRNA allowing for faster elimination of the mRNA. This engineered PPI vector also required an MOI value 4 fold more than AdG3-Track in rH4IIE cells. Despite its lower MOI, AdG3-Track in H4IIE cells exhibited approximately 4.6 fold more insulin secretion than AdG3-2xfur and approximately 103.8 fold more than AdG3-InsTail. The HepG2 cells were transduced with a 6 fold higher MOI of AdG3-2xfur and 4 fold higher MOI of AdG3-InsTail when comparing to AdG3-Track, however, AdG3-Track resulted in higher insulin secretion (2.9 fold more insulin

secretion with AdG3-Track versus AdG3-2xfur and 181.3 fold more with AdG3-Track versus AdG3-InsTail). This difference is more pronounced than that of primary cells *in vivo* which required 1.75 fold more AdG3-InsTail than AdG3-2xfur to restore normoglycemia in mice (unpublished work by Thule et al). Therefore, indicating that AdG3-2xfur secretes 1.75 fold more insulin than AdG3-InsTail and the cell lines exhibited a larger loss of expression than primary cells.

The H4IIE and HepG2 cells transduced with AdG3-Track experienced almost 100% transduction efficiency and GFP expression with MOI values of 1 and 0.17, respectively, when viewed with a fluorescent microscope (Figure 10 and Figure 14). Despite a low MOI value of less than 1 viral particle per cell in HepG2 cells, almost 100% of the cells were expressing GFP. The calculation for MOI is based on the measuring plaque forming units (PFU) and the number of cells per well. The PFU value is not an absolute measure of virus transduction efficiency in cells. Transduction efficiency is dependent on several factors including the cell type, confluency of plated cells and medium conditions. The PFU values for the vectors used here were determined using non-liver cells (Communication with Dr. Peter Thulé). Therefore, it is possible that the original PFU values were not completely accurate before performing the experiments consequently affecting the calculations for the MOI value. Comparison of transduction efficiency of AdG3-Track to the transduction efficiency of AdG3-2xfur and AdG3-InsTail using microscopy is not possible because the two latter vectors do not express fluorescence.

Assuming the calculations for PFU were accurate, composition of the vectors was evaluated to determine is difference there may explain how AdG3-Track secretes a larger amount of insulin and requires a lower MOI than AdG3-InsTail and AdG3-2xfur. The difference between AdG3-Track and AdG3-2xfur is the addition of the downstream CMV promoter and GFP. The CMV incorporated contained an immediate-early

promoter enhancer, which is capable of bidirectional transcription allowing the transcription of genes both upstream and downstream from the promoter (Grzimek, Podlech et al. 1999; Simon, Kuhnapfel et al. 2007). *This phenomenon suggests that AdG3-Track secreted more insulin possibly because the CMV promoter stimulated gene transcription of the upstream PPI gene.*

5.2 Effects of Secretagogues on rH4IIE, rHepG2 and GLUTag-INS cells

5.2.1 Effect of Meat Hydrolysate (MH)

The release of insulin from GLUTag-INS cells is related to the release kinetics of GLP-1 due to their assumed co-localization in secretory granules. To further understand the insulin secretion from these cells, we must have a better understanding of the GLP-1 secretion.

GLP-1 secretion is stimulated by various carbohydrates, fats and proteins (Reimann, Ward et al. 2006). The digestion of proteins in the stomach and intestine yields mainly amino acids and oligopeptides (Cordier-Bussat, Bernard et al. 1997). Peptones stimulate the release of secretory granules containing GLP-1. The mechanisms by which these stimulate GLP-1 secretion is poorly characterized (Reimer, Darimont et al. 2001). One type of protein hydrolysate or peptone, MH, has been shown to stimulate the release of GLP-1 from L cells (Cordier-Bussat, Bernard et al. 1998; Reimer, Darimont et al. 2001; Reimann, Ward et al. 2006; Reimer 2006).

It has been reported that peptones also stimulate proglucagon gene transcription in colonic proglucagon-expressing cell lines such as GLUTag and STC-1 (Cordier-Bussat, Bernard et al. 1998; Reimann, Ward et al. 2006). Peptones directly increase the level of glucagon mRNA though peptone-responsive elements in the promoter (Cordier-

Bussat, Bernard et al. 1997). The posttranslational processing of the proglucagon peptide results in GLP-1.

The increase in proglucagon gene transcription may also explain the effect of MH on hepatic cells. The mechanism for induction of proglucagon gene transcription is through a cascade of events which raise intracellular cAMP levels (Gevrey, Malapel et al. 2004). This is coupled with membrane depolarization and increased intracellular calcium which enhance GLP-1 secretion (Reimann, Ward et al. 2006). *Though the hepatic cells do not express proglucagon, the peptones may have a stimulation effect on the GlRE$_3$ promoter used in the rHepG2 and rH4IIE cells.*

It is important to note that MH was shown by Heather Bara to inhibit glucose induced insulin secretion from primary rat hepatocytes (unpublished data). This phenomenon contrasts the findings in this thesis which showed that MH potentiated glucose induced insulin secretion in hepatic cell lines. The reasoning for this phenomenon is unclear. This could possibly be explained with the differences between primary and immortalized cell lines. It has been reported that immortalized cell lines may experience alterations in glucose sensitivity as seen in some insulinoma cell lines (Reimann and Gribble 2002).

5.2.2 Effect of Glucose

The addition of glucose to stimulate GLUTag-INS cells did not increase the insulin secretion from the cells over stimulation with only MH (Figure 16). These cells are not responsive to glucose at this high (20mM) concentration. This is supported by the work of Reimann and Gribble who analyzed GLUTag cells under increasing concentrations of glucose (2002). It was determined that an increase in the glucose concentration from 0mM to 0.5 mM of glucose resulted in an increase in GLP-1 secretion, but there was no response to increasing concentrations of glucose from 5mM

to 25mM (Reimann and Gribble 2002; Gribble, Williams et al. 2003). This was also seen in primary L cell cultures by Damholt et al (1998). The cell cultures were under 2mM glucose during the basal period which was calculated to be reduced by the cells to 1.5 mM over 24 hours (using a glucose consumption rate of 7.9 $x10^{-5}$ mmol/hr-10^6 cells). Therefore, the cells consistently experienced over 0.5mM of glucose and were not starved before stimulation. Starving of the cells before stimulation may provide an exaggerated increase in insulin secretion after stimulation, which may be a metabolic effect and not due to glucose acting as a secretion agonist. This phenomenon is seen in beta-cells in a buffer solution, which experience lower insulin secretion both basally and after glucose stimulation when compared to cells in cell culture medium (Papas and Jarema 1998).

5.3 Comparison of ISR Between Hepatic and Intestinal cells

In order to compare the kinetics of insulin release in the recombinant hepatic and intestinal cell lines, the previous data were re-analyzed to calculate insulin secretion rate (ISR). It was determined that recombinant hepatic cells transduced with AdG3-InsTail did not secrete enough insulin to be a viable option for combining with the GLUTag-INS cells. The hepatic cells transduced with AdG3-2xfur and AdG3-Track were graphed together to demonstrate the differences in insulin secretion rates.

The GLUTag-INS cells experienced approximately a 2 fold increase in ISR between 26 and 28 hours while the rH4IIE cells transduced with AdG3-2xfur experienced only a 1.25 fold increase (Figure 18). The ISR demonstrates that GLUTag-INS cells respond with faster kinetics, which simulates the first phase of insulin secretion from the beta-cells. This burst of insulin release is due to the presence of secretory granules which are released upon stimulation. The ISR from rH4IIE cells transduced by AdG3-2xfur responded with slower kinetics simulating the second phase of insulin

secretion from beta-cells. This slower response is due to the lack of secretory granules and illustrates the slower kinetics of transcriptional regulation of insulin release. Combining recombinant hepatic and intestinal cell lines further illustrates their varying insulin secretion responses to stimulation. The burst of insulin released from the EE cells should quickly lower the blood glucose levels back to normal. The recombinant hepatic cells should maintain this lowered blood glucose level through its sustained insulin release. Similar trends were seen with HepG2 cells (Figure 19)

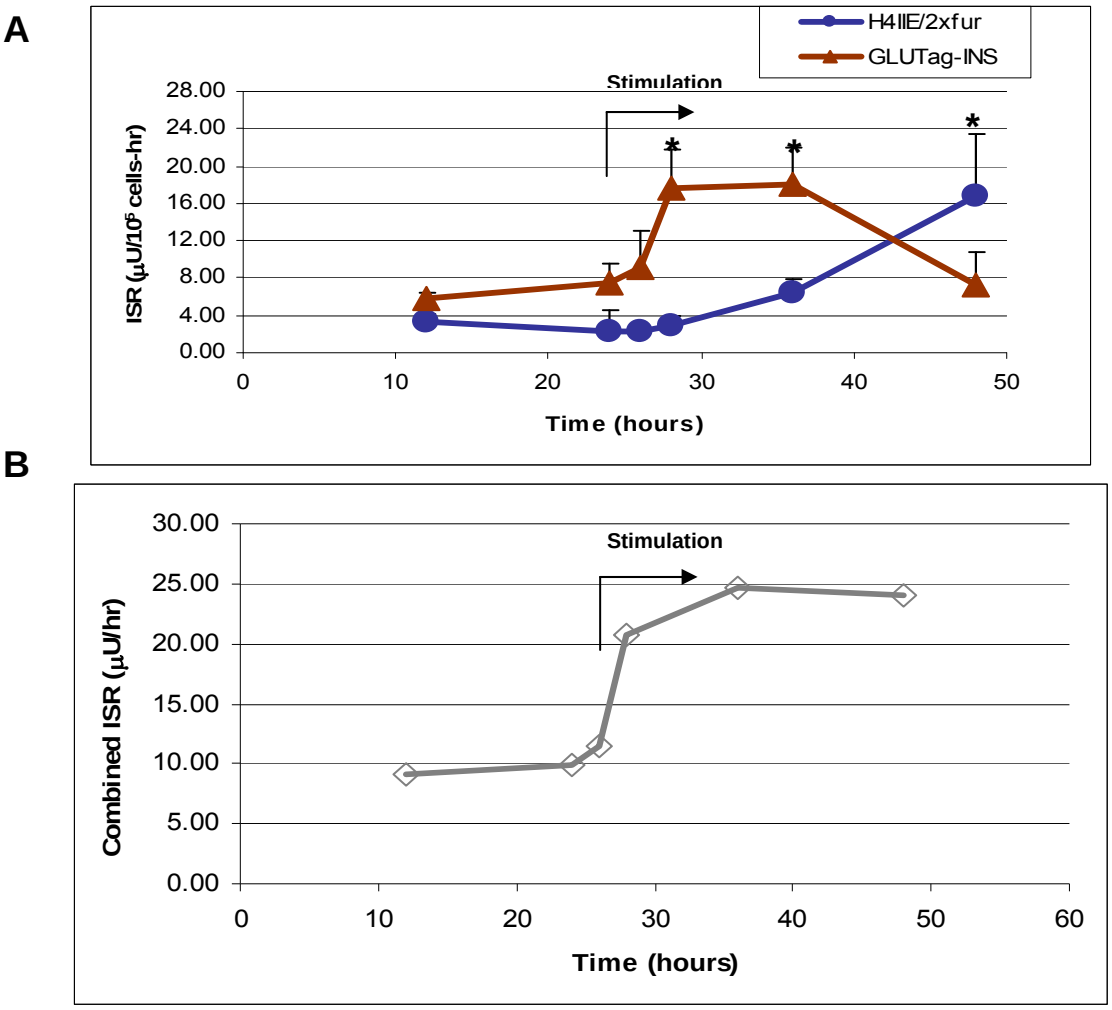

Figure 18: ISR for H4IIE transduced with AdG3-2xfur and GLUTag-INS cells. A) The data presented earlier have been re-analyzed to determine the insulin secretion rate (ISR) of H4IIE cells transduced with AdG3-2xfur and GLUTag-INS cells when stimulated with cocktail medium. B) The combined ISR values of the rH4IIE cells and GLUTag-INS cells were derived from the ISR values in Graph A and added at a cell ratio of 1:1 rH4IIE to GLUTag-INS to determine the total ISR. It was assumed that the two cell types had no interactions and that the total amount of insulin secreted would be the sum of the insulin secreted by each cell population. *$p \leq 0.001$; values are ± SE; n = 3 for each data point.

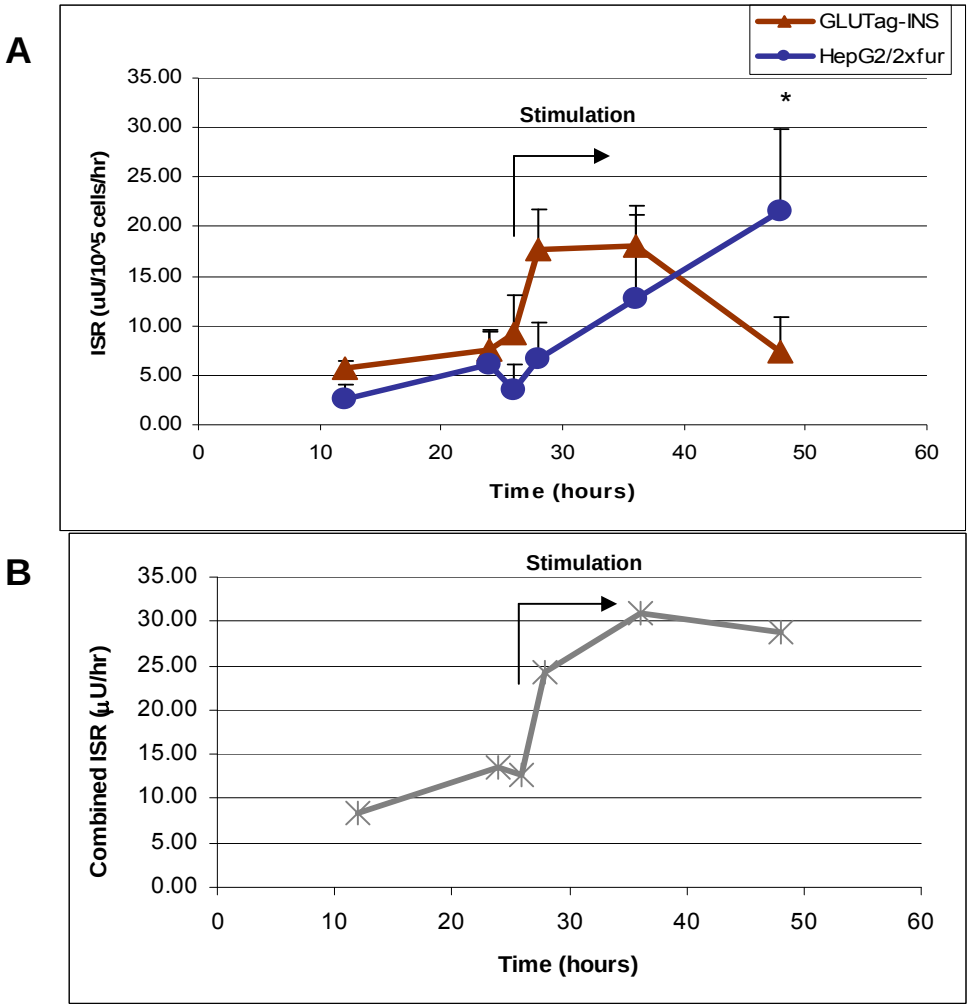

Figure 19: ISR for HepG2 transduced with AdG3-2xfur and GLUTag-INS cells. A) The data presented earlier have been re-analyzed to determine the insulin secretion rate (ISR) of HepG2 cells transduced with AdG3-2xfur and GLUTag-INS cells when stimulated at 24 hours with cocktail medium. B) The combined ISR values of the rHepG2 and GLUTag-INS cells were derived from the ISR values in Graph A and added at a cell ratio of 1:3 rH4IIE to GLUTag-INS to determine the total ISR. It was assumed that the two cell types had no interactions and that the total amount of insulin secreted would be the sum of the insulin secreted by each cell population. *$p \leq 0.001$; values are ± SE; n = 3 for each data point.

When H4IIE and HepG2 cells were transduced with AdG3-Track, they released significantly more insulin than GLUTag-INS cells. The cell number for the combination of cells was adjusted to compensate for the differences in the amounts of insulin accumulation. The AdG3-Track transduced H4IIE cells secreted significantly more insulin than those transduced with AdG3-2xfur (Figure 20). In order to combine the

H4IIE cells transduced with AdG3-Track with the GLUTag-INS cells, a ratio of 9 GLUTag-INS cells to 1 H4IIE cell transduced with AdG3-Track were combined. After adjusting the ratio for the number of cells, the two distinct phases seen by the different cell types was realized. As seen earlier, GLUTag-INS cells experienced a 2 fold increase in ISR between 26 and 28 hours which decreased after 36 hours. The H4IIE cells transduced with AdG3-Track experienced a slower and more sustained increase in insulin secretion which was maintained through 48 hours. Similar trends were seen with HepG2 cells transduced with AdG3-2xfur (Figure 21).

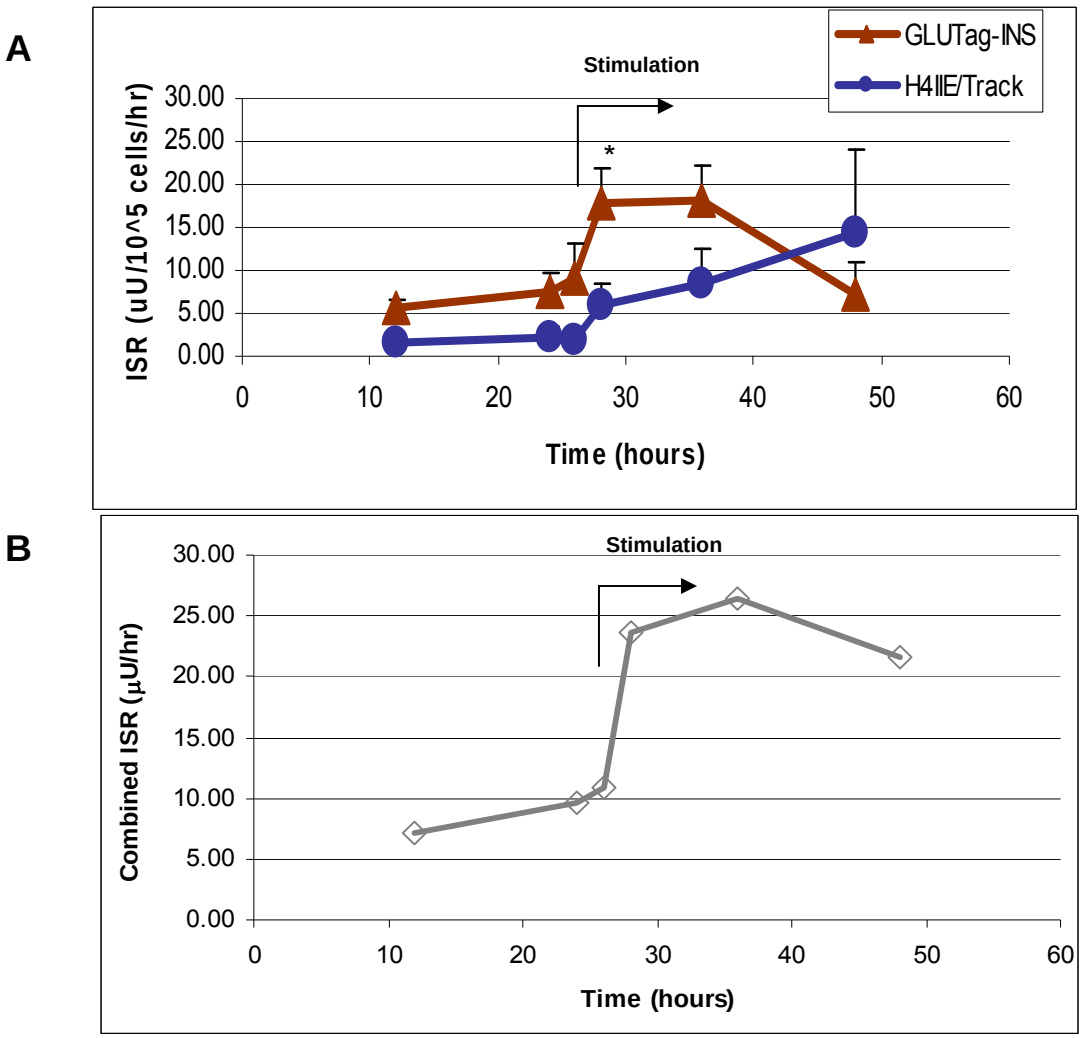

Figure 20: ISR for H4IIE transduced with AdG3-Track and GLUTag-INS cells. A) The data presented earlier have been re-analyzed to determine the insulin secretion rate (ISR) of H4IIE cells transduced with AdG3-Track and GLUTag-INS cells when stimulated at 24 hours with cocktail medium. B) The combined ISR of the rH4IIE cells and GLUTag-INS cells were derived from the ISR values in Graph A and added at a cell ratio of 1:9 rH4IIE to GLUTag-INS to determine the total ISR. It was assumed that the two cell types had no interactions and that the total amount of insulin secreted would be the sum of the insulin secreted by each cell population. *$p \leq 0.001$; values are ± SE; n = 3 for each data point.

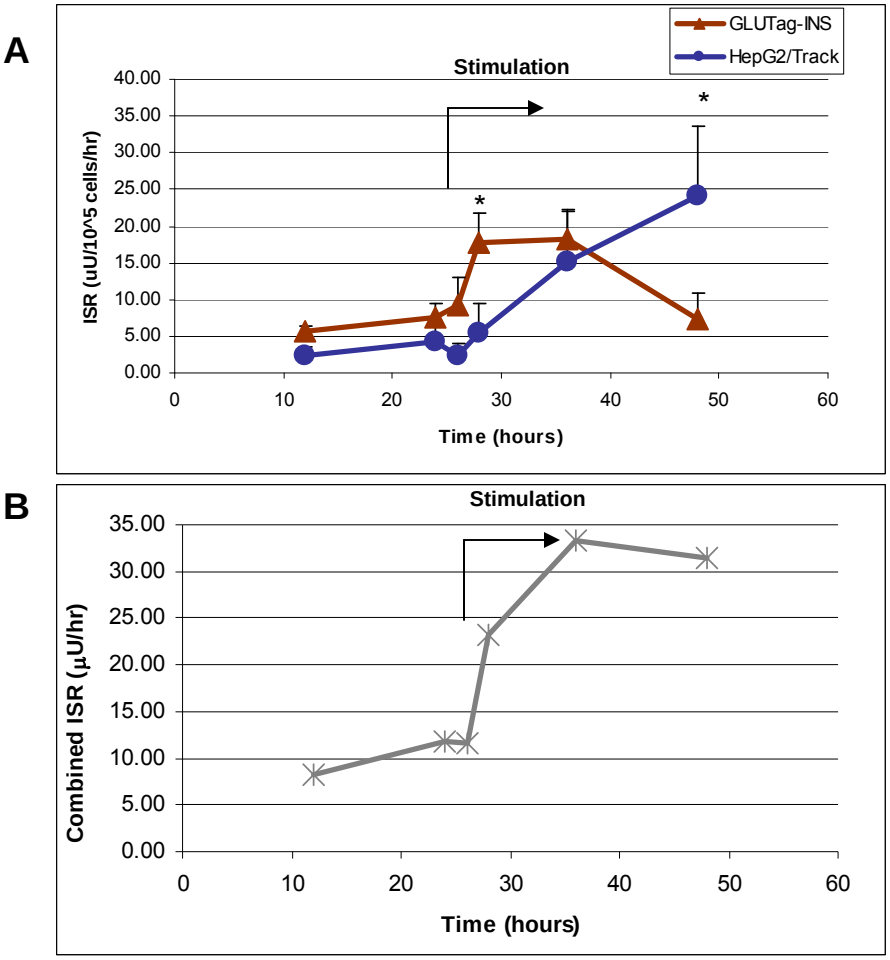

Figure 21: ISR for HepG2 transduced with AdG3-Track and GLUTag-INS cells. A) The data presented earlier have been re-analyzed to determine the insulin secretion rate (ISR) of HepG2 cells transduced with AdG3-Track and GLUTag-INS cells when stimulated at 24 hours with cocktail medium. B) The combined ISR values of the rHepG2 cells and GLUTag-INS cells were derived from the ISR values in Graph A and added at a cell ratio of 1:8 rHepgG2 to GLUTag-INS to determine the total ISR. It was assumed that the two cell types had no interactions and that the total amount of insulin secreted would be the sum of the two individual cells types.*$p \leq 0.001$; values are ± SE; n = 3 for each data point.

CHAPTER 6

CONCLUSIONS

6.1 Conclusions

A combination of recombinant hepatic and intestinal cells offers a promising approach for the treatment of diabetes using engineered non-beta cells. We have described the kinetics of insulin secretion from two recombinant hepatic cell lines transduced with one of three adenovirus vectors, along with a recombinant intestinal cell line stably transfected with an insulin transgene. Assuming only an additive effect, the combination of these cells may better approximate the biphasic insulin secretion dynamics of normal functioning beta-cells than either cell type alone.

6.2 Future Recommendations

The experiments presented in this thesis characterized the kinetics of secretion from recombinant hepatic and enteroendocrine L cell lines; the secretion rates were added up to see how a (otherwise non-interacting) co-culture would secrete. This approach may potentially develop into a treatment for diabetes based on autologous cells as a personalized therapy. The experiments for this thesis were performed using continuous cell lines. To delve further into this treatment option, the intestinal and hepatic cell lines should be combined in a co-culture *in vitro* allowing communication via medium between the two cell types. The same experiment would be performed *in vitro* with primary cells; testing their ability to reproduce the secretion dynamics. At this time, a protocol for the isolation of primary L cells has not been established in our lab and it would require further investigation to determine this method. Once the insulin secretion

dynamics have been confirmed *in vitro*, the experiments should be performed *in vivo* in small animals such as mice.

APPENDIX A

FULL DATA SET

Cell Type	Gene	STIM	Time	Expt 1	Expt 2	Expt 3
				Average cumulative μU/10^5 cells	Average cumulative μU/10^5 cells	Average cumulative μU/10^5 cells
H4IIE	AdG3-2xfur	CONTROL	0	0.12	0.09	0.12
			12	3.04	2.11	1.63
			24-	1.62	3.28	3.57
			24+	0.08	0.11	0.17
			26	0.41	0.55	0.50
			28	0.70	1.15	1.14
			36	1.95	3.30	2.83
			48	3.00	9.29	15.87
H4IIE	AdG3-2xfur	GLUCOSE	0	0.11	0.08	0.12
			12	3.59	2.68	1.90
			24-	0.41	3.14	2.90
			24+	2.99	0.14	0.21
			26	0.77	0.63	0.39
			28	1.13	0.89	0.77
			36	3.35	2.65	2.22
			48	4.51	14.64	15.25
H4IIE	AdG3-2xfur	COCKTAIL	0	0.67	0.12	0.26
			12	3.34	2.77	1.94
			24-	2.51	7.03	3.18
			24+	0.31	0.08	0.33
			26	0.62	0.45	0.56
			28	0.79	0.97	1.08
			36	4.79	4.43	4.12
			48	36.69	18.57	52.85
H4IIE	AdG3Track	CONTROL	0	0.52	0.25	0.87
			12	20.02	12.00	2.76
			24-	21.70	41.69	5.59
			24+	0.39	0.27	1.20
			26	3.32	1.96	1.53

Cell Type	Gene	STIM	Time	Expt 1	Expt 2	Expt 3
				Average cumulative μU/10^5 cells	Average cumulative μU/10^5 cells	Average cumulative μU/10^5 cells
			28	9.20	7.93	2.60
			36	50.61	52.82	37.25
			48	61.83	198.33	40.40
H4IIE	AdG3Track	GLUCOSE	0	0.53	0.46	0.86
			12	15.77	14.37	2.87
			24-	24.90	75.69	4.63
			24+	0.45	0.70	0.90
			26	2.91	2.92	1.41
			28	10.79	12.57	2.35
			36	53.60	34.49	12.03
			48	73.12	207.33	53.83
H4IIE	AdG3Track	COCKTAIL	0	0.57	0.42	1.08
			12	20.68	12.17	3.33
			24-	24.84	42.80	6.05
			24+	0.40	0.45	0.88
			26	2.70	3.33	1.99
			28	12.77	12.59	2.84
			36	48.99	83.49	11.06
			48	107.31	334.92	59.19
H4IIE	AdG3InsTail	CONTROL	0	0.12	0.09	0.19
			12	0.29	0.12	0.23
			24-	0.15	0.21	0.24
			24+	0.24	0.06	0.12
			26	0.12	0.10	0.15
			28	0.16	0.08	0.20
			36	0.23	0.14	0.19
			48	0.22	0.37	0.76
H4IIE	AdG3InsTail	GLUCOSE	0	0.12	0.11	0.16
			12	0.30	0.13	0.20
			24-	0.15	0.20	0.22
			24+	0.62	0.06	0.11
			26	0.18	0.08	0.14
			28	0.17	0.08	0.15

Cell Type	Gene	STIM	Time	Expt 1	Expt 2	Expt 3
				Average cumulative μU/10^5 cells	Average cumulative μU/10^5 cells	Average cumulative μU/10^5 cells
			36	0.33	0.16	0.26
			48	0.29	0.59	1.02
H4IIE	AdG3InsTail	COCKTAIL	0	0.12	0.10	0.16
			12	0.34	0.18	0.19
			24-	0.14	0.40	0.23
			24+	0.28	0.06	0.13
			26	0.12	0.08	0.13
			28	0.14	0.09	0.14
			36	0.26	0.17	0.23
			48	1.79	1.72	1.31
GLUTag-INS	stable	CONTROL	0	0.57	1.09	0.98
			12	3.17	2.66	1.59
			24-	3.11	6.93	4.43
			24+	0.23	0.37	0.41
			26	0.40	0.56	0.54
			28	0.87	0.93	0.74
			36	0.85	2.72	1.14
			48	7.01	10.41	26.75
GLUTag-INS	stable	GLUCOSE	0	0.47	0.76	0.84
			12	4.08	3.26	1.53
			24-	4.49	7.67	3.29
			24+	0.30	0.38	0.74
			26	1.16	0.85	0.53
			28	2.35	1.28	1.27
			36	7.29	4.08	3.74
			48	16.58	22.79	52.65
GLUTag-INS	stable	COCKTAIL	0	0.52	0.77	3.01
			12	3.98	2.78	1.70
			24-	3.44	7.37	5.30
			24+	0.20	0.48	0.75
			26	1.04	1.33	0.88
			28	2.26	2.18	2.07
			36	7.70	8.04	4.90
			48	15.83	39.97	45.71

Cell Type	Gene	STIM	Time	Expt 1	Expt 2	Expt 3
				Average cumulative μU/10^5 cells	Average cumulative μU/10^5 cells	Average cumulative μU/10^5 cells
HepG2	AdG3-2xfur	CONTROL	0	1.49	3.03	0.74
			12	6.54	4.11	15.74
			24-	10.24	9.35	51.56
			24+	1.13	1.02	1.59
			26	2.88	2.12	3.77
			28	3.71	2.68	9.30
			36	14.12	5.84	34.83
			48	84.86	25.79	41.52
HepG2	AdG3-2xfur	GLUCOSE	0	1.70	4.56	1.78
			12	6.68	3.15	15.92
			24-	5.76	11.93	47.05
			24+	7.53	1.05	0.94
			26	2.97	2.01	3.77
			28	4.30	3.06	6.42
			36	17.21	5.74	37.05
			48	95.34	24.84	30.91
HepG2	AdG3-2xfur	COCKTAIL	0	2.06	3.59	0.69
			12	5.72	5.47	16.44
			24-	19.53	17.09	53.61
			24+	2.94	1.62	0.97
			26	2.96	3.29	4.98
			28	4.06	6.57	11.66
			36	24.38	16.16	69.19
			48	221.56	189.48	129.94
HepG2	AdG3Track	CONTROL	0	1.51	1.48	3.63
			12	10.40	26.87	21.69
			24-	37.64	65.42	87.58
			24+	1.48	1.85	0.54
			26	4.29	3.09	5.26
			28	7.11	29.47	18.60
			36	25.61	42.18	61.47
			48	233.50	84.89	150.81
HepG2	AdG3Track	GLUCOSE	0	1.30	1.74	1.11
			12	9.90	23.29	33.94
			24-	37.05	47.59	86.71
			24+	1.15	1.71	0.74

Cell Type	Gene	STIM	Time	Expt 1	Expt 2	Expt 3
				Average cumulative μU/10^5 cells	Average cumulative μU/10^5 cells	Average cumulative μU/10^5 cells
			26	3.74	2.94	4.21
			28	6.60	9.13	13.48
			36	24.82	22.21	81.70
			48	155.45	71.66	130.98
HepG2	AdG3Track	COCKTAIL	0	1.33	4.24	1.23
			12	8.81	30.46	27.56
			24-	36.02	51.66	90.03
			24+	1.06	1.62	1.44
			26	4.36	3.29	7.61
			28	8.00	6.57	22.93
			36	87.88	72.17	158.59
			48	745.40	293.22	528.07
HepG2	AdG3InsTail	CONTROL	0	0.27	0.49	0.12
			12	0.34	0.26	0.27
			24-	0.23	0.51	0.38
			24+	0.25	0.10	0.11
			26	0.24	0.13	0.16
			28	0.25	0.15	0.21
			36	0.44	0.31	2.45
			48	0.91	0.54	1.61
HepG2	AdG3InsTail	GLUCOSE	0	0.26	0.40	0.12
			12	0.37	0.25	0.29
			24-	0.22	0.44	0.65
			24+	0.20	0.10	0.09
			26	0.20	0.16	0.13
			28	0.21	0.19	0.23
			36	0.48	0.30	1.23
			48	0.94	1.05	0.93
HepG2	AdG3InsTail	COCKTAIL	0	0.29	0.20	0.08
			12	0.58	0.31	0.27
			24-	0.21	0.58	0.51
			24+	0.30	0.11	0.10
			26	0.23	0.17	0.17
			28	0.24	0.24	0.27
			36	0.64	0.92	1.74
			48	2.48	3.91	2.24

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