

Genetic Engineering of Non-Beta-Cells for Regulated Insulin Secretion

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LIST OF ABBREVIATIONS

Abbreviation

AAV	adeno-associated virus
AAV2	adeno-associated virus serotype 2
B10 mutation	His-to-Asp mutation at the 10th position in the insulin B chain
cDNA	complementary DNA
CMV	cytomegalovirus
DEX	dexamethasone
DMEM	Dulbecco's modification of Eagle's medium
DOX	doxycycline
EGFP	enhanced green fluorescent protein
EJC	exon-exon junction complex
ES cells	embryonic stem cells
FACS	fluorescence-activated cell sorting
FBS	fetal bovine serum
FIFC	fluorescein isothiocyanate
GIP	glucose-dependent insulinotropic polypeptide
GLP-1	glucagon-like peptide-1
GLUT2	glucose transporter type 2
HEK293 cells	human embryonic kidney 293 cells
hGH	human growth hormone
IDD	insulin-dependent diabetes
ITRs	inverted terminal repeats
MH	meat hydrolystae
MOI	multiplicity of infection
mRNA	messenger RNA
NMD	nonsense-mediated mRNA decay
PC1/3	prohormone convertase 1/3
PC2	prohormone convertase 2
PCR	polymerase chain reaction
polyA	polyadenylation signal
PPI	preproinsulin
P/S	penicillin and streptomycin
rAAV	recombinant adeno-associated virus
rAAV2	recombinant adeno-associated virus serotype 2
RIA	radioimmunoassay
rtTA	reverse tetracycline-responsive transcriptional activator
S14	Spot 14 protein
STZ	streptozotocin
SV40	simian virus 40
TRITC	tetramethylrhodamine isothiocyanate
tTA	tetracycline-responsive transcriptional activator
UTR	untranslated region

SUMMARY

Surrogate β -cells generated by genetic engineering of non- β -cells to produce insulin provide an alternative to islet transplantation in cell-based therapies for treating insulin-dependent diabetes (IDD). Engineered β -cell surrogates are potentially autologous, targeted by gene transfer vehicles in vivo or obtained as a biopsy from the patient and genetically modified ex vivo, and thus greatly relax the immune acceptance problems posed by allogeneic and, more so, xenogeneic islet transplantation. Of particular interest are hepatic cells and intestinal enteroendocrine cells engineered for regulated insulin release. The aim of this work was to use these two cell types as targets to establish a methodology and strategy for insulin gene delivery as well as for improving the dynamics of insulin secretion.

In light of the incorporation of glucose-responsiveness to surrogate β -cells, transcriptionally regulated hepatic cells have received notable attention. However, these cells exhibit sluggish insulin secretion dynamics, which may not be appropriate for achieving euglycemia in IDD patients. To improve the secretion dynamics, we engineered the preproinsulin (PPI) mRNA so as to destabilize it through nonsense-mediated mRNA decay (NMD). When expressed under transcriptional regulation in human HepG2 hepatomas, the engineered PPI mRNA level and the insulin secretion rate declined faster upon switching off transcription, compared to the non-engineered control. This approach provides a simple and straightforward method to improve the dynamics of insulin secretion from transcriptionally regulated cells.

To genetically modify intestinal enteroendocrine cells, we applied recombinant adeno-associated virus serotype 2 (rAAV2)-based vector to establish an engineered human enteroendocrine L-cell model with regulated insulin release. Naturally, intestinal enteroendocrine L-cells are found scattered in the crypts of gut mucosa; they possess a regulated secretory pathway and produce glucagon-like peptide-1 (GLP-1) which, like insulin, is released in a fashion closely related to nutrient ingestion and digestion for normalization of postprandial glycemia. In this research, the engineered L-cell model responded to stimulation by meat hydrolysate, and simultaneously secreted endogenous GLP-1 and recombinant insulin. We used a fusion protein of insulin and enhanced green fluorescent protein (EGFP) to reveal the granule-like compartments of localization of the fusion protein and assess its co-localization with endogenous GLP-1.

Since only approximately 1% of the intestinal epithelial population consists of enteroendocrine cells, there exists a significant challenge in identification of an enteroendocrine L-cell target for insulin production. Among the cell lineages in the intestinal epithelium, enterocytes, which account for digestion and absorption of luminal nutrients, are the predominant cell type. Here, we examined the selectivity and efficiency of rAAV2 transduction in human co-culture models of enteroendocrine L-cells and enterocytes. Our results showed that rAAV2 can differentially modify enteroendocrine L-cells in the co-culture environment while avoiding a prevailing number of enterocytes. The rAAV2 transduced co-culture achieved regulated insulin release in response to stimulation; whereas, a chemically transfected co-culture control failed to respond. This result constitutes an important step towards finding a suitable gene transfer vehicle for producing appropriately secreting intestinal epithelium for regulated insulin release.

Our research has demonstrated the potential of developing appropriate secreting β -cell surrogates based on engineered hepatic cells and intestinal enteroendocrine L-cells. Clearly, the effectiveness of rAAV2 transduction and nonsense-mediated PPI mRNA decay will need to be tested in primary cultured cells and in vivo. While there is still much work to be done, to this end, we have made significant strides toward developing methodologies for engineering potentially autologous non- β -cells for treatment of IDD.

CHAPTER 1

INTRODUCTION AND RATIONALE

1.1 Introduction

Diabetes is a group of diseases characterized by high levels of blood glucose resulting from defects in insulin production, insulin action, or both [1]. The prevalence of diabetes among people under 20 years of age is 0.19% (151,000 people). In order to survive, these juvenile diabetics must have exogenous insulin delivered by injections or a pump. For people of 20 years or older, the prevalence is 8.6% (16.9 million); 22% of them take exogenous insulin, and 11% of them take both insulin and oral medications [1]. For patients who have insulin-dependent diabetes (IDD, the diabetics who need exogenous insulin) and thus need to take insulin injections or infusion, it is important to look for alternatives to improve their life-style and, more importantly, a better way of controlling blood glucose to prevent long-term complications.

Other than taking exogenous insulin, a number of strategies have been proposed of using internal, cell-based insulin delivery to IDD patients. Figure 1 classifies these approaches into three categories: islet or pancreas transplantation, stem cell differentiation for β -cell replacement, and the development of recombinant β -cell surrogates. Among these, only allogeneic islet transplantation and pancreas transplantation have been clinically applied and restored euglycemia. However, after receiving the allograft, the potentially lifetime immunosuppression is still a big concern,

in particular to a young child. In addition, with limited supply of human tissue, it is impossible to meet the demand for millions of IDD patients.

Recent advances in molecular and cellular biology have made possible novel strategies for IDD treatment. Now, we can conceive of stem cell differentiation to replace the damaged pancreatic β -cells by insulin-secreting β -like cells derived from embryonic stem cells or adult tissue stem cells. Or, we could develop surrogate β -cells by genetic engineering of autologous cells to produce insulin (Figure 1). However, in vivo, β -cells are a highly differentiated cell type with a sophisticated phenotype including glucose-sensing apparatus, regulated secretory pathway, and the ability to adjust insulin release according to metabolic cues. Because of these characteristics, investigators have faced a great challenge in using a single gene- or cell-based approach to mimic the intrinsic properties of β -cells. Therefore, it is important that multiple directions of β -cell replacement research proceed simultaneously so as to evaluate different approaches in parallel and also complement each other, in finding a better way for IDD treatment.

In this thesis, the endeavor is devoted to increasing the knowledge of developing recombinant β -cell surrogates for IDD treatment. We genetically modified cells of hepatic and intestinal enteroendocrine origins for regulated insulin secretion. Potentially, these cells can be autologous, targeted by gene transfer vectors or retrieved as a biopsy from the patient and genetically engineered ex vivo, and thus relax the immune acceptance problems existing with allo- and xenogeneic cells.

Genetic engineering of hepatic cells for glucose-responsive insulin secretion is considered by several research groups in developing a cell-based therapy for IDD. Responsiveness to physiologic stimuli is introduced at the gene transcription level by

using promoters up-regulated by glucose and possibly down-regulated by insulin [2-6]. Based on this concept, promising results from in vivo rodent models have been reported [3-6]. However, the sluggishness in secretion dynamics of transcriptionally controlled cells probably makes this approach inappropriate for glycemic regulation in higher animals and, eventually, humans. Of particular significance is the slow dynamics of secretion down-regulation, which result in the cells secreting insulin long after the stimulus has been removed and may thus revert diabetes to hyperinsulinemia and hypoglycemia, a serious pathological condition. To improve the dynamics of insulin secretion from transcriptionally regulated cells, research in CHAPTER 3 aims at modifying the preproinsulin mRNA to become a labile molecule, thereby accelerating its degradation during the down-regulation of insulin secretion.

Recently, the advantages of β -cell surrogates derived from intestinal enteroendocrine cells have received notable attention because of the unique physiologic connection of enteroendocrine cells and β -cells [7-10]. Naturally, enteroendocrine cell families are found scattered in the crypts of gut mucosa; they release incretin hormones such as glucagon-like peptide-1 (GLP-1, from intestinal L-cells) and glucose-dependent insulinotropic peptide (GIP, from gut K-cells) after a meal to potentiate insulin production from pancreas [11]. Both incretin hormone and insulin secretion are closely related to nutrient ingestion and digestion for postprandial glucose normalization [12]. In CHAPTER 4, we describe the development of genetically engineered human intestinal cells for regulated insulin secretion using recombinant adeno-associated virus serotype 2 (rAAV2)-based vectors. We applied rAAV2-mediated insulin gene transfer to genetically modify an enteroendocrine L-cell model, the human NCI-H716 intestinal cell

line, for regulated insulin release. We aim to use this line as a human cellular model to demonstrate that insulin release from the engineered GLP-1-secreting intestinal cells can respond to physiologic stimuli. The implications of our findings in using rAAV2 to produce appropriately secreting enteroendocrine L-cells with regulated insulin release are included.

To genetically modify gut epithelia, viral vectors have shown significant potential in targeting cells lining the gastrointestinal tract [13-15]. However, since only approximately 1% of the intestinal epithelial population consists of enteroendocrine cells, there exists a significant challenge in selectively targeting enteroendocrine L-cells in vivo. Likewise, it is also difficult to isolate a pure L-cell population from intestinal biopsy and perform ex-vivo somatic cell gene delivery. The research presented in CHAPTER 5 extends the research in CHAPTER 4 by using in-vitro models to study the specificity and efficiency of rAAV2-mediated transduction in co-cultures of enterocytes (the predominant cell type in the intestinal epithelium) and enteroendocrine L-cells. The uptake and expression of the insulin gene, as well as the conversion of proinsulin to insulin and the dynamics of insulin secretion were characterized.

1.2 Rationale

This thesis consists of three distinct topics and specific aims in CHAPTERS 3, 4 and 5, all focusing on improving the development of genetically engineered non- β -cells for regulated insulin secretion. Each individual chapter contains detailed motivation, research methods, results and discussion. General information on IDD treatment, including current therapies, and the gene- and cell-based β -cell replacement approaches

are reviewed in CHAPTER 2, the BACKGROUND section. Conclusions and future work are described in CHAPTER 6.

The preliminary work leading to the research presented in CHAPTER 3 is included in APPENDICES A3 to A6, which contain the following: 1) the attempt of using glucose-responsive Spot 14 promoter to control insulin expression, 2) the study of transcriptionally regulated proinsulin secretion from myoblasts, 3) the application of sense-antisense mRNA interaction to accelerate the insulin down-regulation in transcriptionally regulated myoblasts, and 4) the transition from sense-antisense mRNA interaction to nonsense-mediated mRNA decay to destabilize preproinsulin mRNA.

The S14 promoter was initially applied in an attempt to develop glucose-responsive insulin secretion from recombinant β -cell surrogates. However, this line of research was discontinued because our tests of S14-directed transcription indicated little, if any, glucose-induced activation. The details are describes in APPENDIX A.3.

Furthermore, it was realized that even if we were successful at expressing glucose-regulated insulin transgenes in cell hosts, the sluggishness of secretion up- and down-regulation would remain problematic. Thus, the next research effort focused on developing methodologies for improving the insulin secretion dynamics from transcriptionally regulated cells. In the beginning, we tested the possibility of using antisense-mediated mRNA destabilization to accelerate insulin down-regulation. An MMTV (mouse mammary tumor virus) promoter-directed, transcriptionally regulated, insulin-secreting myoblast model was developed with both sense and antisense preproinsulin mRNA expression (APPENDIX A.4). While attempting to elevate the sense-antisense interaction by increasing the copy number of the sense template, we

discovered a much easier and more efficient alternative in destabilizing the preproinsulin mRNA molecule by nonsense-mediated mRNA decay (APPENDIX A.5). This finding led to the research described in CHAPTER 3, which focused on changing the structure of preproinsulin mRNA, thus inducing nonsense-mediated mRNA decay and accelerating the down-regulation of insulin secretion from the recombinant hosts.

Although we were able to generate a more dynamic insulin secretion by using the destabilized preproinsulin mRNA described in CHAPTER 3, there still existed more than hour-long delays in response to up- and down-regulation of insulin secretion. Such dynamics are expected to constitute a problem in achieving euglycemia in higher animals and in humans. To expedite the response time, cells possess regulated secretory pathway are good candidates to be engineered for acute response to stimuli. We therefore focused on exploring the properties of enteroendocrine cells to develop an acute phase of insulin release. We expect that the engineered enteroendocrine cells and hepatic cells can regulate insulin secretion in short- and long-term fashions, respectively. Our findings have led to the concept that a pancreatic substitute based on autologous cells might be a two-compartment tissue, one with peritoneal or hepatic location providing the more constitutive type of insulin secretion and the second with upper intestinal location providing the more acute type of response. The two compartments working together may thus release insulin in a fashion similar to a normally functioning pancreas.

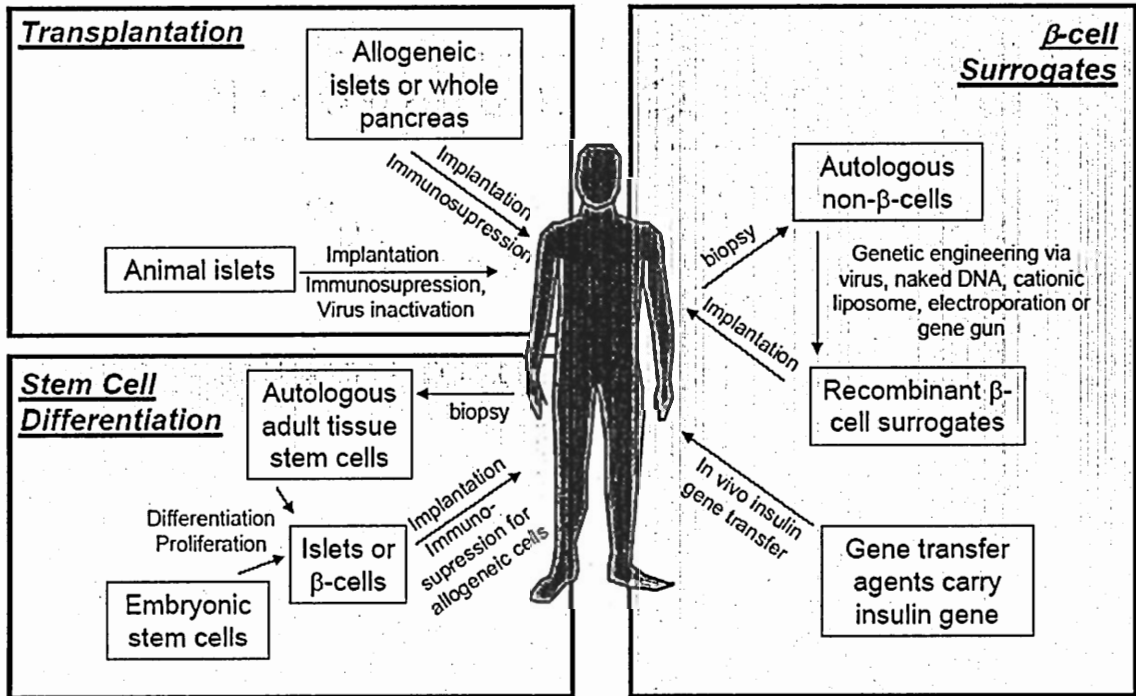


Figure 1. Strategies of cell-based insulin delivery to IDDM patients.

CHAPTER 2

BACKGROUND

2.1 Insulin-dependent diabetes and current treatment

Diabetes mellitus is a group of diseases which have in common the presence of high blood sugar and many other metabolic abnormalities. Two types of diabetes are commonly found. Type 1 diabetes, also called juvenile-onset diabetes, is caused by autoimmune destruction of pancreatic β -cells, which results in insulin deficiency. This form of diabetes, which accounts for roughly 5% to 10% of the overall diabetic population, usually strikes children and young adults, who need exogenous insulin delivery in order to survive [1]. Type 2 diabetes accounts for roughly the other 90% to 95% of diagnosed cases of diabetes [1]. The causes of type 2 diabetes are still a mystery but the symptoms are characterized by the resistance to insulin action on skeletal muscle and adipocytes [16]. As the need for insulin rises because of insulin resistance in type 2 diabetics, their β -cells first elevate insulin secretion levels to compensate the demand, and then might gradually lose the secretion ability over time and, eventually, the compensation fails. Patients with insulin-dependent diabetes (IDD) are those with type 1 and a portion of type 2 diabetes who need exogenous insulin for regulating glucose homeostasis.

Current treatment of IDD involves multiple insulin injections a day or continuous subcutaneous insulin infusion (insulin pump), in addition to a frequent inspection of blood glucose levels [1]. The insulin injections or infusion are cumbersome and

sometimes they need the patients' high level of attention and decision-making ability. Furthermore, due to the fluctuation of physiologic conditions, it is difficult to determine the required insulin dosage, thus causing inefficient control of blood glucose. Although the current treatment is well-accepted by patients and doctors, it cannot avoid incidences of hypoglycemia, a life-threatening condition. More seriously, due to a poor glycemic control, this treatment cannot prevent long-term complications such as heart, kidney and nervous system diseases, stroke and blindness. Since the current insulin treatment is far from ideal, additional cell-based therapies have been proposed to provide a more physiologic regulation of blood glucose levels. These therapies include: 1) **islet transplantation**, 2) **differentiation of pancreatic β -cells from stem cells**, and 3) **engineered β -cell surrogates**.

2.2 Islet transplantation

Islet transplantation is gaining acceptance as a potential therapy for IDD, in particular to patients prone to hypoglycemic incidents. The successes of islet transplantations following the "Edmonton protocol," and the recent follow-up studies have both shown encouraging results [17-19]. The transplant only takes less an hour to perform by injecting islets through a catheter into the liver. The success of the Edmonton protocol primarily depends on appropriate immunosuppression and a new procedure for islet isolation, in addition to a careful screening of recipients [18]. However, with millions of IDD patients, it is impossible to satisfy the demand for islets with limited islet donors (a typical transplant requires 10^6 islets from two average-sized persons). Additionally, patients need to face potentially lifetime immunosuppression. Although

xenogeneic islet transplantation can relieve the shortage of allogeneic islets, the immunological barrier of xenogeneic grafts and the concern of contamination from animal viruses, such as the porcine endogenous retrovirus, have halted the progress [20].

2.3 Differentiation of pancreatic β -cells from stem cells

Differentiation of embryonic stem (ES) cells into pancreatic β -cells constitutes an alternative approach to relax the limited availability of human tissue. Differentiation of human ES cells into insulin-producing cells via the pluripotent human ES cell lines has been reported [21-24]. Advantages of ES cells include their proliferation and differentiation ability. Theoretically, stem cells can be expanded in tissue culture indefinitely, which solves the problem of cell sourcing. Differentiated β -like cells have been transplanted to type 1 diabetic mice and restored euglycemia [25]. Although the preliminary work of differentiating ES cells into β -like cells is promising, a number of questions remain. First, the general concern of using ES-derived grafts is the development of teratomas from undifferentiated cells after implantation; this concern is also applied to the use of ES-derived β -cells. Second, implantation of β -cells alone is different compared with the implantation of islets. Although pure β -cells can replace the function of islets in terms of insulin secretion, it is unclear how the β -cells' phenotype would change after losing their cell-cell contact with the non- β -cells in the islets, especially for those ES-derived cells, which probably possess less phenotypic stability. Third, in type 1 diabetics, differentiated β -cells would still face autoimmune rejection, as is the case with islet allografts, and lifetime immunosuppression is a big concern.

Regeneration of pancreatic β -cells from adult tissue stem cells raises hope of producing insulin-secreting cells from an autologous source [26]. In this scenario, the patients' own stem cells are differentiated to restore the damaged β -cells. Recent reports of differentiation of adult hepatic stem cells into β -like cells and the success of using pancreatic ductal epithelia cells from mice to produce functional islets are encouraging [27,28]. The question of applying adult tissue stem cells is their limited proliferation capacity, which prevents them from large-scale preparation. Nonetheless, this property could also be good as it reduces the risk of unregulated cell growth following implantation. Other than the slow rate of proliferation, the slow rate of differentiation of adult stem cells also needs to be improved before further applications. Again, like ES-derived β -cells, the differentiated β -cells from adult tissue stem cells would face the potential problem of autoimmune rejection in vivo in type 1 diabetic subjects.

2.4 Engineered β -cell surrogates

The concept of using genetically engineered surrogate β -cells for IDD treatment started in 1983 when Moore et al. [29] transferred preproinsulin cDNA to the mouse pituitary AtT20 cell line: The engineered AtT20 cells produced proinsulin, which was sorted to secretory granules and converted to insulin; upon stimulation, regulated insulin secretion was observed. Since then, engineered β -cell surrogates have been derived from a variety of cell types, including fibroblasts [30], skeletal muscle cells [31,32], hepatic cells [2-6] and endocrine cells of different origins [7-9,33-36]. To summarize endeavors in this field during the past 20 years, the implemented approaches consist of three major

steps: 1) **modification of insulin gene**, 2) **delivery of insulin gene**, and 3) **regulation of insulin secretion**.

2.4.1 Modification of insulin gene

Insulin consists of polypeptide chain A (21 amino acids) and polypeptide chain B (30 amino acids), connected by covalent disulphide bonds. These two chains are derived from proinsulin, which includes a polypeptide C between the A and B chains. Proinsulin is derived from preproinsulin, the product after transcription and translation of insulin gene. Preproinsulin carries a secretory signal peptide at the N-terminus to direct its intracellular traffic in β -cells. Following biosynthesis, preproinsulin is cleaved to leave proinsulin during transport into the endoplasmic reticulum. Proinsulin is further processed in secretory granules by prohormone convertases 1/3 (PC1/3) and 2 (PC2) to remove the C peptide and form the mature insulin. PC1/3 cleaves proinsulin at the B-C junction after Arg-32, and PC 2 cleaves at the C-A junction after Arg-65 [37].

Since convertases PC1/3 and PC2 are expressed primarily in neuroendocrine cells, alteration of B-C, C-A junction sites is necessary to facilitate proinsulin processing in other cell types, e.g. hepatocytes, fibroblasts and myoblasts, which do not possess a regulated secretory pathway. Yanagita [38] modified the proinsulin sequence at the B-C and C-A junctions by creating tetrabasic amino acid residues Arg-X-Lys (or Arg)-Arg, thereby allowing the ubiquitous proteolytic enzyme, furin, present in the constitutive secretion pathway to process proinsulin to insulin. This modification is particularly important for non-islet tissues to generate mature insulin; however, for endocrine cells,

such as pituitary cells or enteroendocrine cells which carry PC1/3 and PC2, the modification is not necessary.

Expression of mutated insulin in the B chain at the 10th position allows an elevation of recombinant insulin production in engineered mammalian cells, as well as generates a modified form of insulin with higher activity [39,40]. The His B10-to-Asp mutation increased accumulation of insulin in cellular models, probably by improving the stability of the insulin molecule [41]. Expression of the B10 mutated insulin gene was able to elevate insulin production in cultures of primary rat myoblasts (roughly 6-fold) [42] as well as human embryonic kidney cells (HEK293 cell line, 10-100-fold) [43]. The application of B10 mutated insulin gene greatly increases the insulin production efficiency in the recombinant host, which is particularly useful in increasing the sensitivity of quantifying insulin secretion from engineered β -cell surrogates. This mutation was also applied in the studies described in CHAPTERS 3, 4 and 5. The sequences of the native and mutated preproinsulin peptides and cDNAs are listed in Appendices A.1 and A.2.

2.4.2 Delivery of insulin gene

Delivery of insulin gene to mammalian cells has been performed by viral systems as well as by non-viral gene transfer systems. Before the rapid progress of viral delivery systems in late 1990s, most of the engineered β -cell surrogates were generated via non-viral systems such as, liposome, cation-based gene delivery, electroporation, or gene gun method [44]. Direct intravenous injection of liposome-enclosed plasmid DNA resulted in insulin expression in the liver of rat [45]. Intramuscular injections of naked plasmid

DNA have been shown to result in local expression of insulin in skeletal muscle [46,47]. Although the non-viral delivery methods are simple and do not induce immunologic response, their gene transfer efficiency is relative low [48]. Usually the transgene exhibits transient expression because the gene of interest cannot efficiently integrate with the host chromosome.

The commonly applied viral gene transfer systems are: retrovirus, lentivirus, adenovirus and adeno-associated virus. Their advantages and disadvantages in gene delivery are summarized in Table 1 [49-56]. Generally, viral systems are more efficient in gene transfer than non-viral systems. Key issues in applying viral systems to IDD treatment include: safety, the ability to transduce non-dividing cells, and the ability of maintaining stable expression. Among these, the safety issue has been pivotally enhanced by recent adverse events of retrovirus inducing a lymphoproliferative disorder in treating severe combined immunodeficiency (SCID)-XI disease, and of adenovirus causing a patient death [49].

2.4.2.1 Adeno-associated virus vectors

In CHAPTERS 4 and 5, we applied recombinant adeno-associated virus serotype 2 (rAAV2)-based vector for insulin gene transfer to intestinal enteroendocrine L-cells. Adeno-associated serotype 2, a member of parvovirus, is one of the most promising viral vectors, which have demonstrated its clinical applications in terms of its non-pathogenicity and broad tissue tropism. In addition, AAV2 does not induce cytotoxic effects, cause little, if any, immune response, and has the unique potential of integrating into a specific region of chromosome 19. Nonetheless, with a small packaging capacity

(< 5 kb), rAAV2 cannot carry a lengthy gene of interest, or multiple gene elements to regulate transgene expression [57,58]. This deficiency is partially relieved when rAAV2 is applied to insulin gene transfer. This is because the preproinsulin cDNA only contains 111 codons, i.e. 333 base pairs of nucleotides, which takes less than 10% of the capacity. Another important feature of rAAV2-mediated transgene expression is the expected prolonged transgene expression, as previously demonstrated with other genes and cell hosts [57]. This property is especially important for treating a chronic disease like diabetes. Based on these, the rAAV2-mediated insulin gene delivery has been recognized as a method of gene-based therapy of IDD [4,8,56,59].

2.4.3 Regulation of insulin secretion

The critical part in developing β -cell surrogates for IDD treatment is achieving the property of regulated insulin secretion from the recombinant host. Cells with constitutive insulin release will be dangerous in vivo because of the risk of generating hyperinsulinemia and hypoglycemia, a serious pathologic condition. Physiologically, β -cells are differentiated to possess regulated insulin production and release in response to physiological cues. Therefore, it is worthwhile to review the details of how β -cells control insulin biosynthesis and secretion.

2.4.3.1 Regulation of insulin production and secretion in β -cells

The glucose-stimulated insulin secretion has been extensively studied, due to its importance in regulation of blood glucose levels. When β -cells are pre-incubated in glucose-free or hypoglycemic culture medium and are then exposed to higher levels of

glucose, they exhibit a biphasic secretory response. The first phase of secretion consists of rapid insulin release from intracellular stores and lasts for 5-15 minutes. The second phase is characterized by a steadily increasing secretion rate over a period of several hours, which is due to increased insulin biosynthesis [60]. In normal β -cells, secretion occurs at a minimal, basal level at glucose concentrations below 4 mM, it increases with the glucose concentration for levels between 4 and 15 mM, and it becomes saturated at concentrations above 15 mM. Half-maximal response is observed at 8 mM

Figure 2 is a cartoon representation of β -cells' insulin production from gene to granules. Extracellular glucose concentration can control each step of insulin biosynthesis (transcription, translation, and post-translational modification) and exocytosis. In the transcription step, the insulin promoter is around 400 bp and remarkably complex, with multiple sequence elements along its length [61]. Because each of these sequence elements forms a recognition site for binding of β -cell transcription factors, a transcriptional activation complex can only be formed by binding of the promoter and a unique set of transcription factors in the β -cell nucleus [61,62]. This uniqueness makes the insulin promoter hard to be applied to non- β -cells for insulin biosynthesis control.

Short-term (less than 2 h) glucose-stimulated proinsulin biosynthesis is mainly regulated at the translational level [63-64]. Up to 10-fold increase of proinsulin biosynthesis induced by glucose can be observed in 1 hour. Only 2- to 5-fold elevation in preproinsulin mRNA levels is seen around 60-90 minutes [65,66]. Although in the short term preproinsulin mRNA levels do not change much in response to glucose, the translation of preproinsulin mRNA increases significantly as a result of enhancing: (i) the

ratio of membrane-bound polysomal pool of preproinsulin mRNA (the major site of proinsulin biosynthesis in β -cells) to the free preproinsulin mRNA in the cytosol [67], (ii) preproinsulin mRNA stability [68], (iii) translation initiation [69], and (iv) translation elongation [69,70]. Since an increased proinsulin biosynthesis needs an increase on conversion of proinsulin to insulin, the β -cell regulates the biosynthesis of prohormone convertase enzymes PC1/3 and PC2 by glucose stimulation also at the translation level along with that of proinsulin [71].

The control of glucose-stimulated insulin release from granules is generally accepted to occur via ATP-sensitive potassium channels, which couple cell metabolism to electrochemical activity [72,73]. Pancreatic β -cells use glucose transporter isotype II (GLUT2) and glucokinase to control the first two steps of glycolysis, thus regulating cell metabolism. These two enzymes are called the sensing apparatus of glucose, both have high Michaelis constant (K_m ; GLUT2 \sim 17, glucokinase \sim 8) [74,75] which can change the rate of glycolysis according to physiological glucose levels. So, when the surrounding glucose concentration rises, the glucose uptake rate and the metabolic activity of the β -cell also increase. This results in an increase of the intracellular ATP or of a combination of ATP-related compounds, which in turn closes ATP-sensitive potassium channels. The closure of ATP-sensitive potassium channels produces a membrane depolarization, which activates voltage-dependent Ca^{2+} channels, increases Ca^{2+} influx into the β -cell, and triggers the acute phase of insulin release [76].

2.4.3.2 Regulation of insulin secretion from engineered β -cell surrogates

Although β -cells are complicated and difficult to mimic, significant progress has been made in using transcription and exocytosis to regulate recombinant insulin secretion from β -cell surrogates.

2.4.3.2.1 Transcriptionally regulated insulin secretion from engineered hepatic cells

Transcriptionally regulated insulin release has been developed primarily in hepatic cells as a strategy for IDD treatment. The advantages of targeting liver as the insulin-secreting surrogate organ are: 1) liver is the major target for insulin action, and actively participates in regulating glucose homeostasis via the counteractive balance between glycogenesis, and glycogenolysis/gluconeogenesis; 2) hepatocytes possess glucose-sensing ability involving the glucokinase and glucose transporter type 2 (GLUT2), which is similar to that of β -cells; and 3) several liver-specific gene elements coupled with carbohydrate metabolism have been identified, which include glucose-responsive enhancer/promoter that can be applied to direct insulin gene expression [77].

Many liver-specific genes have been found to respond to an increase of plasma glucose by up-regulating their transcriptional activity, which include genes encoding L-type-pyruvate kinase (L-PK) [78-80], glucose-6-phosphatase (G6Pase) [81], Spot 14 [82] and phosphoenolpyruvate carboxykinase (PEPCK) [83]. The enhancer/promoter regions of these genes were applied in the liver with an intention to control the insulin transgene expression thereby up- and down-regulating insulin release according to glucose levels. Lee et al. [4] and Chen et al. [6] directly applied L-PK promoter and G6Pase promoter, respectively, to drive transgenic insulin expression in the liver. Thulé et al. [2,3] connected glucose-responsive elements from L-PK gene into the insulin-suppressive

basal promoter of the rat insulin-like growth factor binding protein-1 gene to produce a promoter that is active in hepatocytes, inhibited by insulin and stimulated by glucose. Lu et al. [54,84] applied the connection of PEPCK promoter and thymidine kinase promoter, or β -actin promoter (as a basal promoter) to control insulin gene transcription which can be up-regulated by cAMP and down-regulated by insulin. By doing so, these investigators reported induction of insulin secretion in rodent models, in primary cultures of hepatocytes, or in cultures of hepatoma cell lines. Significantly, in vivo hepatic insulin transgene expression has demonstrated its effectiveness in correcting streptozotocin (STZ)-induced diabetic rodents, and non-obese diabetic (NOD) mice [3-6].

Although publications describe the potential of hepatic insulin production for IDD treatment, concerns have been raised on the sluggish dynamics of transcriptionally regulated insulin [85,86]. Of particular importance is the slow secretion down-regulation, which results in the cells secreting insulin long after the stimulus has been removed and may thus revert diabetes to hyperinsulinemia and hypoglycemia, a serious pathological condition. It has been pointed out that preproinsulin (PPI) mRNA, the intermediate molecule encoding the insulin peptide, is the key for the slow dynamics. When transcription is activated via physiologic stimuli, PPI mRNA levels are elevated and more preproinsulin is translated for down-stream processing. However, when transcription stops, the down-regulation of preproinsulin neogenesis strongly depends on the stability of PPI mRNA. It has been demonstrated that, after being synthesized, PPI mRNA is very stable over a period of 24 h in both primary β -cells and transformed β cell lines [65,87]. Although the half-life of PPI mRNA is shorter in recombinant hepatocytes and hepatomas (around 6 to 9 h) [88,89], its time-frame is still too long for applications in

higher animals and humans. To expedite the dynamics of secretion down-regulation, translation needs to stop soon after transcription has been turned off.

To relieve this crucial limitation, PPI mRNA needs to be destabilized before its application in regulation of insulin secretion. The topic of modulation of mRNA stability, in general, is currently under intense investigation [90-92]. Specifically with PPI mRNA, to accelerate the turnover rate, the use of antisense RNA [93] or connecting the insulin gene with the 3'-untranslated region (3'-UTR) of some labile mRNAs, such as those encoding cytokines, have been considered [77]. In CHAPTER 3 of this thesis, we report a novel way to destabilize PPI mRNA through nonsense-mediated mRNA decay (NMD) and investigate its effect on the dynamics of insulin secretion from a recombinant human hepatoma cell line.

2.4.3.2.1.1 Nonsense-mediated mRNA decay

Gene expression requires delicate coordination and integration of multiple processes such as transcription, splicing, polyadenylation and translation. Substantial activities of gene expression happen routinely in cells, thereby incidents of mistakes are inevitable. Many of the errors lead to premature translation termination, also called nonsense mutation, in which case a stop codon forms in the middle of an mRNA molecule. Nonsense mutation can also be introduced by DNA replication while cells divide, and it has been estimated that nonsense mutation accounts for roughly 30% of genetic diseases [94]. The truncated peptides encoded by the mutant mRNA can be very reactive and potentially toxic to the host cell; therefore, perhaps through evolution, a

mechanism called mRNA surveillance, or nonsense-mediated mRNA decay, has evolved to target the unfinished mRNA molecule and accelerate its degradation [95].

Although researchers have focused on mRNA surveillance and NMD for years, details of how a nonsense mutant mRNA is marked are still under investigation. Studies have shown that during the splicing of an mRNA in the nucleus, proteins are deposited in 20-24 nucleotides upstream of the exon-exon junction to form the exon-exon junction complex (EJC) [96]. The EJC is thought to enhance mRNA export, and in addition it might also serve as an mRNA marker [97]. It has been hypothesized that the first round of translation can displace EJC proteins; thus if translation stops prematurely in the middle of the transcript leaving the EJC proteins downstream of the nonsense codon untouched, EJC proteins can recruit the cells' surveillance machine for mRNA degradation [98]. However, recent data show that mammalian mRNAs lacking introns downstream of the nonsense codon can also be degraded via NMD [99]. Also, splicing of downstream introns is not always required for NMD [100], and downstream introns are not always sufficient to specify NMD [101]. In CHAPTER 3, our results do not fall into the model that the EJC initiates NMD, but supports the idea that introns may not absolutely required for NMD [99]. Details of the difference between the native preproinsulin (PPI) mRNA and the engineered PPI mRNA through NMD are discussed.

2.4.3.2.2 Regulated insulin secretion from pituitary cells through exocytosis

In parallel with the engineering of hepatic cells for insulin production, other investigators are targeting endocrine cells which possess the regulated secretory pathway to control insulin release. In transgenic mouse models and cell lines, recombinant

pituitary cells have been shown to respond to secretagogues with acute release of insulin through exocytosis [33-35]. Since pituitary cells lack responsiveness to postprandial stimuli, attempts have been made to engineer glucose responsiveness by expressing glucokinase (GK), or the type II glucose transporter GLUT2, or both, in these cells [36]. Also, recently, Wu et al. [34] engineered pituitary cells to express glucagon-like peptide-1 (GLP-1) receptors on the cell surface in response to the postprandial GLP-1 release from the gut. However, since pituitary cells naturally do not participate in glucose metabolism in the body, recombinant pituitary cells secrete not only insulin but also their endogenous hormones such as adrenocorticotrophic hormone, and serious disorders may result if these hormones are abnormally up-regulated after meals.

2.4.3.2.3 Regulated insulin secretion from enteroendocrine cells through exocytosis

Recently, the advantage of targeting intestinal enteroendocrine cells for regulated insulin release has received notable attention [7-10]. The intestinal enteroendocrine system, consisting of endocrine cells in the lining of the gastrointestinal tract, is the largest system of endocrine cells in the body in terms of the cell number and the variety of hormones released [102]. Incretin hormones such as glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP) are naturally released from intestinal enteroendocrine L-cells and gut K-cells after a meal to potentiate insulin secretion from the pancreas. K-cells are mostly found in the upper part of the digestive tract (stomach, duodenum and jejunum), and L-cells are located primarily in the lower gut (jejunum, ileum and colon) [11]. In humans, the secretion dynamics of GLP-1 and GIP closely parallel those of insulin, in the sense that all of them rise a few minutes after

glucose ingestion, and return to basal levels in 2-3 h depending on the glucose dosage [12, also in Appendix A8]. Chueng et al. [7] explored this unique connection between incretins and insulin, and used a transgenic mouse model to demonstrate that regulated insulin release from recombinant gut K-cells in vivo can restore euglycemia in type 1 diabetic mice. Nonetheless, an effective gene transfer method for delivery of insulin gene to somatic enteroendocrine cells still needs to be developed.

In CHAPTERS 4 and 5 of this thesis, we present a methodology which can efficiently transfer the insulin gene to somatic enteroendocrine cells, specifically to the enteroendocrine L-cells. The enteroendocrine cell families contain different cellular sub-lineages. It has been hypothesized that they are all derived from multi-potential stem cells in crypt of Lieberkuhn. The same stem cells also give rise to other cell types in the intestinal epithelium such as enterocytes, goblet cells and Paneth cells [103,104]. After differentiation, the enteroendocrine lineages, including L-cells, are mostly localized around the crypt area, while other lineages might undergo terminal differentiation as they migrate to the villus tip or the bottom crypt. The apical microvilli of L-cells reach the intestinal lumen, suggesting they can sense luminal concentration of dietary nutrients; their secretory vesicles reside next to the basal lamina, offering evidence of the L-cells' ability to discharge their granular contents into circulation [105]. The GLP-1 released from L-cells is a potent secretagogue towards pancreatic β -cells for insulin secretion. The therapeutic effect of GLP-1 on treating type 2 diabetes has been widely recognized and is under intense investigation [106-108]. Clearly, this underscores the compatibility of GLP-1 and insulin in regulating glucose homeostasis, and the potential of developing GLP-1 secreting L-cells as β -cell surrogates for IDD treatment.

In addition to the enteroendocrine families, there are three other main cellular lineages in the gut mucosa, including enterocytes, goblet cells and Paneth cells [109]. Among these cells, the enterocytes, which account for digestion and absorption of luminal nutrients, are the predominant cell type. Gut epithelium is primarily composed of enterocytes forming a barrier with tight junctions to separate the gut lumen from the basolateral domain. Because the enteroendocrine cells constitute only 1% of the intestinal epithelium population, it is difficult to identify an enteroendocrine target *in vivo* or in isolated mucosal cell culture. Also, to genetically modify enteroendocrine L-cells, inevitably, the gene transfer agents will interact with the surrounding enterocytes. In CHAPTER 5, the details of using co-culture models to evaluate the selectivity and efficiency of rAAV2 transduction to target L-cells are presented. The use of additional elements to possibly further enhance the specificity of transduction is discussed.

Table 1. Comparison of commonly used viral vectors [49]. SCID: severe combined immunodeficiency

Viral vector	Stability of expression	Transduction of non-dividing cells	Safety	Other notable features	Reported use in insulin gene transfer
Retrovirus	Advantage - Stable transgene expression	Disadvantage - Only infect dividing cells	Disadvantage - Inducing lymphoproliferative disorder in treating SCID-XI disease (2002-2003)	Advantage - Low inflammatory potential	[32,50,51]
Lentivirus	Advantage - Stable transgene expression	Advantage - Infect dividing and non-dividing cells	Disadvantage - HIV-derived virus	Disadvantage - Integration might cause oncogenesis	--
Adenovirus	Disadvantage - Transient transgene expression	Advantage - Infect dividing and non-dividing cells	Disadvantage - Caused patient death because of strong immune response (1999)	Advantage - High viral titers and high levels of transgene expression	[2,3,6,52-55]
Adeno-associated virus	Advantage - Stable transgene expression	Advantage - Infect dividing and non-dividing cells	Advantage - Not related to human diseases - Low immunogenicity	Advantage - Potential site specific chromosomal integration Disadvantage - Limited transgene insertion capacity (< 5 kb)	[4,8,56]

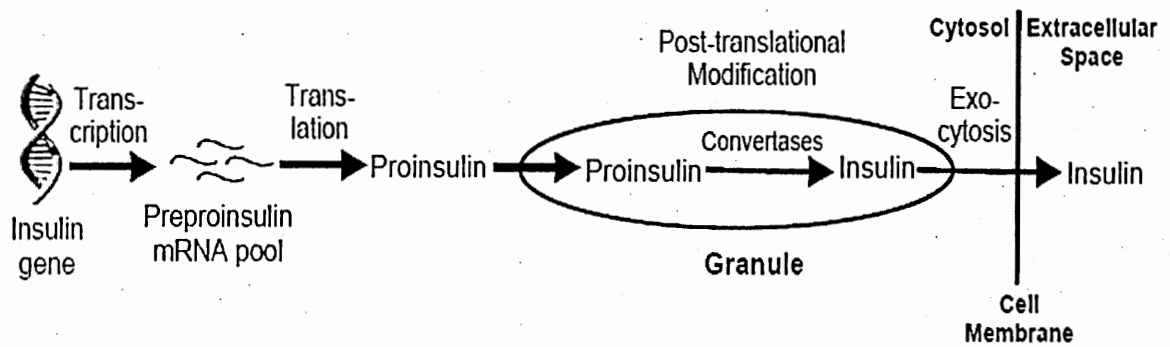


Figure 2. β -cells' insulin production: from gene to granules

CHAPTER 3

Preproinsulin mRNA Engineering and its Application to the Regulation of Insulin Secretion from Human Hepatomas

3.1 Abstract

Cell-based therapies for treating insulin-dependent diabetes (IDD) can provide a more physiologic regulation of blood glucose levels in a less invasive fashion than daily insulin injections. Promising cells include non- β -cells genetically engineered to secrete insulin in response to physiologic cues; responsiveness can be introduced at the transcriptional level to regulate preproinsulin (PPI) mRNA biosynthesis. However, these cells exhibit sluggish secretion dynamics, which are not appropriate for achieving euglycemia in higher animals and, eventually, humans. In this work, we have engineered the PPI mRNA so as to destabilize it through nonsense-mediated mRNA decay (NMD). When expressed under transcriptional regulation in HepG2 hepatomas, the engineered PPI mRNA level and the insulin secretion rate declined faster upon switching off transcription, compared to the one-copy non-engineered control. Our work provides a simple and straightforward method to improve the dynamics of transcriptionally regulated insulin secretion, which can be a useful tool in developing cell-based therapies for IDD.

3.2 Introduction

Genetic engineering of non- β -cells for glucose-responsive insulin secretion offers significant promise in developing a cell-based therapy for insulin-dependent diabetes. Responsiveness to physiologic stimuli is introduced at the gene transcription level by using promoters up-regulated by glucose and possibly down-regulated by insulin [2-6,32]. Based on this concept, glucose-regulated insulin expression has been achieved in streptozotocin (STZ)-induced diabetic rodents [3-6,32]. Although these results are promising, the sluggishness in secretion dynamics of transcriptionally controlled cells makes them inappropriate for glycemic regulation in higher animals and, eventually, humans. Of particular significance is the slow dynamics of secretion down-regulation, which result in the cells secreting insulin long after the stimulus has been removed and may thus revert diabetes to hyperinsulinemia and hypoglycemia, a serious pathological condition.

It has been suggested that the prolonged stability of preproinsulin (PPI) mRNA causes the sluggishness of secretion down-regulation [77,86]. Although prior reports on the PPI mRNA half-life in non- β -cells are limited, data from normal and transformed β -cells strongly indicate that the stability of PPI mRNA is a limiting factor in expediting secretion down-regulation. In isolated primary rat islets, the half-life of PPI mRNA was estimated to be 77 h under high glucose (17 mM) and 29 h under low glucose concentration (3.3 mM) [87]. In β TC-3 insulinomas, there was only marginal PPI mRNA degradation over 24 hours after transcription was stopped [65], while the half-life of PPI mRNA in RIN-5F insulinomas was found to be 58 h and 26 h under high (20 mM) and low (3 mM) glucose concentration, respectively [87].

Recently, nonsense-mediated mRNA decay (NMD) has received significant attention because of its biological and medical importance [95,98,110,111]. Mutant mRNAs with premature stop codons can be detected by cells via a surveillance mechanism, and are subjected to NMD [112,113]. Although NMD has probably evolved to eliminate erratic mRNAs, it also offers an interesting approach to destabilize genetically engineered mRNA.

In this work, we investigated the destabilization of PPI mRNA through engineering nonsense-mediated mRNA decay, and its effect on the dynamics of insulin secretion from recombinant HepG2 hepatomas. The implications of our findings in producing appropriately secreting non- β -cells with transcriptional regulation of insulin expression are discussed.

3.3 Materials and methods

3.3.1 Plasmids for insulin expression

Vectors A-E in Figure 3 are five plasmids constructed to systematically increase insulin expression in HepG2 cells. Human preproinsulin cDNA wild-type, furin-compatible with furin cleavage sites at the B-C and C-A junctions, and furin-compatible with His B10-to-Asp mutation (B10 mutation) were all generous gifts from Genentech, Inc (San Francisco, CA) [43]. The sequences of the native and mutated preproinsulin peptides and cDNAs are listed in Appendices A.1 and A.2. The backbone of insulin expression plasmids originated from an 1822 bp BglII/BamHI fragment of plasmid pRL/Null (Promega, Madison, WI). A 508 bp XbaI/BamHI restriction fragment containing both the Simian Virus 40 (SV40) late polyadenylation and SV40 enhancer

signals from plasmid pGL3-control (Promega) was used to replace the SV40 late polyadenylation signal in pRL/Null to prepare the plasmid with the SV40 enhancer. The human cytomegalovirus (CMV) promoter used to drive insulin gene was obtained from an 883 bp BglII/NheI restriction fragment of plasmid pcDNA3.1(+) (Invitrogen, Carlsbad, CA). The CMV promoter was connected to the BglII and SpeI sites of pRL/Null to prepare the plasmid with an intron, or to the BglII and NheI sites to prepare the plasmid without an intron. Different versions of XbaI/XbaI preproinsulin cDNA fragment were connected to the backbones using NheI and XbaI sites.

The backbone of the tet-responsive insulin expression plasmid was obtained from a 2074 bp XhoI/XbaI fragment of plasmid pRL/Null (Promega). The tetracycline-responsive promoter was obtained from a 448 bp XhoI/EcoRI restriction fragment of plasmid pTRE2pur (Clontech, Palo Alto, CA). This promoter was inserted into the multiple cloning sites of pRL/Null. For the control plasmid containing one copy of insulin gene, a 360 bp XbaI/XbaI restriction fragment containing the furin-compatible insulin gene with B10 mutation was connected to the backbone using NheI and XbaI sites (vector F). For the engineered plasmid, two additional copies of the insulin gene were consecutively inserted to the backbone via the XbaI site with the same head-tail configuration (vector G). After transcription, the engineered PPI mRNA would contain three consecutive copies of the insulin gene with stop codons in the middle of the transcript. Translation is expected to stop in the middle of the engineered PPI mRNA and induce NMD. The XhoI/XhoI restriction fragments of the Clontech plasmids cat# K1620-A and K1621-A were used to prepare plasmids pTet-Off and pTet-On, respectively.

3.3.2 Cell culture and transfection

HepG2 human hepatoma cells (ATCC, Manassas, VA) were grown in DMEM supplemented with 10% fetal bovine serum, 1.1 mg/ml sodium pyruvate and 100 units/ml penicillin/streptomycin at 37°C in a 5% CO₂ / 95% air humidified atmosphere. Approximately 10⁶ cells were seeded in each 9.6 cm² well of 6-well plates and fed with 2 ml of culture medium for 8-12 h prior to transfection. All transfections were carried out with FUGENE 6 reagent (Roche, Indianapolis, IN) following the manufacturer's directions. For each well, HepG2 cells were transfected for 24 h with the plasmid DNA cocktail (2.1 µg or 2.25 µg) and 6 µl of FUGENE 6 reagent. Unless otherwise specified, transfected cells were incubated in culture medium for another 24-36 h for recovery. Cells were then washed and incubated with culture medium for two consecutive 1-h periods to stabilize insulin secretion before any tests.

3.3.3 Systematic increase of insulin expression

HepG2 cells were transiently transfected using 2 µg of test plasmid (Figure 3, vector A, B, C, D or E) and 0.1 µg of plasmid pGL3-Control (Promega) for luciferase expression as internal standard according to the procedure described in Section 3.3.2. Cells were then incubated for 1 h in culture medium, which was collected to assay for secreted insulin. Cells were detached using 2.5 ml 0.25% trypsin solution supplemented with 5 mg of collagenase type II for luciferase assay and cell counting.

3.3.4 Dynamic insulin expression

HepG2 cells were transiently co-transfected using 2 μg of plasmid pTet-Off (or pTet-On) and 0.25 μg of the control or engineered PPI mRNA expression plasmid (Figure 3, vectors F, G). Tet-Off transfected cells in parallel cultures were incubated in doxycycline (DOX)-free medium or in medium with 1 $\mu\text{g}/\text{ml}$ DOX for 24-36 h. Media were renewed, and cells were incubated for an additional 1 h during which insulin secretion was measured; cells were then lysed for quantitative mRNA assay. To study the dynamics of insulin gene down-regulation, Tet-Off transfected cells were switched from DOX-free medium to 1 $\mu\text{g}/\text{ml}$ DOX medium for 8 h. Every hour, culture medium was renewed and collected for insulin assay. Every two hours, cells were lysed for quantitative mRNA assay. To dynamically regulate insulin secretion using Tet-On system, Tet-On transfected cells were switched from DOX-free medium to 1 $\mu\text{g}/\text{ml}$ DOX medium for 1 h, washed four-times then incubated with DOX-free medium for 7 h, and the steps were repeated. Every hour, culture medium was renewed and collected for insulin assay.

3.3.5 Translation inhibition test

Tet-Off transfected HepG2 cells were washed once, and then exposed to DOX-free medium with 28 $\mu\text{g}/\text{ml}$ cycloheximide for 4 h [99]. The time of addition of cycloheximide-containing medium was time 0. To resume translation, cells were washed four times, and then incubated with DOX-free, cycloheximide-free medium for two consecutive 4-h periods. At 0, 4, 8, and 12 h, cells were lysed for quantitative mRNA assay.

3.3.6. Assays

Firefly luciferase activity from the internal control vector pGL3-Control was measured using the luciferase assay system (Promega) following the manufacturer's protocol and using an LS 5000 scintillation counter (Beckman, Fullerton, CA). Secreted insulin was measured by human insulin specific radioimmunoassay (RIA) kit (LINCO Research, St. Charles, MI). The primary antibody in the kit cross-reacts with proinsulin at less than 0.2%. Radioactivities were determined in Auto-Gamma Counting System, Cobra II (Packard, Meriden, CT). The change of intracellular PPI mRNA level was quantified by the TaqMan real-time PCR technique (Applied Biosystems, Foster City, CA). Total RNA was isolated using RNeasy Mini Kit (Qiagen, Valencia, CA); cDNA was then synthesized using Avian myoblastosis virus (AMV) Reverse Transcriptase Kit (Promega) to prepare templates for TaqMan real-time PCR. The expression levels of PPI mRNA and of the internal reference tTA (tetracycline-responsive transcriptional activator, from co-transfected plasmid pTet-Off) mRNA were measured using probes labeled with 6FAMTM and VICTM (Applied Biosystems), respectively, in separate tubes. The primers and probes (Table 2) were designed using Primer Express software (Applied Biosystems). Measuring preproinsulin mRNA-FAM and tTA mRNA-VIC permitted correction of discrepancies from differences in sample preparation and transfection efficiencies. PCRs were performed with the TaqMan Universal PCR Master Mix and the ABI PRISM 7700 Sequence Detection System (Applied Biosystems) using the following thermal cycle routine: 50°C for 2 min, 95°C for 10 min, and then 40 cycles of 95°C for 15 s, followed by 60°C for 1 min. A comparative threshold cycle (C_T) method (User

Bulletin Number 2; Applied Biosystems) was used to determine relative gene expression. Relative quantification of PPI mRNA was done by normalizing the PPI mRNA with the tTA mRNA signal and by assigning a value of 100 to the normalized sample level at time zero.

3.4 Results

3.4.1 Increase of insulin expression from transfected HepG2 cells

Figure 3 shows the plasmids constructed to improve insulin expression. With the CMV promoter, vector D resulted in insulin secretion rates of 720 ± 130 fmole/(hr \cdot 10⁶cells) from transiently transfected HepG2 hepatomas (Figure 4). This was 32.0 ± 9.4 fold higher than the secretion rate achieved with the furin-compatible insulin gene without any gene attachments (Figures 3 and 4, vector A), and 27.5 ± 8.0 fold higher relative to the wild-type insulin gene with the chimeric intron and SV40 enhancer (Figures 3 and 4, vector E). However, because the SV40 enhancer is not compatible with the tet-responsive system, vectors applied for tet-responsive insulin expression did not contain this sequence, but they did contain the B-C and C-A junction and B10 His-to-Asp mutations, as well as the chimeric intron.

3.4.2 Preproinsulin mRNA engineering and expression

The control and engineered PPI mRNA expression plasmids (vectors F and G, Figure 3) were each transiently co-transfected with plasmid pTet-Off in HepG2 cells. Use of the engineered PPI mRNA decreased the insulin expression level to 109 ± 43 fmole/(hr \cdot 10⁶cells) from 320 ± 55 fmole/(hr \cdot 10⁶cells) (n=4) of the control construct, or

by 66%. Evidently, the decreased stability of engineered mRNA reduced intracellular mRNA levels and thus expression, as observed in many other nonsense mutations [112,113]. However, cells with both engineered and control plasmids were regulated in a similar fashion by DOX (Figure 5). With the control plasmid, transfected cells had a 24.8 ± 5.2 -fold higher mRNA expression and a 20.7 ± 3.8 -fold higher insulin secretion rate in the absence relative to the presence of DOX. With the engineered plasmid, the corresponding values were 20.7 ± 3.9 -fold higher mRNA and 19.2 ± 4.8 -fold higher insulin secretion rate in the absence vs. presence of DOX.

3.4.3 Dynamics of down-regulation of insulin expression

The down-regulation of insulin gene expression was tested using the Tet-Off expression system. In the dynamic test, transcription was down-regulated by exposing cells at time zero to culture medium with $1 \mu\text{g/mL}$ DOX (Figure 6). The engineered PPI mRNA exhibited a faster decline with a half-life of less than 4 hours relative to the control which had a half-life of more than 8 hours. The decline of insulin secretion rate followed the similar trends. Thus, immunoreactive insulin can be synthesized from the engineered PPI mRNA, and that the dynamics of down-regulation upon switching off transcription at both the mRNA and secreted protein levels are faster with the engineered PPI mRNA relative to control.

3.4.4 Regulation of insulin secretion using Tet-On system

Up- and down-regulation of insulin secretion from transfected HepG2 cells was tested using the Tet-On system, in which DOX induces insulin gene expression. Using 1

hour of induction in an 8-hour cycle, insulin production from the engineered PPI mRNA responded to the transcriptional switches (Figure 7). On the other hand, insulin production from the control PPI mRNA failed to decline during the 7-hour basal periods of exposure to DOX-free medium.

3.4.5 Translation inhibition test

A control experiment was performed to elucidate whether NMD was indeed involved in shortening the half-life of the engineered PPI mRNA. Since normal mRNA and nonsense mutants are distinguished from each other via translation, the effect of inhibiting translation on the levels of control and engineered PPI mRNA was examined. HepG2 hepatomas transiently transfected with control or engineered PPI mRNA under Tet-Off control were maintained in DOX-free medium and exposed to cycloheximide at time 0 (Figure 8). The engineered/control PPI mRNA ratio at time 0 was defined as 100%. After 4-h of cycloheximide treatment, this ratio increased to 490% compared to the cycloheximide-free culture. Upon withdrawing cycloheximide to resume translation, the engineered/control PPI mRNA ratio decreased toward the basal level.

3.5 Discussion

Cell sourcing constitutes a critical issue in developing a cell-based therapy for treatment of IDD. Non- β -cells, primarily cells of hepatic origin, are considered by several research groups as hosts for recombinant insulin expression under transcriptional regulation [2-6]. A major advantage of these cells is that they are potentially autologous, retrieved as a biopsy from the patient. A disadvantage is that transcriptionally controlled

cells exhibit sluggish secretion dynamics and thus may not be suitable, as such, for achieving normoglycemia in higher diabetic animals and humans. To expedite the dynamics of secretion down-regulation, translation needs to stop soon after transcription has been turned off.

The topic of modulation of mRNA stability is currently under intense investigation [90-92]. Specifically with PPI mRNA, to accelerate the rate of mRNA turnover, the use of antisense RNA [93] or connecting the insulin gene with the 3'-untranslated region (3'-UTR) of some labile mRNAs, such as those encoding cytokines, have been considered [77]. Compared to these, our approach involving NMD is simple and straightforward. It is also expected to be generic and applicable to different host cells, including primary cells.

NMD improved the secretory response of HepG2 cells but also reduced intracellular PPI mRNA levels and thus insulin expression. To ameliorate this problem, we increased insulin expression before applying NMD. HepG2 hepatomas transiently transfected with vector D (Figure 3) secreted insulin at a rate of 720 fmole/(hr•10⁶cells), which is higher than the basal insulin secretion rate of 380 fmole/(hr•10⁶cells) exhibited by β TC-3 mouse insulinomas [114]. For tet-responsive insulin expression, although the SV40 enhancer was not applied, the insulin secretion from Tet-Off transfected HepG2 in DOX-free medium was 320 fmole/(hr•10⁶cells) and 109 fmole/(hr•10⁶cells) when the control and engineered PPI mRNA, respectively, were used.

The specificity of the Tet-Off system and the sub-toxic DOX concentration used in this study [115] increase the fidelity of mRNA half-life measurements compared to experiments with non-specific inhibitors [116]. One concern in determining insulin

secretion dynamics with the tet-responsive system is the pharmacokinetics of DOX. It has been shown that the Tet-Off system and its transcription repressor tetracycline can be applied to measure the stability of a spliced intron with a half-life as low as 6 min [117]. Hence, it appears that tetracycline quickly diffuses into cells and blocks transcription. We expect DOX, a tetracycline isomer and analogue, to have similar kinetics in regulating insulin gene expression in HepG2. However, the reverse process of decline of intracellular DOX concentration may be more complicated since, besides diffusion out, processes such as dissociation of DOX from the DOX-protein complex, distribution and accumulation of DOX in organelles, and metabolism of DOX may be occurring and contributing to the delays in insulin secretion down-regulation exhibited by the Tet-On system (Figure 7). It is possible that with a metabolizable secretagogue, e.g., glucose, the intrinsic kinetics of transcription are closer to the kinetics of secretion, especially during the down-regulation process.

In Figure 7, we applied two DOX square waves to induce the Tet-On system, and obtained a 5-fold increase of insulin secretion from the single copy plasmid. We have also performed an 8-h induction with the same Tet-On system, and obtained an approximately 30-fold increase of insulin secretion. This result is similar to the findings of Gossen et al. [115] using luciferase as the reporter to test the kinetics of the Tet-On system. Due to the stability of the one-copy PPI mRNA plasmid, there was no decline in insulin secretion rate during the two 7-h DOX-free basal periods. However, the increase in insulin secretion from the second induction was significantly lower than the first. If the experiment continued in the same fashion, we would expect the insulin secretion to not exhibit any net increase after some maximum, as any (small) additional increase in

secretion rate upon induction would be compensated by a decline during the subsequent basal period.

This work constitutes a first step towards developing potentially autologous, genetically engineered, transcriptionally controlled non- β -cells for treatment of IDD. Clearly, the NMD-mediated improvement of secretion dynamics will need to be validated in primary cells expressing insulin under a glucose-responsive promoter. In this, it is important that the host cells and the insulin gene be of the same species, as species mismatch may alter the stability of PPI mRNA considerably. It should be noted, however, that there exists no evidence that the NMD methodology would not be applicable to primary cells. Finally, the recombinant cells will need to be studied in an in vivo situation, as the insulin secretion dynamics might be different in culture and in vivo.

Table 2. Probe and primer sequences for TaqMan real-time PCR technique.

Gene	Probe and primer sequence (5' → 3')
Insulin	FAM probe: TCCGACCTGGTGGGAAGCTCTCTACCTAGTG Forward: TTTGTGAACCAACACCTGTGC Reverse: GGGTGTGTAGAAGAAGCCTCGTT
tTA	VIC probe: CCCGTAAACTCGCCCAGAAGCTAGGTGT Forward: GGTCGGAATCGAAGGTTTAACA Reverse: TGCCAATACAATGTAGGCTGCT

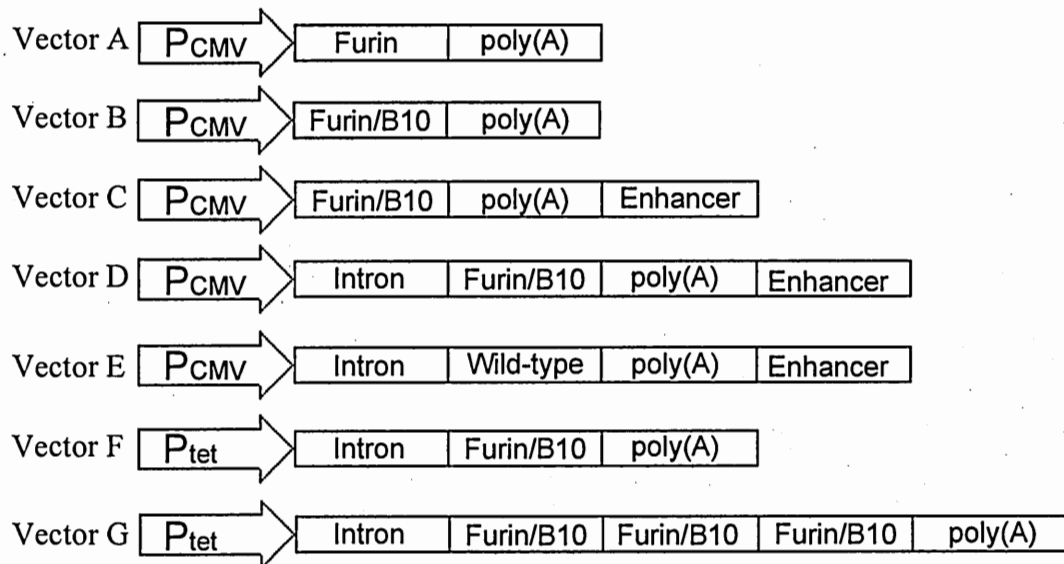


Figure 3. Plasmid structures for systematic increase of insulin gene expression in HepG2 cells. Vectors A, B, C, D and E were constructed for evaluating elements that increase insulin expression. Vector F and G are the tet-responsive plasmids with one and three copies, respectively, of PPI cDNA.

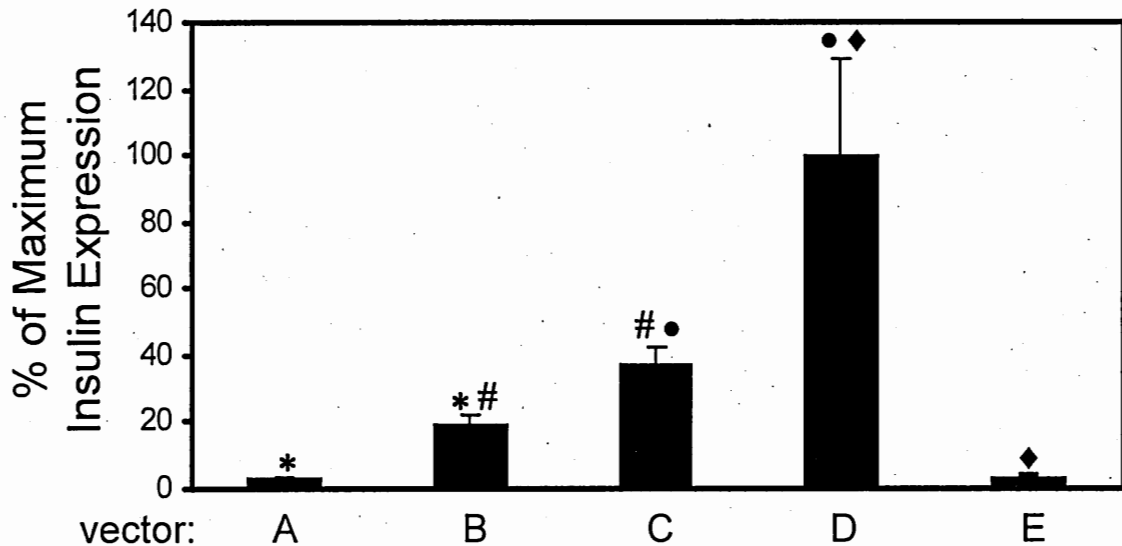


Figure 4. Systematic increase of insulin expression from transfected HepG2 hepatomas. Cells were transiently co-transfected with the test vector shown in Figure 3 (A, B, C, D or E) and the internal control plasmid pGL3-control. Luciferase, expressed through pGL3-control, was used to normalize the insulin secretion rate and thus correct for variations in transfection efficiency. The normalized insulin secretion rate from vector D (highest insulin expression vector) was assigned a value of 100. Experiments were performed in triplicate wells. *, # and ♦ indicate P values < 0.02, and • indicates P value < 0.04. P values were calculated using a one-tailed t-test, assuming unequal variances. Bars indicate standard deviation.

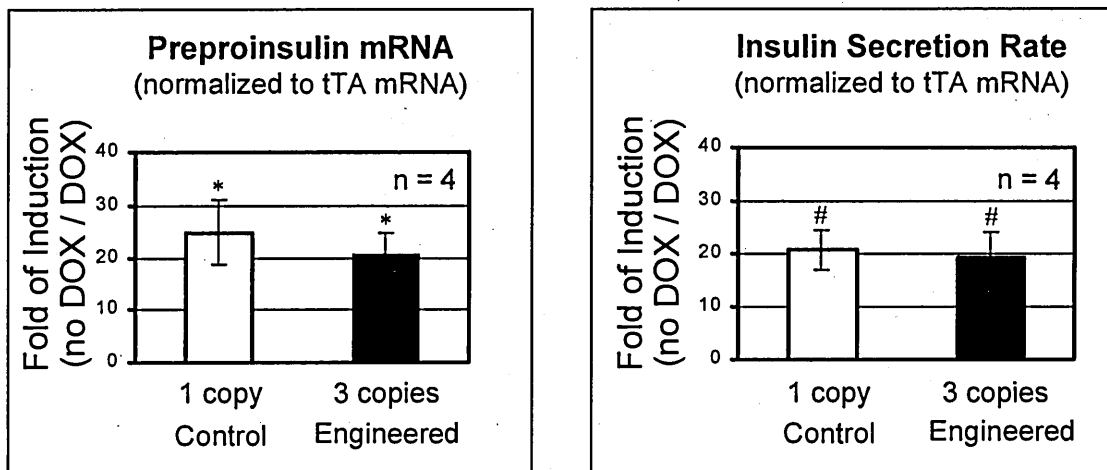


Figure 5. Regulation of insulin gene expression using Tet-Off system with control (open bar) and engineered (closed bar) PPI mRNA expression. tTA mRNA was used as an internal standard for both quantitative mRNA measurement and insulin secretion rate measurement. Doxycycline (DOX) repressed insulin gene expression. Each experiment involved 4 independent tests. * indicates P value = 0.15, and # indicates P value = 0.32. Bars indicate standard deviations.

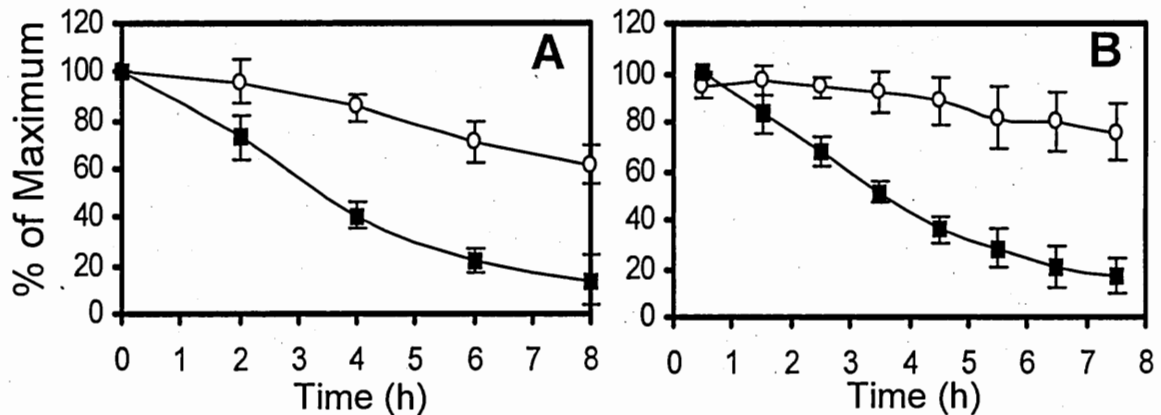


Figure 6. Dynamics of down-regulation of insulin gene expression using Tet-Off system with control (open circles) and engineered (filled squares) PPI mRNA expression. A: Down-regulation of PPI mRNA. B: Down-regulation of insulin secretion rate. tTA mRNA was used as an internal standard for quantitative PPI mRNA assay. In each independent test, the PPI mRNA was normalized by designating the sample without DOX treatment ($t = 0$) as the calibrator and setting it at 100%; insulin secretion rates were normalized by designating the sample with the highest rate as the calibrator and setting it at 100%. After 8-h down-regulation, the control and the engineered PPI mRNA decreased to $61 \pm 8 \%$ ($P < 0.007$) and $14 \pm 10 \%$ ($P < 0.003$), respectively, and the insulin secretion rates to $75 \pm 12 \%$ ($P < 0.05$) and $17 \pm 7 \%$ ($P < 0.002$), respectively. Each experiment involved 3 independent tests. Bars indicate standard deviations.

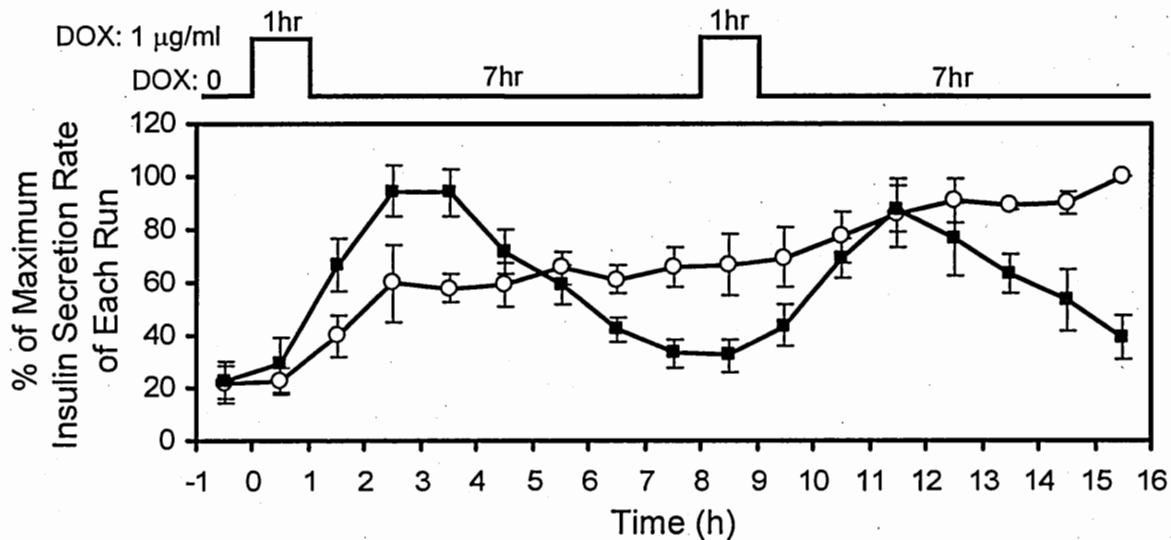


Figure 7. Regulation of insulin secretion from transfected HepG2 cells using Tet-On system with control (open circles) and engineered (filled squares) PPI mRNA expression. In each independent test, the insulin secretion rates were normalized by designating the sample with the highest rate as the calibrator and setting it at 100%. Each experiment involved 3 independent tests. Bars indicate standard deviations.

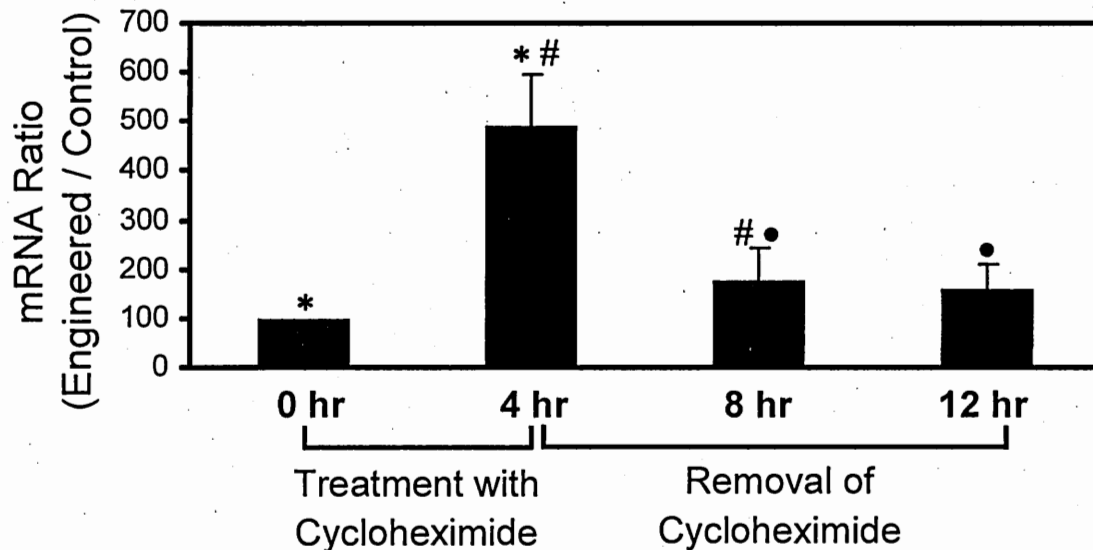


Figure 8. Effect of translation on PPI mRNA stability (evidence of nonsense-mediated mRNA decay). HepG2 hepatomas were transiently co-transfected with pTet-Off and the control or engineered PPI mRNA expression plasmid. tTA mRNA was used as an internal standard for quantitative PPI mRNA assay. In each independent test, the PPI mRNA ratios (engineered / control) were normalized against the sample without cycloheximide treatment (t = 0), which was set at 100%. Each experiment involved 4 independent tests. * and # indicate P values < 0.003, and • indicates P value = 0.36. Bars indicate standard deviations.

CHAPTER 4

Development of Genetically Engineered Human Intestinal Cells for Regulated Insulin Secretion Using rAAV-Mediated Gene Transfer

4.1 Abstract

Cell-based therapies for treating insulin-dependent diabetes (IDD) can provide a more physiologic regulation of blood glucose levels in a less invasive fashion than daily insulin injections. Promising cells include intestinal enteroendocrine cells genetically engineered to secrete insulin in response to physiologic stimuli; responsiveness occurs at the exocytosis level to regulate the acute release of recombinant insulin. In this work, we established a human cellular model to demonstrate that meat hydrolysate can simultaneously stimulate glucagon-like peptide-1 (GLP-1, an enteroendocrine cell-derived incretin hormone) and recombinant insulin secretion from the engineered human NCI-H716 intestinal cell line. Cells were genetically modified using the recombinant adeno-associated virus (rAAV)-mediated insulin gene transfer. Recombinant cells were then differentiated to display endocrine features, in particular the formation of granule-like compartments. A fusion protein of insulin and enhanced green fluorescent protein (EGFP) was designed to reveal the compartments of localization of the fusion protein and assess its co-localization with endogenous GLP-1. Our work provides a unique human cellular model for regulated insulin release through genetic engineering of GLP-1 secreting intestinal cells, which is expected to be useful for cell-based therapies of IDD.

4.2 Introduction

Cell-based therapies for treating insulin-dependent diabetes (IDD) can provide a more physiologic regulation of blood glucose levels in a less invasive fashion than daily insulin injections. Promising cells include non-beta-cells genetically engineered to secrete insulin in response to physiologic cues [2-7,9]. These cells are potentially autologous, targeted by gene transfer vectors or retrieved as a biopsy from the patient and genetically engineered *ex vivo*, and thus relax the immune acceptance problems existing with allo- and xenogeneic cells.

Enteroendocrine cell families, which are scattered in the lining of the digestive tract, are good candidates to be engineered for regulated insulin secretion [7,9,10,118,119]. Incretins such as glucagon-like peptide-1 (GLP-1) and glucose-dependent insulintropic polypeptide (GIP) are naturally released from enteroendocrine L- and K-cells after a meal to potentiate insulin production from pancreas [11]. Incretin and insulin secretions are closely related to nutrient ingestion and digestion for normalization of postprandial glycemia. Based on this, Cheung et al. [7] used a transgenic mouse model to show that regulated human insulin production by the genetically engineered GIP-secreting gut K-cells is sufficient to correct streptozotocin (STZ)-induced diabetes.

To genetically modify enteroendocrine cells for IDD treatment, it is essential to have a reliable gene deliver method, which should efficiently transfer the insulin gene, maintain stable insulin expression in the host cells, and cause little, if any, immune response in the patient. Among the available gene transfer techniques, the recombinant adeno-associated virus, or rAAV, -based vectors are currently generating promising

results in phase II clinical trials involving targeting the epithelial tissues of the lungs for treating cystic fibrosis [120]. Thus, rAAV is an intriguing gene delivery vehicle for targeting other tissues. In this work, we applied rAAV-mediated insulin gene transfer to genetically modify a model cell type, the human NCI-H716 intestinal cell line, for regulated insulin release. This line is derived from a poorly differentiated caecal adenocarcinoma [121], and has been described to exhibit enteroendocrine L-cell-like characteristics, in particular formation of secretory granules and regulated GLP-1 secretion after differentiation [122-124]. We aim to use this line as a human cellular model to demonstrate that insulin release from the engineered GLP-1-secreting intestinal cells can respond to physiologic stimuli. The implications of our findings in using rAAV to produce appropriately secreting non- β -cells with regulated insulin release are discussed.

4.3 Materials and methods

4.3.1 Cell culture

Human NCI-H716 cells (ATCC, Manassas, VA) were grown in suspension in RPMI 1640 medium supplemented with 10% fetal bovine serum and 100 units/ml penicillin/streptomycin for proliferation and maintenance. Adhesion and endocrine differentiation were initiated by growing the cells on Matrigel (Becton Dickinson, Bedford, MA)-coated tissue culture surfaces in differentiation medium (DMEM supplemented with 10% fetal bovine serum) [122-124]. Human embryonic kidney 293 (HEK293) cells (ATCC) used for AAV production and human HT-1080 fibrosarcoma cells (ATCC) used for AAV titer determination were grown in DMEM supplemented

with 10% heat-inactivated fetal bovine serum and 100 units/ml penicillin/streptomycin. Cell cultures were all maintained at 37°C in a 5% CO₂ / 95% air humidified atmosphere.

4.3.2 Recombinant AAV vector plasmid

Figure 9A shows the structure of the rAAV vector plasmid constructed for insulin and EGFP expression. The backbone of the plasmid originated from plasmid pAAV-MCS (Stratagene, La Jolla, CA), which contains inverted terminal repeats (ITRs) of AAV serotype 2 (AAV2). Human preproinsulin cDNA with the His B10-to-Asp mutation (B10 mutation) was a generous gift from Genentech, Inc (San Francisco, CA) [43]. The 5'-end of this gene was connected to a chimeric intron obtained from plasmid pRL/Null (Promega, Madison, WI) for optimal splicing. The 3'-end of this gene was connected to a fragment originated from plasmid pGL3-control (Promega) containing Simian Virus 40 (SV40) late polyadenylation signal for transcription termination and SV40 enhancer to elevate gene expression. The human cytomegalovirus (CMV) promoter provided by the backbone plasmid pAAV-MCS was used to constitutively drive the insulin gene expression. To include an EGFP expression cassette for reporter assays, a gene fragment containing EGFP cDNA and a synthetic intron from plasmid pEGFP-IRESpuro (Clontech, Palo Alto, CA) was connected to the SV40 promoter (originated from plasmid pGL3-control) then inserted to the 3'-end of the insulin expression cassette. The human growth hormone (hGH) polyadenylation signal provided by the backbone plasmid pAAV-MCS supported transcription termination of EGFP expression. The finished construct contained insulin and EGFP expression cassettes flanked by AAV2 ITRs.

4.3.3 Recombinant AAV vector production, purification, titration and transduction

Production of the rAAV vector, AAV/insulin/EGFP, encoding insulin and EGFP expression cassettes was accomplished using the AAV Helper-Free System (Stratagene) as described in the manufacture's protocol. Briefly, sixty 100-mm dishes of approximately 70% confluent, low-passage HEK293 cells were co-transfected with the constructed insulin and EGFP expression plasmid (Figure 9A), plasmid pAAV-RC (Stratagene) and plasmid pHelper (Stratagene). Transfections were carried out with FUGENE 6 reagent (Roche, Indianapolis, IN) following the manufacturer's directions. For each 100-mm dish, HEK293 cells were transfected with 2.5 μg of each of the three plasmids and 15 μl of FUGENE 6 reagent. Transfected cells were harvested 3 days after transfection. Purification of rAAV particles followed the protocol developed by Auricchio et al. [125] using a single-step heparin column chromatography. Titration of infectious rAAV particles (infectious units) was performed by fluorescence-activated cell sorting (FACS) following the protocol offered by Stratagene (Instruction Manual of AAV Helper-Free System) using HT-1080 cells as targets for transduction. FACS analysis was carried out using a Becton Dickinson LSR benchtop flow cytometer (BD Bioscience, Lexington, KY). The number of infectious units per ml of viral stock was approximately 7×10^7 . Transduction of NCI-H716 cells was performed in suspension using a multiplicity of infection (MOI) of 10 infectious units per cell in a 6-well-plate with 10^6 cells/well. Prior to transduction, NCI-H716 cells were centrifuged, washed with L-RPMI (RPMI supplemented with 2% FBS) then centrifuged again. A viral suspension prepared in L-RPMI was added to the cell pellet, mixed, then passed through a 21G needle several times. The cell-virus suspension was transferred to a 6-well-plate with 1.5-ml suspension

per well. After 2-h incubation at 37°C, 1.5 ml of H-RPMI (RPMI supplemented with 18% FBS) were added to each well. Images of EGFP expression in NCI-H716 cells were taken 3 days after transduction using OLYMPUS IX70 fluorescence microscope (Lake Success, NY). Transduction efficiencies were determined at the same time using FACS with EGFP as the reporter.

4.3.4 Immunochemical staining of insulin/proinsulin

AAV/insulin/EGFP transduced NCI-H716 cells were examined by immunochemical staining for insulin expression 3 days after transduction. Prior to the staining procedure, aggregates of transduced cells were disrupted by passing the cell suspension through a 21G needle several times. Cells were then seeded on a poly-L-lysine coated 4-chamber slide, fixed with 4% paraformaldehyde and permeabilized with 0.5% Triton X-100 in PBS. The DAKO ARK kit (DAKO, Carpinteria, CA) and mouse monoclonal anti-human insulin/proinsulin antibody (Sigma, cat # I-2018; diluted 1:1000) were applied to detect the presence of insulin/proinsulin in the fixed monolayer following the manufacturer's directions. This kit incorporates the avidin-biotin complex with peroxidase and uses diaminobenzidine as the chromogen.

4.3.5 Secretion studies

Three million of AAV/insulin/EGFP transduced NCI-H716 cells were differentiated by growing on Matrigel-coated, 60-mm culture wells in 4 ml of differentiation medium for two days. The culture medium was then switched to basal medium [DMEM (GIBCO, cat.# 23800-022, Grand Island, NY) supplemented with 5

mM glucose and 1% fetal bovine serum] overnight before secretion studies. On the day of the experiment, parallel cultures were washed and incubated in basal medium for two consecutive 1-h periods to stabilize the basal secretions of insulin and GLP-1. The secretion test was initiated by incubating stabilized monolayers in basal medium for one hour to determine the basal secretion rate. Monolayers were then exposed to 2% (w/v) meat hydrolysate (Sigma, cat. # P-7750, supplemented in basal medium) to stimulate GLP-1 and insulin secretion (test) or maintained in basal medium (control) for one hour. Cultures were then washed three times with basal medium and maintained in basal medium for another four hours. Every hour, the culture medium was renewed and samples were collected for insulin, proinsulin and GLP-1 assays.

4.3.6 Assays

Insulin and proinsulin concentrations were measured by human insulin specific radioimmunoassay (RIA) kit (LINCO Research, St. Charles, MI, cat. # HI-14K) and human proinsulin RIA kit (LINCO research, cat. # HPI-15K), respectively, according to the manufacturer's protocols. The human insulin specific RIA kit cross-reacts with proinsulin at less than 0.2%. The human proinsulin RIA cross-reacts with human insulin at less than 0.1%. Radioactivities were determined in Auto-Gamma Counting System, Cobra II (Packard, Meriden, CT). GLP-1 concentration was measured by GLP-1 ELISA kit (LINCO research, cat. # EGLP-35K). This kit measures only active forms of GLP-1, i.e., GLP-1 (7-36) and GLP-1 (7-37), and does not cross-react with other forms of GLP-1, including GLP-1 (1-36), GLP-1 (1-37), GLP-1 (9-36) and GLP-1 (9-37) (manufacturer's

specifications). The fluorochrome generated from the ELISA assay was quantified using a Spectra Max Gemini plate reader (Molecular Devices, Sunnyvale, CA).

4.3.7 Expression of insulin-EGFP fusion protein

Figure 9B shows schematically the rAAV vector plasmid designed for the expression of insulin-EGFP fusion protein. The backbone of the plasmid is the same as the plasmid in Figure 9A except the gene of interest is insulin-EGFP fusion protein. EGFP encoded from the plasmid pEGFP-N2 (Clontech) is located at the C terminus of preproinsulin. Figure 9C shows the DNA sequence at the junction of preproinsulin-EGFP cDNA fusion and the restriction enzymes used for the connection of preproinsulin cDNA and EGFP cDNA. Production, purification and titration of the rAAV vector, AAV/FUSION, encoding the insulin-EGFP fusion protein for infecting NCI-H716 cells followed the same procedure as previously described for preparation of AAV/insulin/EGFP.

4.3.8 Fluorescence microscopy of insulin-EGFP fusion protein and GLP-1 staining

AAV/FUSION transduced then differentiated NCI-H716 cells were detached and seeded on a poly-L-lysine coated 4-chamber glass slide. Seeded cells were imaged immediately by the OLYMPUS IX70 fluorescence microscope, or fixed (4% paraformaldehyde in PBS) for confocal microscopy, or fixed and permeabilized (0.5% Triton X-100 in PBS) for GLP-1 staining. GLP-1 staining was achieved by incubating the monolayer with primary antibody, rabbit-anti-GLP-1 (Aviva, San Diego, CA; diluted 1:50), at 4°C overnight then revealed with TRITC-conjugated anti-rabbit IgG (Sigma;

diluted 1:160). Localization of the insulin-EGFP fusion protein and GLP-1 immunofluorescence staining was performed using a Zeiss LSM510 laser-scanning confocal microscope (Carl Zeiss, Thornwood, NY). An argon laser at 488-nm and a 505-530-nm filter were used for detecting insulin-EGFP fusion protein. A helium/neon laser at 543-nm and a long pass 585-nm filter were used for TRITC detection.

4.4 Results

4.4.1 AAV/insulin/EGFP transduction of NCI-H716 cells

Human intestinal NCI-H716 cells grown in suspension as floating aggregates were transduced with the rAAV vector, AAV/insulin/EGFP, before differentiation. Twenty-four hours after transduction, some EGFP-positive cells could be identified under the fluorescence microscope. Figure 10 shows a typical result of EGFP expression in cells 3 days after transduction. To quantify the transduction efficiency, fluorescence-activated cell sorting (FACS) was applied to detect EGFP-positive cells. FACS analysis indicated that the percentage of EGFP-positive cells in a pool of transduced cells was 31% - 43% with an average of $37 \pm 5\%$ (n=4); the latter can be considered as the average transduction efficiency. To further characterize the insulin expression, insulin and proinsulin in transduced cells were immunochemically stained as shown in Figure 11. A brown-colored precipitate indicates the intracellular presence of insulin and/or, proinsulin antigens. These results show that the insulin and EGFP genes were successfully expressed in NCI-H716 cells via rAAV-mediated gene transfer.

4.4.2 Regulated GLP-1 and insulin secretion from the AAV/insulin/EGFP transduced and differentiated NCI-H716 cells

Endogenous GLP-1 and recombinant insulin secretion was studied from transduced, differentiated cells by exposing them to a square wave of 2% meat hydrolysate (MH) for 1 hour. MH has previously been shown to induce a 5-fold increase of GLP-1 release over a 2-h period [122]. Figure 12 shows the MH profile and the GLP-1 and insulin secretion rates, averaged over 1-h periods. GLP-1, insulin and proinsulin assays were all performed on the same samples collected during the secretion study. A 4.2-fold (± 1.1 , $n=4$) increase of the GLP-1 secretion rate was achieved with MH stimulation. Upon removal of MH, the GLP-1 secretion rate quickly decreased toward the basal level. The insulin release exhibited similar kinetics as the GLP-1, with a 2.7-fold (± 0.4 , $n=4$) increase after the stimulation (Figure 12B). Since the radioimmunoassays used to measure the concentrations of insulin and proinsulin are highly specific (see Materials and Methods for cross-reactivities), it was possible to estimate the conversion of proinsulin to insulin by calculating the ratio $(\text{insulin}) \times 100\% / (\text{insulin} + \text{proinsulin})$ using the amounts of secreted polypeptides. An approximately 80% conversion of proinsulin to insulin was consistently maintained throughout the test (Figure 12C), indicating that the engineered NCI-H716 cells possess the necessary proteolytic enzymes to process proinsulin to immunoreactive insulin.

Table 3 compares the insulin secretion rates from engineered NCI-H716 cells, mouse β -TC3 insulinomas [114], and engineered, insulin-secreting mouse pituitary AtT-20 tumor cells [126]. Both the basal and stimulated insulin secretion rates from recombinant NCI-H716 cells are lower by roughly an order of magnitude than those from

β -TC3 cells, but they are comparable to the insulin secretion rates from engineered AtT-20 cells.

4.4.3 Intracellular localization of insulin-EGFP fusion protein and of GLP-1

A control experiment was performed to reveal the secretory granules in the engineered NCI-H716 cells using the insulin-EGFP fusion protein. The plasmid constructed for the production of the rAAV vector, AAV/FUSION, encoding the insulin-EGFP fusion chimera is shown schematically in Figure 9B. This plasmid was designed to tag EGFP at the C terminus of preproinsulin by fusing the 3' end of the preproinsulin cDNA with the 5' end of the EGFP cDNA (Figure 9C). By this design, we expected that the secretory signal peptide from preproinsulin would direct the intracellular traffic of the fusion protein to the secretory granules, and the EGFP could reveal the localization of the fusion protein.

The AAV/FUSION transduced and differentiated cells were first examined by a conventional fluorescence microscope after being seeded on a poly-L-lysine coated glass slide (Figures 13A and 13B). In cells expressing insulin-EGFP fusion protein, small vesicular structures inside the cells can be visualized as shown in Figure 13B. Figure 14A shows a confocal laser-scanning micrograph of a single cell in the fixed monolayer expressing the insulin-EGFP fusion protein. The granule-like compartments were clearly revealed inside the cell. This kind of compartment cannot be visualized in the AAV/insulin/EGFP transduced then differentiated cells, which express separately insulin and EGFP. This is because EGFP naturally does not include any secretory peptide signal in its protein sequence; thus EGFP was homogeneously distributed in the cytosol, as

shown in Figure 14B. Figure 14C is a confocal laser-scanning micrograph of two cells expressing the insulin-EGFP fusion protein; Figure 14D shows the same two cells observed for GLP-1 staining. The co-localization of green (Figure 14C) and red (Figure 14D) fluorescence demonstrates the co-localization of recombinant insulin-EGFP fusion protein and endogenous GLP-1 in the granule-like compartments. These findings provide evidence that the engineered NCI-H716 cells possess secretory granules after differentiation, and that these granules are available for storage of endogenous GLP-1 as well as recombinant insulin. This is consistent with the similarity of GLP-1 and insulin secretory responses against MH (Figure 12).

4.5 Discussion

A cell-based therapy of IDD based on autologous cells relaxes the immune acceptance requirements posed by allogeneic and, more so, xenogeneic islets and β cell lines. A limitation of this approach, however, consists of achieving appropriate insulin secretion dynamics from engineered cell hosts. Recently, cells of enteroendocrine origin are receiving increased attention due to their potential for fast release of recombinant insulin in response to physiologic stimuli [7,9,10,118,119]. In this work, we examined the genetic engineering of the human NCI-H716 intestinal cell line as an enteroendocrine L-cell model for regulated insulin release. We demonstrated that rAAV can transduce and express the insulin gene in these cells which, after differentiation, secrete endogenous GLP-1 and recombinant insulin with the same acute dynamics following stimulation by MH. In accord with this, a fusion insulin/EGFP chimera was found to co-localize with

endogenous GLP-1 in the same granule-like compartments displaying the endocrine features of the cells.

After meals, dietary nutrients stimulate GLP-1 secretion mainly from the lower gut (jejunum, ileum, colon and rectum) where L-cells are relatively abundant [127-128]. Specifically, oral administration of glucose has shown a dose-dependent effect on circulative GLP-1 levels and parallel secretions of GLP-1 and insulin in humans. Both hormones rise within a few minutes after glucose ingestion, and return to the basal levels in 2 to 3 h depending on the glucose dosage [12, also in Appendix A8]. In this study, our engineered L-cell model demonstrated an acute release of both the recombinant insulin and the endogenous GLP-1 against stimulation. Cultures promptly responded to MH stimulation and up-regulated insulin and GLP-1 release within 1 h; once MH was removed, insulin and GLP-1 release decreased to essentially basal levels also within 1 h. Interestingly, the proinsulin conversion to insulin remained consistently high at 80%, indicating that there is a sufficient concentration of proteolytic enzymes recognizing proinsulin in the vesicular structures where proinsulin localizes. In the enteroendocrine cell model of this study, the correspondence of GLP-1 and insulin release against MH stimulation actually resembles the parallel secretion of postprandial GLP-1 and insulin in healthy humans. Thus, for IDD treatment, we expect that the engineered L-cells will quickly adjust between pre- and postprandial conditions to meet the different insulin demands.

To genetically modify gut epithelia, viral vectors have shown significant potential in targeting cells lining the gastrointestinal tract [13-15]. In particular, rAAV vectors have been used to infect gut epithelia through oral gavage administration resulting in

phenotypic change in a rat model over a 6-month period [15]. L-cells face the gut lumen, so it is expected that they could be directly accessed via noninvasive administrations in the gastrointestinal tract. However, since only about 1% of the intestinal epithelial population consists of enteroendocrine cells, there exists a significant challenge in selectively targeting L-cells to express insulin. Recent work by Nian et al. [129] using a transgenic mouse model to demonstrate that the human proglucagon promoter regulates tissue-specific gene expression in L-cells opens the possibility that recombinant insulin expression can be limited specifically in L-cells via the selective activity of the proglucagon promoter.

In parallel with the engineering of enteroendocrine cells for regulated insulin release, other investigators are using promoters up-regulated by glucose and possibly down-regulated by insulin to drive insulin expression in liver or muscle cells [2-6]. Using these glucose-regulated transgenes, insulin expression has been achieved in streptozotocin (STZ)-induced diabetic rodents with promising results. However, transcriptional regulation of insulin expression results in sluggish secretion dynamics which may not be appropriate for glycemic regulation in higher animals and, eventually, humans. Destabilization of preproinsulin mRNA by nonsense-mediated decay improves the secretion dynamics from transcriptionally regulated cells but not to the point of achieving an acute secretory response [89]. In a different approach, investigators are engineering insulin expression in other endocrine cells, such as pituitary cells, which possess the regulated secretion pathway; this is done in both transgenic mouse models and cell lines [33-36]. Recombinant pituitary cells respond to secretagogues with acute release of the insulin through exocytosis. Since pituitary cells lack responsiveness to

postprandial stimuli, attempts have been made to engineer glucose responsiveness by expressing glucokinase (GK), or the type II glucose transporter GLUT2, or both, in these cells [36]. However, recent data have shown that this approach may lead to glucose-induced toxicity and result in glucose-dependent apoptotic cell loss [130]. In addition, recombinant pituitary cells secrete not only insulin but also their endogenous hormones, and serious disorders may result if these hormones are abnormally up-regulated after meals.

Since an average person releases about 0.5 ~ 0.7 units of insulin per kg per day [131], the number of engineered L-cells needed to produce the same amount of insulin is estimated as 7.9×10^{10} to 15.8×10^{10} cells for a 70-kg person, after doing the following calculation:

$$\frac{\frac{0.5 \sim 0.7 \text{ unit}}{\text{day} \times \text{kg}} \times 6 \times 10^6 \frac{\text{fmole}}{\text{unit}} \times 70 \text{ kg}}{79 \frac{\text{fmole}}{10^6 \text{ cells} \times \text{h}} \times 24 \frac{\text{h}}{\text{day}}} = 1.1 \sim 1.6 \times 10^{11} \text{ cells,}$$

where $79 \text{ fmole}/(10^6 \text{ cells} \times \text{h})$ is the basal insulin secretion rate of recombinant NCI-H716 cells. In another calculation to estimate the number of engineered L-cells to maintain the basal insulin level in the body, the dynamic insulin balance can be written as:

$$\Delta(Vc) / \Delta t = N \times q - k \times Vc,$$

where $\Delta(Vc)$ is the change of insulin amount in the body over time period Δt ; N is the number of cells; q is the specific insulin secretion rate; and k is the reaction constant of insulin degradation. First order degradation of insulin in the body is assumed. We also assume 150 fmole/ml of basal insulin level in the circulation, 5 L of blood and 5-min

half-life of insulin [12, 131]. To obtain the reaction constant k of insulin degradation, we do the following calculation:

$$\Delta(V_c) / \Delta t = -k \times V_c,$$

where zero generation of insulin and only insulin degradation occurs. Integrating, we obtain $\ln(V_c/V_{c0}) = -k \times t$, where V_c/V_{c0} is 1/2 when $t = 5$ min; therefore, k is 0.139 min^{-1} or 8.34 h^{-1} . Assuming insulin concentration reaches steady state at the basal level, the number of required engineered L-cells to maintain the basal insulin level is:

$N = k \times V_c / q = (8.34 \text{ h}^{-1} \times 5 \text{ L} \times 150 \text{ fmole/ml} \times 1000 \text{ ml/L}) / 79 \text{ fmole}/(10^6 \text{ cells} \times \text{h}) = 7.9 \times 10^{10}$ cells. The result is within the range of the first estimation, thus validating the approximate equivalence of the two approaches. Following the second approach, we estimate the number of enteroendocrine L-cells in the body by doing the following calculation:

$\ln(V_c/V_{c0}) = -k' \times t \Rightarrow k' = 8.34 \text{ h}^{-1}$, where k' is the reaction constant of GLP-1 degradation and we assume 5-min half-life of GLP-1 in the circulation [105], and N' (the number of L-cells in the body) = $k' \times V_{c'} / q' = (8.34 \text{ h}^{-1} \times 5 \text{ L} \times 1 \text{ pmole/L}) / 9.5 \text{ pmole}/(10^6 \text{ cells} \times \text{h}) = 4.4 \times 10^9$ cells (assuming 1 pmole/L of the basal GLP-1 level, 5 L of blood [12] and the averaged basal GLP-1 secretion rate, $9.5 \times 10^{-3} \text{ pmole}/(10^6 \text{ cells} \times \text{h})$, obtained via the secretion study of NCI-H716 cells in the result section), which is less than the number required to maintain the basal insulin level by a factor of 25 ~ 36. Thus, to overcome the deficiency, a higher insulin secretion rate from the engineered L-cells is necessary to reduce the required L-cell number. Potentially, this could be done by improving the transduction efficiency, such as using a higher viral titer as well as

multiple transductions, which would increase the percentage of transduced cells and the gene dosage per cell.

In summary, we have established a regulated insulin-secreting cellular model by rAAV-mediated insulin gene transfer to a human GLP-1-secreting intestinal cell line. Results demonstrated similar secretion dynamics of recombinant insulin and endogenous GLP-1. Our study thus suggests that engineering intestinal L-cells to produce insulin may constitute a feasible approach for IDD treatment in terms of the dynamic response and the compatibility of GLP-1 and insulin in glycemic normalization.

Table 3. Comparison of insulin secretion rates. BrcAMP: 8-bromo-cyclic AMP.

	Engineered human NCI-H716 cells		Mouse β -TC3 insulinomas [114]		Engineered mouse pituitary AtT-20 cells [126]	
	basal	stimulated	basal	stimulated	basal	stimulated
	0% MH	2% MH	0 mM glucose	25 mM glucose	0 mM BrcAMP	5 mM BrcAMP
Insulin secretion rate [fmole/(10 ⁶ cells×h)]	79 (\pm 29, n=4)	204 (\pm 44, n=4)	384 (\pm 210, n=4)	3930 (\pm 264, n=2)	60	360

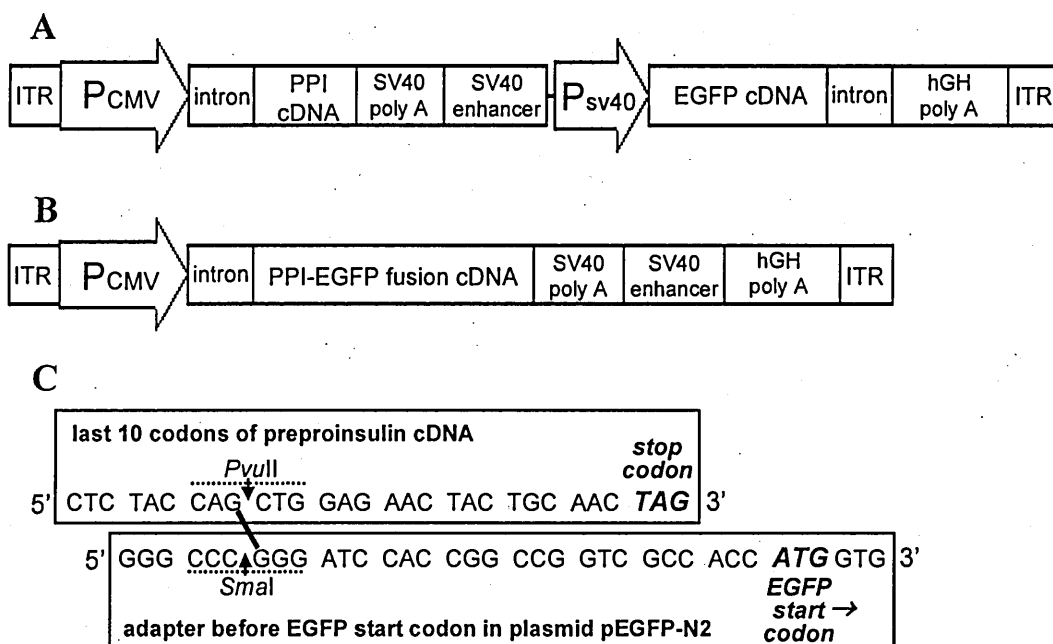


Figure 9. Structures of the rAAV vector plasmids for expression of insulin and EGFP (A), and insulin-EGFP fusion protein (B). (C), DNA sequence at the junction of the preproinsulin-EGFP fusion cDNA in (B). *PvuII* restriction endonuclease was used to generate a preproinsulin cDNA fragment with a 3'-blunt end. *SmaI* restriction endonuclease was used to generate an EGFP cDNA fragment from plasmid pEGFP-N2 with a 5'-blunt end. The two blunt ends were then ligated to generate a chimeric fusion cDNA encoding insulin-EGFP fusion protein. PPI: preproinsulin.

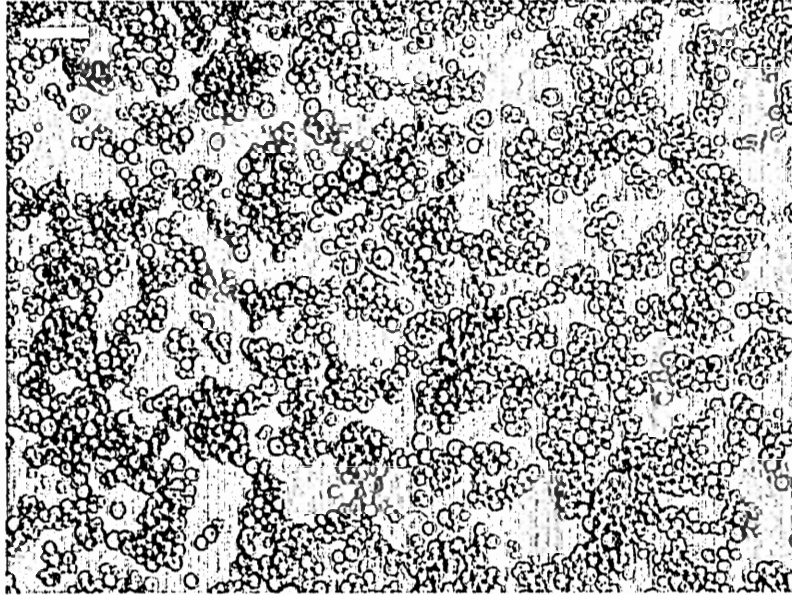
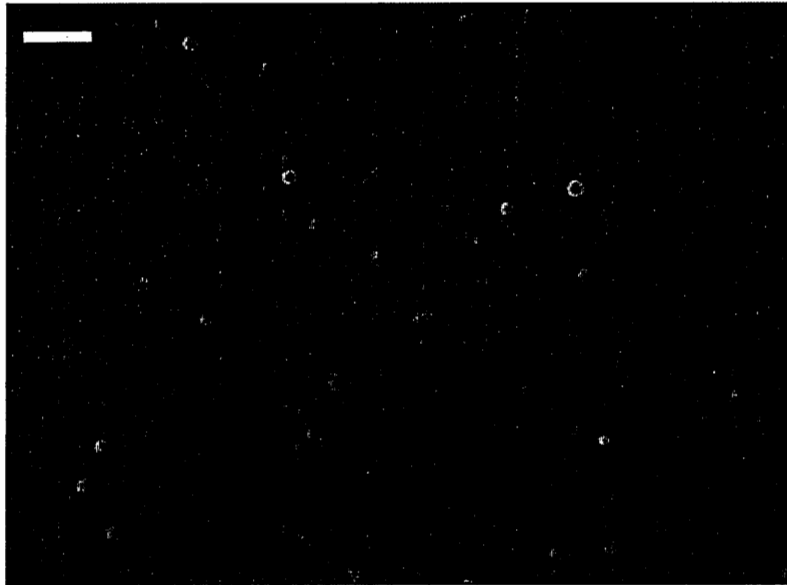
A**B**

Figure 10. Phase-contrast (A) and fluorescence (B) micrographs of the AAV/insulin/EGFP transduced NCI-H716 cells in suspension. (A) and (B) were taken under the same view. EGFP used as a reporter for rAAV-mediated gene transfer. Bar = 100 μ m.

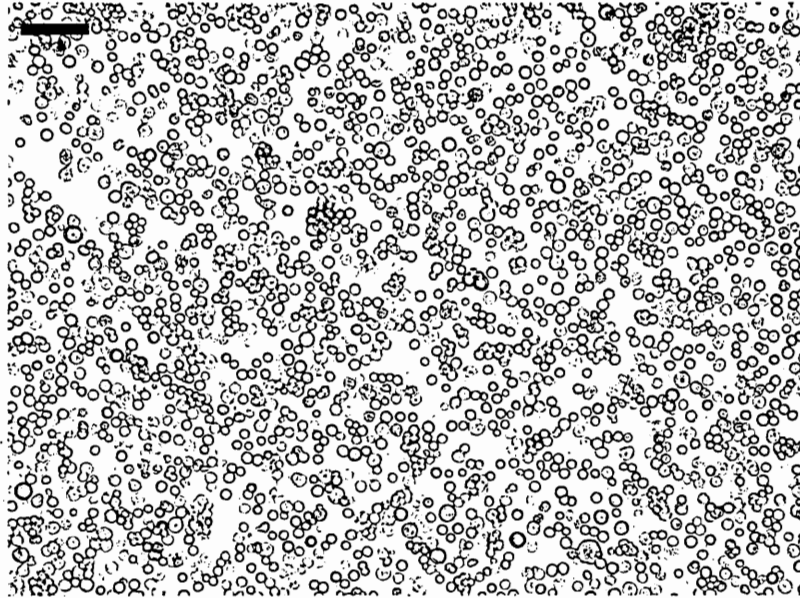
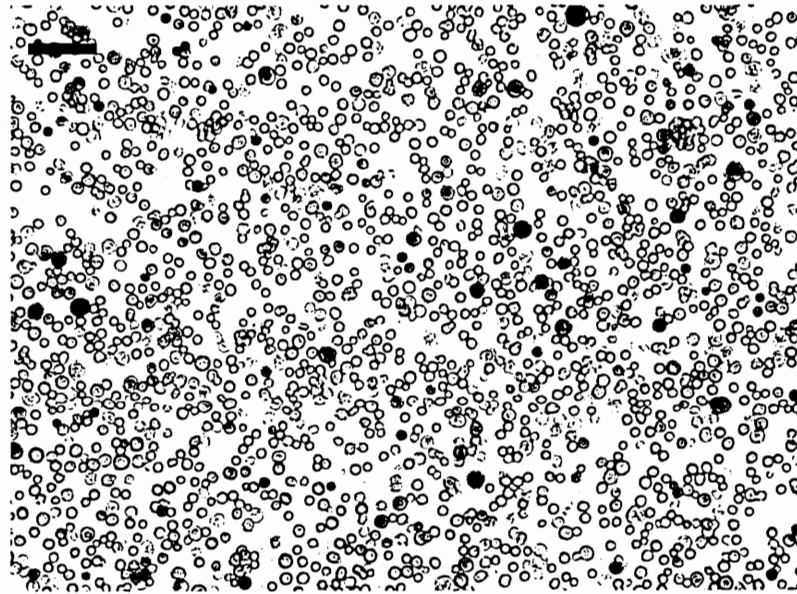
A**B**

Figure 11. Immunochemical staining of insulin/proinsulin in the AAV/insulin/EGFP transduced NCI-H716 cells. (A) Control: parental cells without transduction. (B) Test: cells transduced with AAV/insulin/EGFP. Use of avidin-biotin complex with peroxidase and 3,3'-diaminobenzidine as the chromogen resulted in a brown-colored precipitate at the antigen site. Bar = 100 μ m.

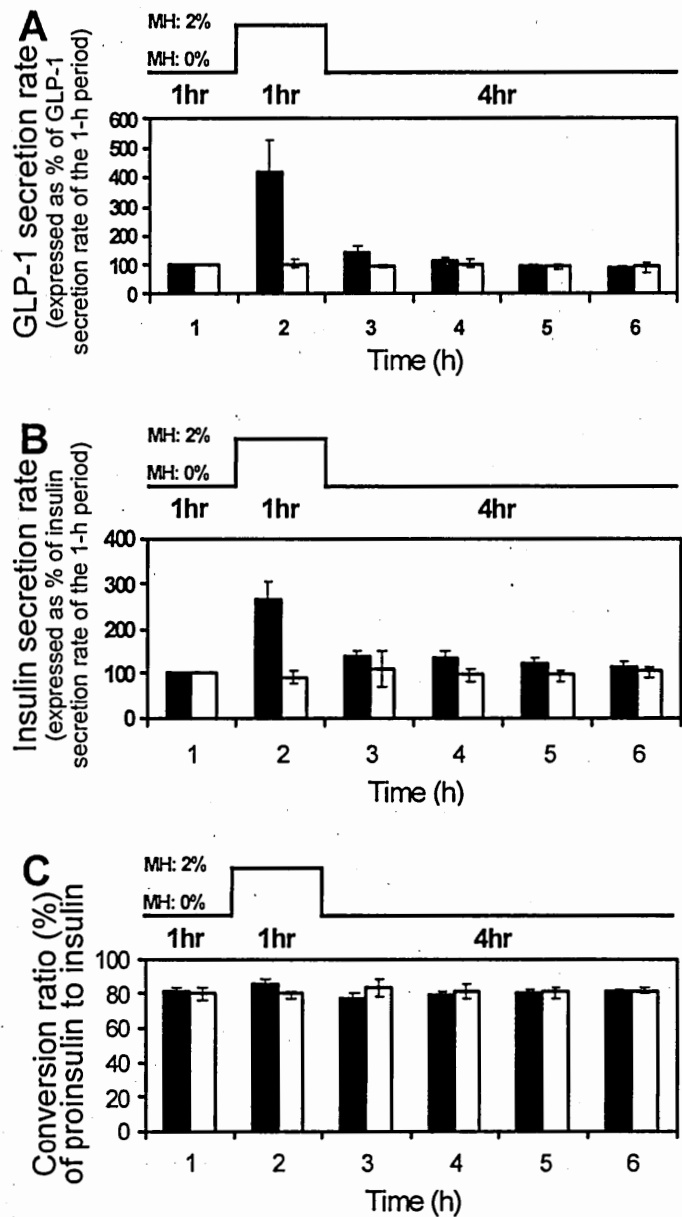


Figure 12. Secretion study of the engineered NCI-H716 cells against MH stimulation. Solid bars (test) are for monolayers incubated in the basal medium for 1 h, exposed to MH for 1 h, then switched back to the basal medium for another 4 h. Empty bars (control) are for monolayers treated with basal medium throughout the test. In each independent test of (A) and (B), the GLP-1 and the insulin secretion rates were normalized against the 1-h sample, which was set at 100%. (C), conversion of proinsulin to insulin. During each 1-h period of the secretion study, proinsulin and insulin concentrations in the samples were determined by proinsulin and the insulin radioimmunoassays each with very low cross-reactivity against the other species (see Materials and Methods). The percent conversion of proinsulin to insulin was calculated as $(\text{insulin}) \times 100\% / (\text{insulin} + \text{proinsulin})$. Each experiment involved 4 independent tests. Bars indicate standard deviations.

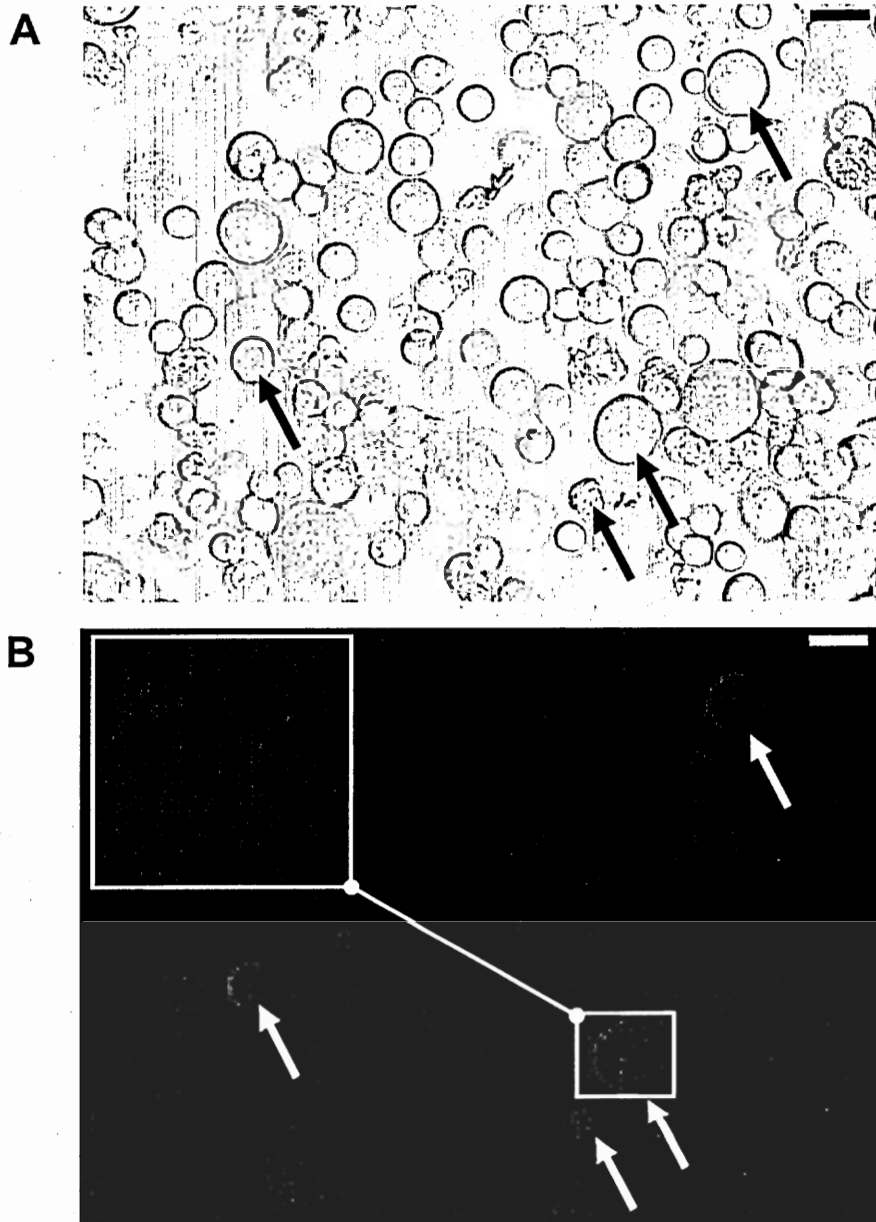


Figure 13. Phase-contrast (A) and fluorescence (B) micrographs of the same AAV/FUSION transduced, differentiated NCI-H716 cells. Arrows in (A) indicate cells with strong fluorescence, which appear clearly in (B) where they are also indicated by arrows. A typical fluorescence-positive cell is enlarged and displayed in the corner of (B). More fluorescence-positive cells could be seen under the same view by adjusting the focus; these appear vaguely in the background of (B). Insulin-EGFP fusion protein was used to reveal the secretory granules in the engineered cells. Secretory granules appeared as green, small vesicular structures inside the cells. Bar = 10 μ m.

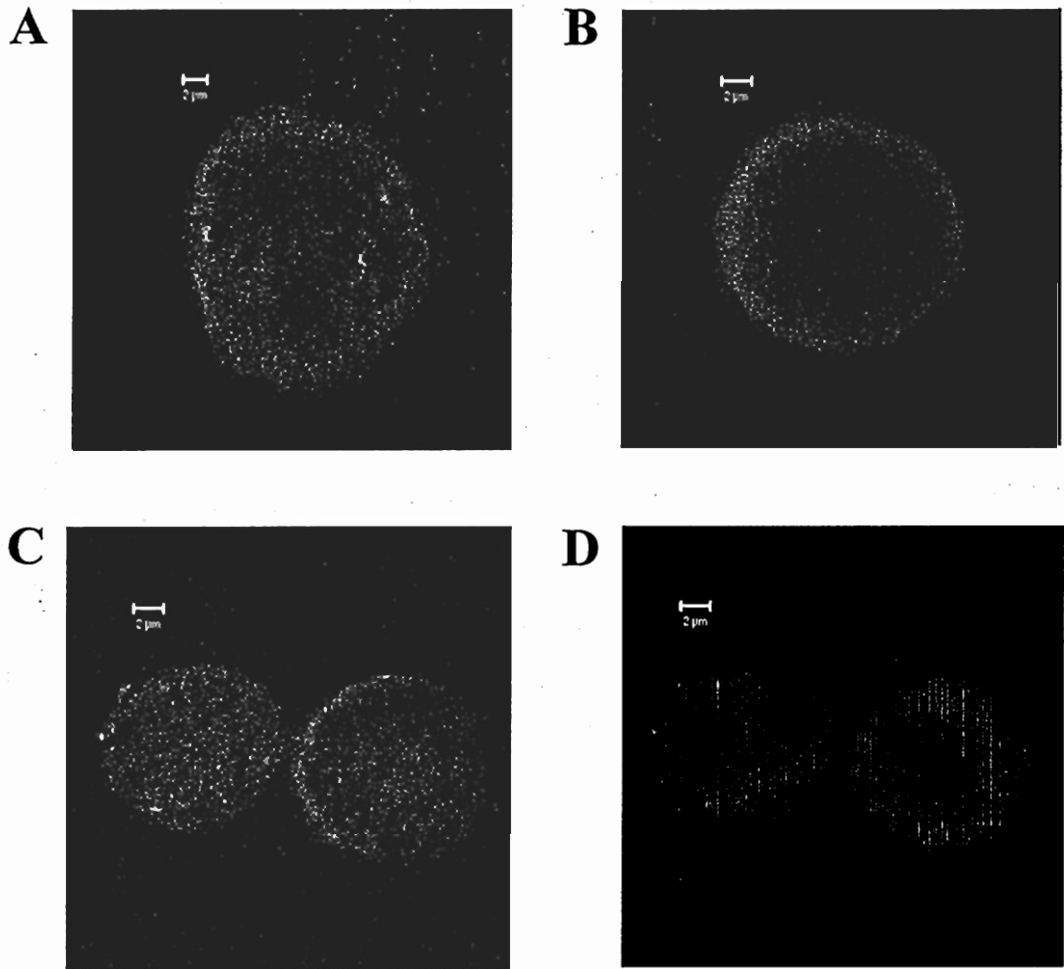


Figure 14. Confocal laser-scanning microscopy of the engineered NCI-H716 cells. (A) A single AAV/FUSION transduced, differentiated NCI-H716 cell. Dense-core, granule-like compartments can be clearly identified. (B) A single AAV/insulin/EGFP transduced, differentiated NCI-H716 cell. EGFP homogeneously distributed in the cytosol. (C) and (D) are views of the same AAV/FUSION transduced, differentiated NCI-H716 cells expressing insulin-EGFP fusion protein (green fluorescence) and immunostained for GLP-1 (red fluorescence). (C) and (D) were taken under the same view using different fluorescence detection settings (see Materials and methods). Co-localization of insulin-EGFP fusion protein with GLP-1 is demonstrated by the green fluorescence in (C) occupying the same compartments as the red fluorescence in (D). Bar = 2 μ m.

CHAPTER 5

Differential rAAV2 Transduction Efficiencies and Insulin Secretion Profiles in Pure and Co-Culture Models of Human Enteroendocrine L-Cells and Enterocytes

5.1 Abstract

Cell-based therapies for treating insulin-dependent diabetes (IDD) can provide a more physiologic regulation of blood glucose levels in a less invasive fashion than insulin injections. Previously, we developed an engineered human enteroendocrine L-cell model for regulated insulin release via recombinant adeno-associated virus serotype 2, or rAAV2, transduction. The aim of this study was to evaluate the efficiency and selectivity of rAAV2-mediated insulin gene delivery to enteroendocrine L-cells in co-culture with a prevailing number of enterocytes, which are the predominant cell type in intestinal epithelium. We tested rAAV2 transduction in pure and co-culture models of human cell lines of enterocytes (Caco-2 and T84 cells lines) and enteroendocrine L-cells (NCI-H716 cell line). Non-viral, chemical-mediated transfection was used as a control. Transduced and transfected co-cultures were subjected to insulin secretion studies. In pure cultures, rAAV2 exhibited low transduction efficiency towards both Caco-2 and T84 enterocytes, as opposed to a strong reporter expression in permissive NCI-H716 L-cells. In co-cultures of NCI-H716 L-cells and Caco-2 or T84 enterocytes, rAAV2 exhibited differential transduction efficiency with a strong preference towards NCI-H716 L-cells. The rAAV2 transduced co-culture achieved regulated insulin release against stimulation, whereas the chemically transfected co-culture failed to respond. This study demonstrated

that rAAV2-mediated insulin gene transfer can differentiate human intestinal cell types in vitro, in particular enterocyte and enteroendocrine L cell lines. We consider the AAV2 vector a useful tool in developing enteroendocrine L-cell specific insulin gene delivery for IDD treatment, in terms of AAV2 avoiding enterocytes and targeting selectively L-cells.

5.2 Introduction

Surrogate β -cells generated by genetic engineering of non- β -cells to produce insulin provide an alternative to islet transplantation in cell-based therapies for treating insulin-dependent diabetes (IDD). Engineered β -cell surrogates are potentially autologous, targeted by gene transfer vehicles in vivo or obtained as a biopsy from the patient and genetically modified ex vivo, and thus greatly relax the immune acceptance problems posed by allogeneic and, more so, xenogeneic islet transplantation.

A variety of cell types have been engineered to produce insulin, including fibroblasts [30], hepatic cells [2-4,6], muscle cells [5,31] and endocrine cells of different origins [33-36]. While developing recombinant β -cell surrogates, the advantages of targeting intestinal enteroendocrine cells have received notable attention [7-10]. Naturally, enteroendocrine cell families are found scattered in the crypts of gut mucosa; they release incretin hormones such as glucagon-like peptide-1 (GLP-1, from intestinal L-cells) and glucose-dependent insulinotropic polypeptide (GIP, from gut K-cells) after a meal to potentiate insulin production from pancreas [11]. Both incretin hormone and insulin secretion are closely related to nutrient ingestion and digestion for normalization of postprandial glycemia [12]. Because of the unique connection between incretins and

insulin, engineering of enteroendocrine cells for regulated insulin secretion constitutes a feasible approach for IDD treatment in terms of the dynamic release of insulin, as well as the compatibility of incretins and insulin in glycemic normalization.

Although promising results from *in vitro* and *in vivo* models have demonstrated the potential of using the engineered enteroendocrine cells to correct IDD [7-9], the identification of an enteroendocrine cell target for insulin production remains a big hurdle. This is because only approximately 1% of the intestinal epithelial population consists of enteroendocrine cells; therefore, there exists a significant challenge in selectively targeting enteroendocrine cells *in vivo*. Likewise, it is also difficult to isolate a pure enteroendocrine population from intestinal biopsy and perform *ex-vivo* somatic cell gene delivery.

Along the digestive tract, the intestinal epithelium consists of four major cell types: enterocytes, goblet cells, Paneth cells and enteroendocrine cells. Among these cells, the enterocytes, which account for digestion and absorption of luminal nutrients, are the predominant cell type. Because of the prevailing enterocytes in the gut epithelium, any method intending to modify enteroendocrine cells will inevitably interact with the surrounding enterocytes. Previously, we applied rAAV2-mediated transduction to establish a recombinant human enteroendocrine L-cell model with regulated insulin release [8, or CHAPTER 4]. However, it is unclear whether the same process remains effective under the presence of a majority of enterocytes. Here, prior to *in-vivo* experiments, we studied the efficiency and specificity of rAAV2-mediated transduction in co-cultures of human enterocyte and enteroendocrine L cell lines. The uptake and expression of the insulin gene, as well as the conversion of proinsulin to insulin and the

dynamics of insulin secretion were characterized. The implications of our findings towards using rAAV2-mediated gene transfer to produce appropriately secreting, potentially autologous enteroendocrine L-cells for IDD treatment are discussed.

5.3 Materials and methods

5.3.1 Cell lines and culture conditions

Human Caco-2 cells (ATCC, Manassas, VA; an enterocyte model [132]) and Human HT-1080 fibroblasts (ATCC) were cultured in Dulbecco's modification of Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% nonessential amino acids. Human T84 cells (ATCC; an enterocyte model [133]) were cultured in a 1:1 mixture of Ham's F12 medium and DMEM supplemented with 5% of FBS. Human NCI-H716 cells (ATCC) as the enteroendocrine L-cell model [122,134] were grown in suspension in RPMI medium supplemented with 10% FBS. Adhesion and endocrine differentiation of NCI-H716 cells were initiated by growing the cells on Matrigel (Becton Dickinson, Bedford, MA)-coated surfaces in DMEM supplemented with 10% fetal bovine serum [124]. Human embryonic kidney 293 (HEK293) cells (ATCC) used for rAAV2 production were grown in DMEM supplemented with 10% heat-inactivated FBS. Co-cultures of NCI-H716 cells and Caco-2 cells were grown in the Caco-2 culture medium; co-cultures of NCI-H716 cells and T84 cells were grown in the T84 culture medium. Growth media were all supplemented with 100 U/ml penicillin and 100 µg/ml streptomycin. Cell cultures were all maintained at 37°C in a 5% CO₂ / 95% air humidified atmosphere.

5.3.2 Plasmids and Virus

The rAAV vector plasmid, pInsulin-EGFP (Figure 15A), carrying the expression cassettes of insulin (CMV-driven) and EGFP (SV40-driven), was constructed as previously described [8, or CHAPTER 4]. This plasmid was used with two other plasmids, pAAV-RC (Stratagene, La Jolla, CA) and pHelper (Stratagene), for rAAV2 production following the manufacturer's protocol (AAV Helper-Free System, Stratagene). Purification and titration of rAAV2 were performed as previously described [8, or CHAPTER 4]. Plasmid pInsulin-EGFP, alone, was also used in FUGENE6 (Roche, Indianapolis, IN)-mediated transfection as a control.

5.3.3 Co-culture systems

Two co-culture systems of enterocyte and enteroendocrine L cell lines were applied. In the first co-culture system, a total of 2.5 million NCI-H716 L-cells and enterocytes (Caco-2 cells or T84 cells), were mixed in a ratio of 1:10 and seeded onto a 35-mm tissue-culture dish. One day later, the co-culture was washed twice using culture medium. Unlike enterocytes, NCI-H716 cells attached poorly to tissue culture plastic; therefore, after the washing step, more than 80% of NCI-H716 cells were detached and removed. The remaining NCI-H716 cells with enterocytes were then subjected to transduction or transfection as described below.

In the second co-culture system, serial seeding of Caco-2 enterocytes and NCI-H716 cells to a 35-mm tissue-culture dish was applied. First, a 5-mm cloning disc (Scienceware, Pequannock, NJ) was used to transfer Matrigel to the center of a 35-mm dish. This created a Matrigel-coated circular area for the adhesion of NCI-H716 cells

[124]; the ratio of the Matrigel-coated region to the surface area of a 35-mm dish is 1/49 (Figure 15B). The dish was then placed in a 37°C incubator for 1 h. One ml of the Caco-2 culture medium was added to the dish, and care was taken not to wet the disc region at the center. One hour later, the culture medium was replaced by a suspension of 2.5 million Caco-2 cells in 1 ml culture medium. Again, care was taken not to wet the disc region, so Caco-2 cells grew around the Matrigel disc, but not into the disc region. Twelve hours later, the disc was removed and a suspension of 2 million NCI-H716 cells in 1.5 ml medium was added to the dish and covered the entire surface. The dish was then placed in a 37°C incubator for settling and adhesion of NCI-H716 cells to the Matrigel. Ten minutes later, the unattached cells were re-suspended by tapping and swirling the dish. Following another 10 min of settling and adhesion, the unattached cells were removed by washing with culture medium twice. Approximately $0.5 - 1 \times 10^5$ NCI-H716 cells adhered. Pure culture controls were prepared in the same way, i.e., enterocytes around a disc and enteroendocrine cells by themselves in the disc area only. Transduction and transfection of the co-cultures (Figure 15C) and of the pure culture controls were performed one day later.

5.3.4 Transduction and transfection

Recombinant AAV2 transduction of the pure cultures of Caco-2 cells, T84 cells or HT-1080 cells was done in a 35-mm tissue culture dish at 60% to 80% of confluency using a multiplicity of infection (MOI) of 10 infectious units per cell. Transduction followed the procedure suggested by the Instruction Manual of AAV Helper-Free System (Stratagene) for HT-1080 transduction, except that genotoxic agents, hydroxyurea and

sodium byruvate were not added. Transduction of the pure culture of NCI-H716 cells in suspension used a MOI of 10 as previously described [8, or CHAPTER 4]. Transduction of the co-culture of enterocytes and NCI-H716 cells followed the same procedure as that for the pure cultures of enterocytes.

Plasmid transfection was carried out with FUGENE6 reagent (Roche) following the manufacture's directions for adherent cells (Caco-2, T84, HT-1080 cells and the co-cultures) or suspension cells (NCI-H716 cells). For each transfection of cells in a 35-mm dish, 5 µg of the plasmid DNA and 12 µl of FUGENE6 reagent were used.

5.3.5 Immunofluorescent staining

Caco-2 cells and HT-1080 cells were seeded on 2-well chamber slides and cultured overnight. NCI-H716 cells were seeded to poly-L-lysine-coated 2-well chamber slides right before the experiment. Recombinant AAV2 transduction of cells on the slides was performed at a 37°C incubator for 2.5 h. Cells were then washed with culture medium, fixed with 4% paraformaldehyde in PBS for 15 min, permeabilized with 0.5% Triton X-100 in PBS for 10 min, and blocked in 10% horse-serum PBS for 1 h. Mouse A20 monoclonal antibody against the intact particle of AAV2 (Maine Biotechnology Services, Portland, ME; diluted 1:5) was incubated with the monolayer at 4°C overnight, then revealed with TRITC-conjugated goat-anti-mouse IgG (Sigma; diluted 1:30). Visualization of the AAV2 immunofluorescent staining was performed using a Zeiss LSM510 laser-scanning confocal microscope (Carl Zeiss, Thornwood, NY). A helium/neon laser at 543-nm and a long-pass 560-nm filter were used for TRITC detection.

5.3.6 Secretion study

The co-culture prepared by serial seeding of Caco-2 cells and NCI-H716 cells, as well as the corresponding pure culture controls were subjected to rAAV2 transduction or FUGENE6-mediated transfection for insulin expression. Two days later, the culture medium was switched to basal medium [DMEM (GIBCO, cat.# 23800-022, Grand Island, NY) supplemented with 5 mM glucose and 1% fetal bovine serum], and cultures were incubated overnight. On the day of the experiment, parallel cultures were washed and incubated in basal medium for two hours to stabilize the basal secretion of insulin. The secretion test was initiated by incubating stabilized monolayers in basal medium for two hours to determine the basal secretion rate. Monolayers were then exposed to basal medium supplemented with 2% (w/v) meat hydrolysate (Sigma, cat. # P-7750) to stimulate insulin secretion for two hours. Cultures were finally washed three times with basal medium and incubated in basal medium for another two hours. When switching medium from one type to another, samples were collected for insulin and proinsulin assays.

5.3.7 Analytical techniques

EGFP expression was visualized using a Zeiss LSM510 laser-scanning confocal microscope (Carl Zeiss) with an argon laser at 488 nm and a band-pass 500-550-nm filter. Fluorescence-activated cell sorting (FACS) analysis was carried out using a Becton Dickinson LSR benchtop flow cytometer (BD Bioscience, Lexington, KY) with an argon laser at 488 nm and a FITC window to detect EGFP. Insulin and proinsulin

concentrations were measured by human insulin-specific radioimmunoassay (RIA) (LINCO Research, St. Charles, MI, cat. # HI-14K) and human proinsulin RIA (LINCO research, cat. # HPI-15K), respectively, according to the manufacturer's protocols. The human insulin-specific RIA cross-reacts with human proinsulin at less than 0.2%, whereas the human proinsulin RIA cross-reacts with human insulin at less than 0.1% (Manufacturer's specifications). Radioactivities were determined in Auto-Gamma Counting System, Cobra II (Packard, Meriden, CT).

5.4 Results

5.4.1 Recombinant AAV2 transduction in pure cultures

The ability to transduce NCI-H716, Caco-2 and T84 cells by AAV2 was first investigated in pure cultures using the viral vector derived from plasmid pInsulin-EGFP (Figure 15A). Human HT-1080 fibroblasts, known for their permissiveness to AAV2 transduction, were used as positive controls. The transduction efficiency, indicated by EGFP expression, was visualized by confocal microscopy and quantified by FACS. As seen in Figure 16, NCI-H716 and HT-1080 cells expressed EGFP strongly, indicating permissiveness to rAAV2 transduction. On the other hand, the Caco-2 and T84 cell cultures exhibited only sparse green fluorescence, indicating low transduction efficiencies. FACS analysis showed that enterocytes were transduced at less than 1% on average ($0.9 \pm 0.5\%$ for Caco-2 and $0.7 \pm 0.3\%$ for T84, $n = 3$), whereas NCI-H716 cells were transduced at approximately 37% ($\pm 5\%$, $n = 4$).

5.4.2 Chemical reagent (FUGENE6)-mediated transfection in pure cultures

In parallel with rAAV2-mediated transduction, FUGENE6-mediated transfection with plasmid pInsulin-EGFP (Figure 15A) was used as control. Results are shown in Figure 17. NCI-H716 L-cells exhibited little permissiveness to FUGENE6 transfection (Figure 17A), lower than to rAAV2 transduction (Figure 16A). On the other hand, Caco-2 and T84 enterocytes were both efficiently transfected and expressed EGFP (Figures 17B and 17C). This finding shows that the low EGFP expression in enterocytes after rAAV2 transduction (Figure 16B and 16C) was caused by the low permissiveness of these cells to rAAV2, and not by the low expression of the EGFP cassette. HT-1080 cells were transfected efficiently with FUGENE6 (Figure 17D), similar to rAAV2 (Figure 16D).

5.4.3 Immunofluorescent staining of transduced monolayers

The staining of AAV2 particles in transduced monolayers supported the hypothesis that AAV2 has different entry efficiencies in Caco-2 relative to permissive cells. Figures 18A and 18B show that, in permissive NCI-H716 L-cells and HT-1080 fibroblasts, entry, endocytosis and aggregation of AAV2 have taken place 2.5 h post-transduction. In comparison, in the transduced Caco-2 monolayer, the fluorescence signals of AAV2 capsids did not reveal similar aggregation (Figure 18C); in fact, the immunofluorescent staining of transduced Caco-2 cells was indistinguishable from that of untransduced controls (Figures 18D).

5.4.4 Recombinant AAV2 transduction and FUGENE6 transfection in co-cultures

Although NCI-H716 L-cells are permissive to rAAV2 transduction in the pure-culture condition, it is unclear whether L-cells can be effectively transduced when surrounded by a large number of low-permissive enterocytes. To test this, the two in vitro co-culture systems described in Materials and Methods were used. The first system, consisting of a monolayer of NCI-H716 cells and Caco-2 or T84 enterocytes randomly dispersed at a ratio of 1:50 to 1:100, was transduced by rAAV2 in experiments and was transfected by FUGENE6 in parallel controls. Figure 19 shows the two different patterns of EGFP expression in the two cultures. In the rAAV2-mediated transduction (Figures 19A and 19B), only a limited number of cells was transduced to exhibit green fluorescence; most of them were spherical, identifiable as NCI-H716 L-cells by comparison with the cells in Figure 16A. On the other hand, in the FUGENE6-transfected cultures, a greater number of cells expressed EGFP (Figures 19C and 19D), and these cell clusters appeared morphologically similar to enterocytes transfected under pure-culture conditions (Figures 17B and 17C). These findings indeed indicate that rAAV2-mediated gene transfer avoids the low-permissive enterocytes and selectively targets NCI-H716 L-cells in this co-culture system, whereas specific targeting of L-cells cannot be achieved with the non-viral, FUGENE6-mediated gene transfer.

The second co-culture system consisted of a 5-mm island of NCI-H716 L-cells on Matrigel in a 35-mm dish surrounded by enterocytes. In this system, the L-cells were strongly attached and easily withstood the washing and mixing steps in the insulin secretion studies. Figure 15C shows this co-culture system one day after the seeding was completed. Results from transduction by rAAV2 and transfection via FUGENE6, visualized using confocal microscopy, are shown in Figure 20. Similar to what was

found with the previous co-culture system (Figure 19), rAAV2 transduction was able to preferentially modify NCI-H716 L-cells at the central island, whereas FUGENE6-mediated transfection mainly modified Caco-2 enterocytes in the surrounding area with very low efficiency towards the L-cells (Figure 20).

5.4.5 Insulin secretion profiles of transduced and transfected co-cultures

Recombinant insulin secretion was studied from rAAV2-transduced co-cultures and pure cultures, as well as from FUGENE6-transfected co-cultures and pure cultures. Monolayers were exposed to basal medium for 2 hours, followed by medium with 2% meat hydrolysate (MH) for 2 hours to stimulate insulin secretion, followed by 2 hours of basal medium. MH has previously been shown to be a secretagogue to parental and recombinant NCI-H716 L-cells and induce acute release of endogenous glucagon-like peptide-1 (GLP-1) and recombinant insulin [8,122]. Figure 21 shows the insulin secretion rates and the conversion ratio of proinsulin to insulin, averaged over each 2-h period for both the co-cultures and the pure culture controls. Measurements of insulin and proinsulin were both performed on the same samples collected during the secretion study. MH exposure increased the insulin secretion rate to (1.73 ± 0.22) -fold and (1.99 ± 0.38) -fold of the basal rate for the rAAV2-transduced NCI-H716 L-cells and the co-culture, respectively. On the other hand, the insulin released from rAAV2-transduced Caco-2 cells was too low to be detected (Figure 21A). In comparison, FUGENE6-transfected Caco-2 cells and the co-culture failed to respond to MH stimulation, and the insulin released from the transfected NCI-H716 L-cells was too low to be quantified (Figure 21B).

Since the radioimmunoassays used to determine the insulin and proinsulin concentrations are highly specific (see Materials and Methods for cross-reactivities), it was possible to estimate the conversion of proinsulin to insulin by calculating the ratio $(\text{insulin}) \times 100\% / (\text{insulin} + \text{proinsulin})$. Previously, we have shown that NCI-H716 L-cells possess the necessary proteolytic enzymes to process proinsulin to immunoreactive insulin [8, or CHAPTER 4]. Here, we found that rAAV2-mediated insulin delivery can target NCI-H716 L-cells in the co-culture and achieve an average of $66 \pm 12\%$ conversion of proinsulin to insulin over the 6-h secretion study, similar to the conversion achieved in pure NCI-H716 cell cultures ($76 \pm 6\%$, Figure 21C). In comparison, FUGENE6-mediated transfection only generated an average of $25 \pm 3\%$ and $28 \pm 3\%$ of proinsulin conversion over the same period of study from the transfected Caco-2 pure culture and co-culture systems, respectively (Figure 21D). This low conversion ratio indicates that Caco-2 cells do not express any significant levels of endoproteases converting recombinant proinsulin to immunoreactive insulin.

5.5 Discussion

Cell-based therapies with enteroendocrine cells engineered to secrete insulin have been considered as a potential treatment of IDD [7-10]. With regard to enteroendocrine L-cells in particular, advantages include: 1) their endogenous incretin hormone, GLP-1, is compatible with insulin in regulating glucose homeostasis; 2) L-cells, like pancreatic β -cells, carry prohormone convertases 1/3 and 2 [135], which are necessary for the post-translational processing of proinsulin to insulin; and 3) the engineered L-cells respond to physiologic stimuli and secrete recombinant insulin with the same kinetics as endogenous

GLP-1 [8, or CHAPTER 4]. The obstacle of this approach, however, is the specific targeting of L-cells while performing the insulin gene delivery. In this work, we examined the selectivity and efficiency of rAAV2 transduction in human in-vitro models of enteroendocrine L-cells and enterocytes. Results demonstrated that rAAV2 can genetically modify NCI-H716 L-cells in the co-culture environment in spite of a prevailing number of Caco-2 or T84 enterocytes. The rAAV2-transduced co-culture achieved regulated insulin release in response to stimulation; on the other hand, the co-culture subjected to non-viral chemical-mediated transfection failed to generate such a response.

Recombinant AAV2 is capable of transgene delivery to a broad spectrum of host cells, but the transduction efficiency may vary widely [136-138]. The different efficiency towards L-cells and enterocytes identified in this study was not due to the expression cassette, as evidenced by the results from the chemical transfection. Rather, by comparing the virus immunostaining in transduced monolayers of enterocytes and permissive cells, the different efficiency appears to be due to an inefficient binding and/or endocytosis of rAAV2 in enterocytes. Clearly, to understand the differences in AAV2 transduction efficiency among various cell types, the population size and distribution of AAV2 surface receptors, as well as the AAV2 intracellular trafficking dynamics, need to be characterized.

It has been proposed that different cell types in the intestinal epithelium, including enterocytes and enteroendocrine cells, are derived from a multi-potential stem cell population found at or near the base of crypt of Lieberkuhn [139,140]. The enterocyte lineage undergoes terminal differentiation as the cells migrate from the crypt to the villus

tip, whereas the enteroendocrine lineage, including L-cells, remains localized around the crypt area. To selectively modify enterocytes or a sub-lineage of enteroendocrine cells, transgenic mice derived from mouse embryos transfected with a cell-specific promoter driving the expression of the transgene have frequently been applied [7,129,141]. However, for somatic gut epithelium, further studies are needed to elucidate the interaction between cell lineages and gene transfer agents in order to develop an appropriate vehicle for intestinal gene delivery.

In this research, we demonstrated that rAAV2 can efficiently transduce L-cells while avoiding a predominant number of enterocytes. However, there are many other cell types in the mucosa and in the surrounding tissues that we did not include in our simplified in vitro system. Indeed, administration of rAAV2 through oral gavage to the rat small intestine indicated that the viral particles were capable of crossing the epithelium barrier and infecting the lamina propria [15]. This result is consistent with a low rAAV2 permissiveness of enterocytes relative to other cells in the lamina propria, e.g. fibroblasts, also demonstrated in this study (Figure 16). Since fibroblasts lack a regulated secretory pathway and do not express the prohormone convertases for proinsulin to insulin processing, the potential penetration of insulin-encoded rAAV2 to the lamina propria would likely cause unregulated proinsulin production. To avoid this undesired event, additional strategies need to be implemented for targeting L-cells for insulin expression to the exclusion of connective tissue. This could be accomplished by enriching L-cells in isolated primary mucosal culture [142], transducing them by rAAV2 ex vivo, then implanting them back to the host. A challenge here would be to ensure that,

following implantation, L-cells remain responsive to postprandial signals and retain their endocrine phenotype, including secretion of GLP-1 and insulin in the blood.

A significant advantage offered by rAAV2-mediated insulin gene delivery is the expected prolonged transgene expression, as previously demonstrated with other genes and cell hosts [143]. This feature is especially important for treating a chronic disease like IDD. However, since the gut epithelium renews frequently, it is possible that transduced L-cells might gradually lose their transgene expression due to their limited life-span. A possible solution to this problem consists of using rAAV2 to target mucosal stem cells. Previous results with peroral rAAV2 administration to rats demonstrated phenotypic change of intestinal mucosa over a period of 6 months [15]. Although direct experimental confirmation is lacking, it appears that in the study of During et al. rAAV2 was able to infect mucosal stem or progenitor cells to achieve the prolonged transgene expression *in vivo*. To transduce mucosal stem cells and maintain insulin production exclusively in the L-cell lineage, insulin gene expression will need to be directed by an L-cell specific promoter, such as the proglucagon promoter [129], so as to prevent nonspecific and unregulated proinsulin/insulin secretion from other cell lineages in the epithelium.

This study constitutes an important step towards finding a suitable gene transfer vehicle for producing appropriately secreting intestinal epithelium for regulated insulin release. The rAAV2-mediated insulin gene transfer offers the advantage of selectivity and efficiency, as indicated by our results, and expected long-term duration. Clearly, these characteristics will need to be tested in primary cultured cells and *in vivo*. In this, it should be noted that results from animal models cannot always be applied to humans

because the species mismatch often alters the physiology of intestinal cells as well as the results of rAAV2 transduction.

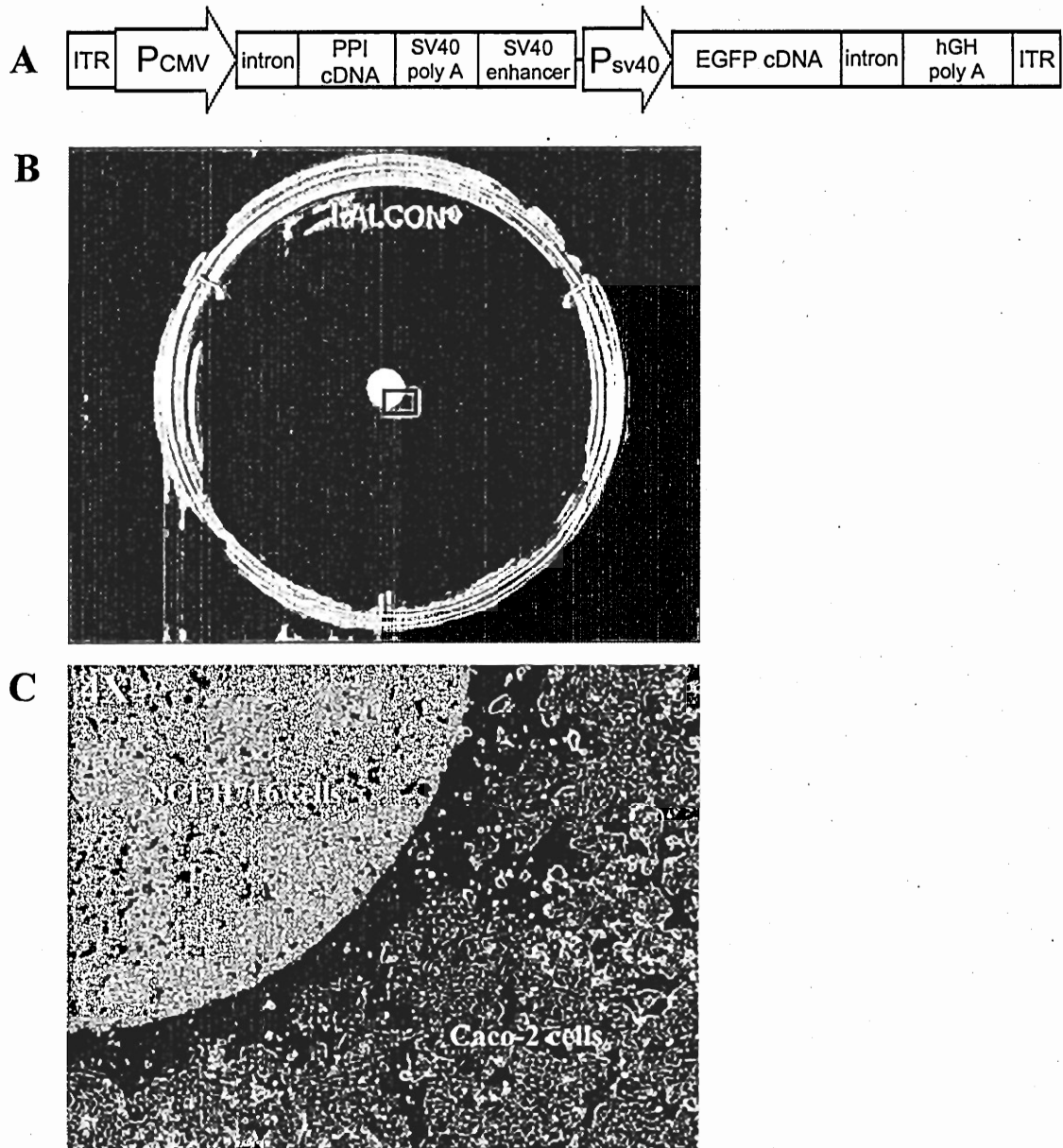


Figure 15. (A) The rAAV2 vector plasmid, pInsulin-EGFP, for expression of insulin and EGFP. ITR: inverted terminal repeat. PPI: preproinsulin. (B) Coating Matrigel at the center of a 35-mm tissue-culture dish via a cloning disc (white, round paper). Disc diameter: 5 mm. (C) Co-culture of NCI-H716 L-cells and Caco-2 enterocytes, one day after seeding. The position of the image is indicated by the box in (B). Magnification: $\times 4$.

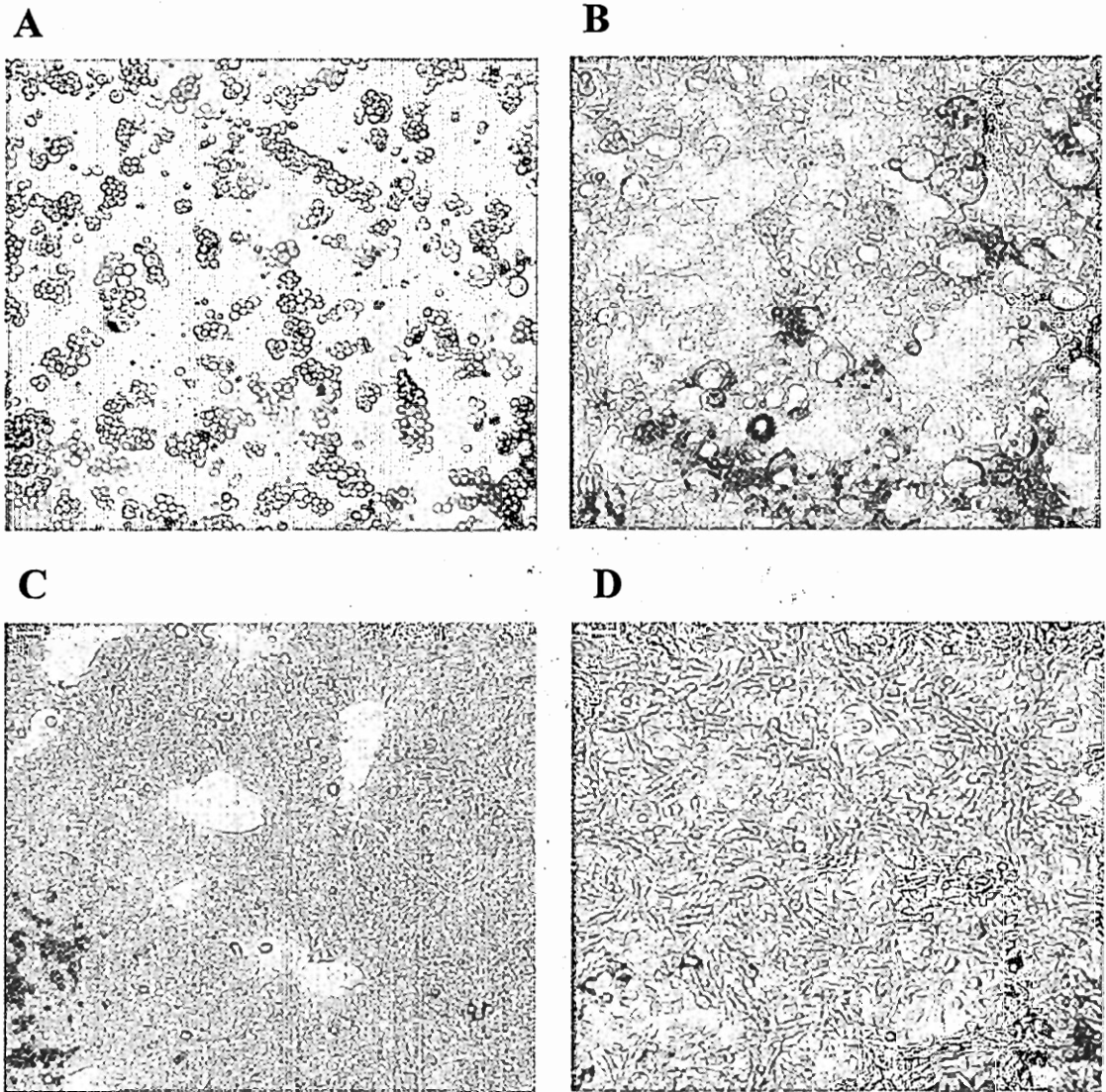


Figure 16. Recombinant AAV2 transduction in pure cultures of (A) NCI-H716 cells, (B) Caco-2 cells, (C) T84 cells and (D) HT-1080 cells. Reporter: EGFP. Bar = 50 μ m.

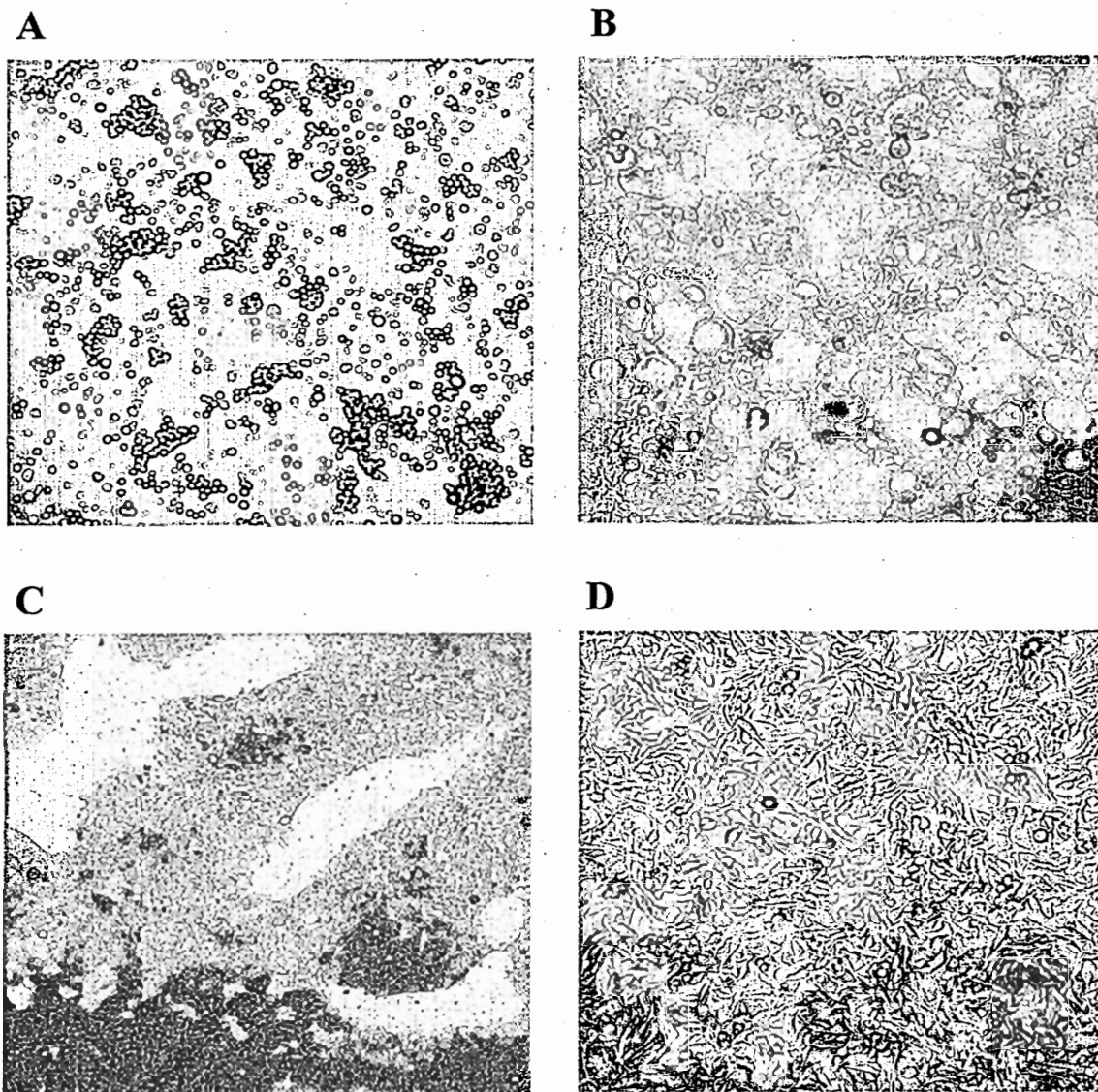


Figure 17. Chemical reagent (FUGENE6)-mediated transfection in pure cultures of (A) NCI-H716 cells, (B) Caco-2 cells, (C) T84 cells and (D) HT-1080 cells. Reporter: EGFP. Bar = 50 μ m.

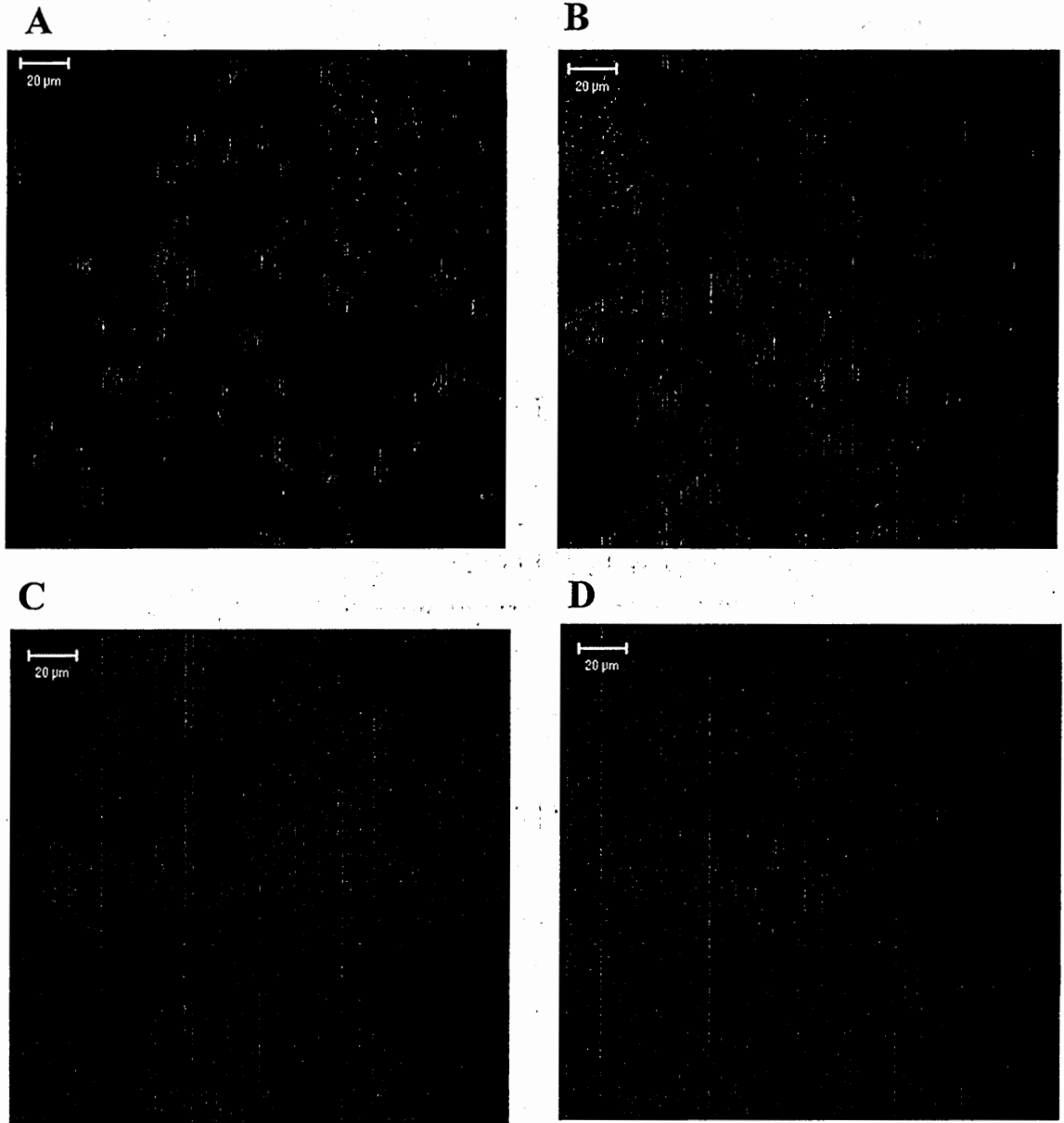


Figure 18. Immunofluorescent staining of AAV2 particles in (A) transduced NCI-H716 cells, (B) transduced HT-1080 cells, (C) transduced Caco-2 cells, and (D) parental Caco-2 cells without transduction. Bar = 20 μm .

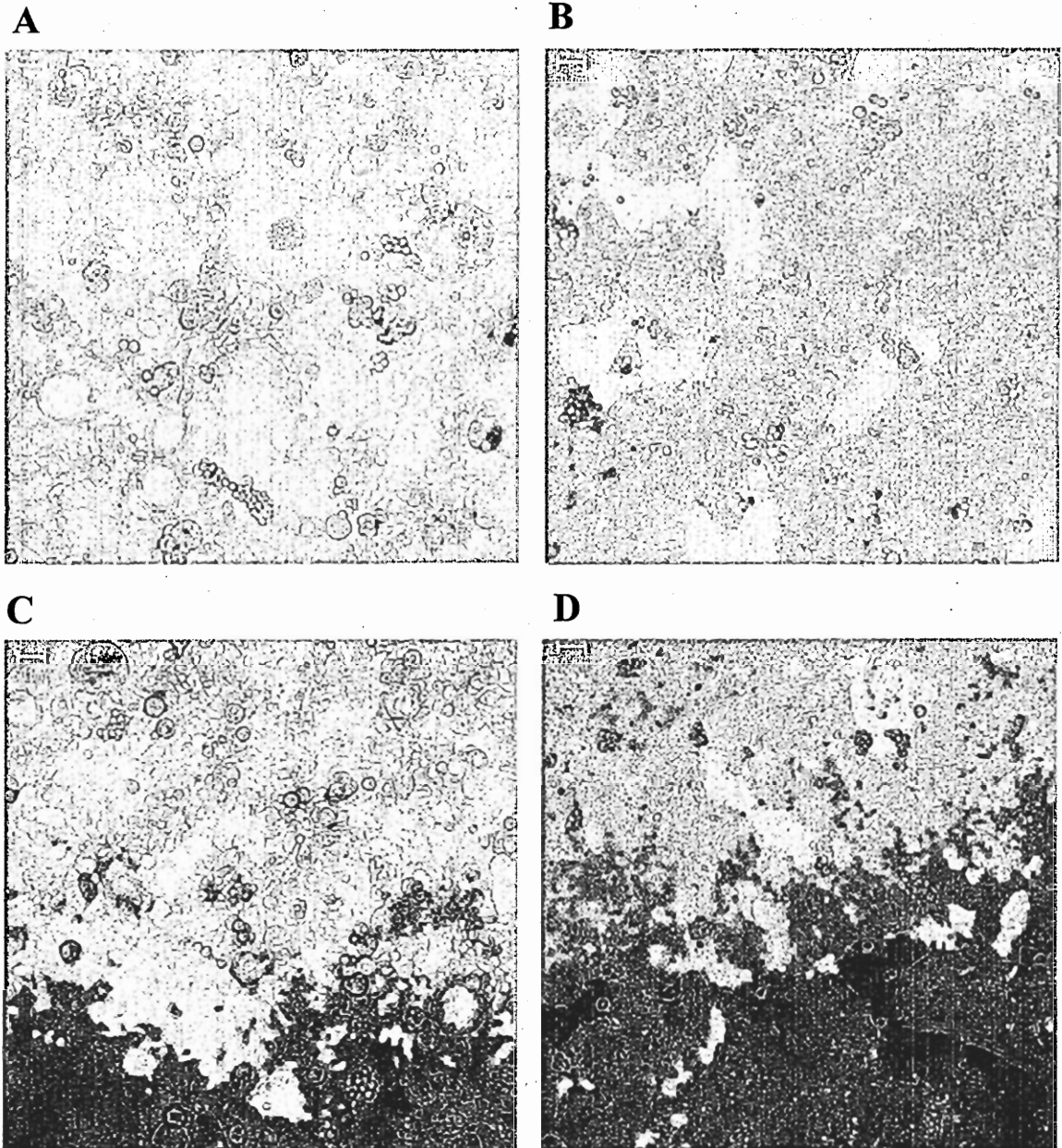


Figure 19. Recombinant AAV2 transduction and FUGENE6-mediated transfection of the co-culture with randomly mixed NCI-H716 L-cells and enterocytes. (A) rAAV2-transduced co-culture of NCI-H716 cells and Caco-2 cells. (B) rAAV2-transduced co-culture of NCI-H716 cells and T84 cells. (C) FUGENE6-transfected co-culture of NCI-H716 cells and Caco-2 cells. (D) FUGENE6-transfected co-culture of NCI-H716 cells and T84 cells. Reporter: EGFP. Bar = 50 μ m.

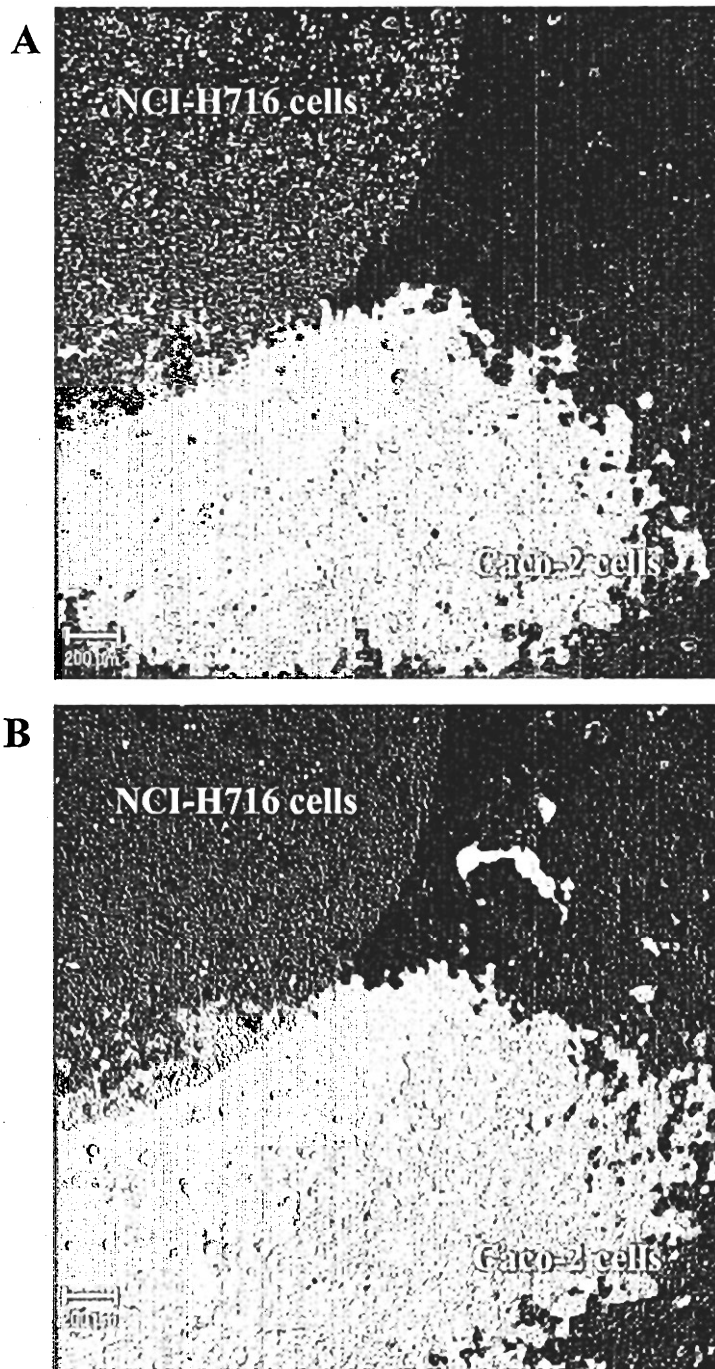


Figure 20. Recombinant AAV2 transduction and FUGENE6-mediated transfection of the co-culture with NCI-H716 L-cells and Caco-2 enterocytes in defined regions. (A) rAAV2-transduced co-culture. (B) FUGENE6-transfected co-culture. Reporter: EGFP. Bar = 200 µm.

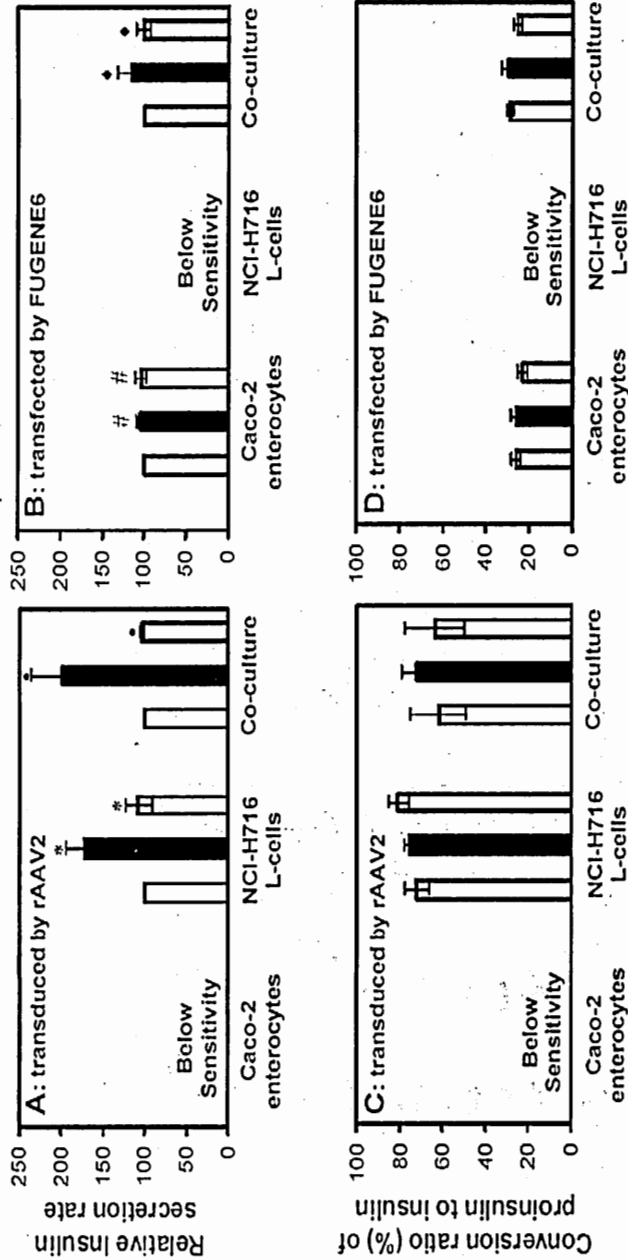


Figure 21. Insulin secretion from the engineered co-cultures against MH stimulation. Empty and solid bars are for monolayers treated with basal medium and 2% MH medium, respectively. Monolayers were treated with basal medium for 2 h (empty bar), exposed to MH for 2 h (solid bar), and then switched back to the basal medium for another 2 h (empty bar). (A) and (B) Insulin secretion rates averaged over 2-hour periods. In each independent test, secretion rates were normalized against the first 2-h sample, which was set at 100%. Below sensitivity means the insulin concentration in the sample was lower than the sensitivity of the assay (2 μ U/ml, i.e. 12 pM). (C) and (D) Conversion of proinsulin to insulin. During each 2-h period of the secretion study, proinsulin and insulin concentrations in the samples were determined by proinsulin and insulin radioimmunoassay, each with very low cross-reactivity against the other species (see Materials and Methods). The percent conversion of proinsulin to insulin was calculated as $(\text{insulin}) \times 100\% / (\text{insulin} + \text{proinsulin})$. Each experiment involved 4 independent tests. Error bars indicate standard deviations. *, \bullet , #, and \blacklozenge indicate pair-wise statistical comparisons using a one-tailed t-test, assuming unequal variances. * and \bullet : $P < 0.01$; # : $P = 0.40$; and \blacklozenge : $P = 0.10$. There were no statistical differences between the proinsulin-to-insulin conversion ratio during each induced period and the ratio during the corresponding prior and subsequent basal period.

CHAPTER 6

CONCLUSIONS AND FUTURE WORK

6.1 Conclusions

The development of recombinant β -cell surrogates for IDD treatment has been investigated over the last two decades. In this thesis, we have established intriguing technologies that add insight to this field. First, we have engineered preproinsulin mRNA so as to destabilize it through nonsense-mediated mRNA decay (NMD). The engineered preproinsulin mRNA carries three insulin gene copies with stop codons in the middle of the mRNA molecule to induce NMD. In human hepatomas, when expressed under transcriptional regulation, the engineered preproinsulin mRNA level and the insulin secretion rate declined faster upon switching off transcription, compared to the one-copy non-engineered control. This work provides a simple and straightforward method to destabilize mRNA, specifically the preproinsulin mRNA. This methodology is expected to be generic and applicable to different host cells, including primary cells.

In the second part of this thesis, we explored a suitable cell type for regulated insulin release. In this, an engineered human intestinal enteroendocrine L-cell model, the NCI-H716 cell line, was established to demonstrate that recombinant insulin and endogenous GLP-1 can simultaneously be released upon stimulation. Cells were genetically modified by recombinant adeno-associated virus serotype 2 (rAAV2)-based vector and then were differentiated to display endocrine features, in particular the formation of granule-like compartments. A fusion protein of insulin and enhanced green

fluorescent protein was designed to reveal the granule-like compartments and the co-localization of fusion protein with endogenous GLP-1. This study suggests that engineering of intestinal enteroendocrine L-cells for regulated insulin release constitutes a feasible approach for IDD treatment, in terms of the dynamic release of recombinant insulin as well as the compatibility of GLP-1 and insulin in regulating glycemic homeostasis.

In the final portion, we investigated rAAV2 transduction in distinguishing different intestinal cell types, specifically the enterocyte and enteroendocrine L cell lines. The selectivity and efficiency of rAAV2-mediated gene transfer toward enteroendocrine L-cells, as opposed to enterocytes, were demonstrated by co-culture models of enteroendocrine L-cells with a prevailing number of enterocytes. Upon stimulation, the rAAV2 transduced co-culture achieved regulated insulin release; whereas, the chemically transfected controls failed to respond. This study suggests that the AAV2 vector is a valuable tool in developing enteroendocrine L-cell specific insulin gene delivery in terms of AAV2 avoiding enterocytes and targeting selectively L-cells.

6.2 Future work

6.2.1 Regulation of insulin secretion via insulin biosynthesis

Although we were able to shorten the half-life of preproinsulin (PPI) mRNA from more than 8 h to roughly 3 h (Figure 6A), the dynamics of transcriptionally controlled insulin secretion are still too sluggish to be satisfactory for regulating glucose homeostasis because of the serious lag time before and after stimulation (Figure 7). The under production of insulin in the beginning of stimulation will create a window of

hyperglycemia; whereas, when stimulation is removed, the long-lasting insulin production due to the prolonged activity of PPI mRNA would cause dangerous hypoglycemia episodes. It is possible to further engineer PPI mRNA to become a labile molecule with its half-life within minutes instead of hours. However, since insulin production rates are correlated to PPI mRNA levels, the decrease of PPI mRNA stability would reduce the levels of insulin production. In this, how to achieve fast secretion dynamics and maintain enough insulin production from transcriptionally regulated cells represent a big challenge.

Alternatively, using translation to modulate insulin biosynthesis so as to regulate insulin secretion is a possible direction. In this, it is expected that without the involvement of up- and down-regulation of the long half-life PPI mRNA, the secretion dynamics of translational control would be faster compared to those of transcriptional control. It is known that, at the translational level, glucose stimulates proinsulin synthesis up to 10 fold within one hour in β -cells [87]. Although this induction is β -cell specific, recently a more generic translational enhancer, the 5'-UTR (un-translated region) of human mRNA encoding Hsp70, has been identified [144]. Since the liver naturally responds to glucose stimulation, it would be of interest to identify glucose-regulated translational elements in hepatic cells and test any translational enhancer for elevation of preproinsulin mRNA translation.

6.2.2 Regulated insulin release from intestinal enteroendocrine cells

Intestinal enteroendocrine L- and K-cells are suitable targets for insulin gene transfer because incretin secretion from L- and K-cells is compatible with insulin in

postprandial secretion dynamics as well as in physiologic effects. Currently, no other cell types have been identified with similar characteristics. However, because gut epithelium renews frequently, L- and K-cells have limited life-span in mucosa. In order to maintain a constant insulin release, it is necessary to target mucosal stem cells for insulin gene transfer. In this, an additional strategy is needed to use L- and K-cell specific promoter to confine insulin gene expression in these two cellular lineages to prevent nonspecific and unregulated insulin or proinsulin secretion from other cell types in the gut epithelium.

The L-cell specific proglucagon promoter and the K-cell specific GIP promoter have both been identified and applied to derive transgenic mice for tissue-specific transgene expression [7,130]. However, to genetically modify somatic gut mucosal stem cells, the challenges are: 1) a direct administration of gene transfer vectors in vivo would be interfered by differentiated cells in the gut epithelium as well as by other cell types in the surrounding tissues, which would cause low levels of gene transfer efficiency to mucosal stem cells [15]; 2) currently, there are no differentiation markers for gut mucosal stem cells, therefore no tools are available for identification and isolation of mucosal stem cells for ex-vivo somatic cell gene delivery [145]; and 3) if mucosal stem cells can be isolated and genetically modified ex vivo, the engraftment of engineered cells to the recipient's mucosal wall would still be challenging. Alternatively, a proof-of-concept experiment can be conducted by isolating and transducing intestinal crypts (rich in stem cells) ex vivo, and then implanting them subcutaneously in an immunocompromised mouse for stem cell differentiation [145] as well as transgene expression to evaluate the gene transfer efficiency and cell-specific activity of proglucagon or GIP promoter. Clearly, more research needs to be done in specifying surface markers for stem cell

identification and isolation, or for the design of ligand-targeting vectors for intestinal gene transfer [146].

APPENDICES

A.1 Amino acid sequences of native and mutated preproinsulin

The amino acid sequence corresponding to the **native** human preproinsulin is:

Met-ala-leu-trp-met-arg-leu-leu-pro-leu-leu-ala-leu-leu-ala-leu-trp-gly-pro-asp-pro-ala-ala-ala-phe-val-asn-gln-his-leu-cys-gly-ser-his-leu-val-glu-ala-leu-tyr-leu-val-cys-gly-glu-arg-gly-phe-phe-tyr-thr-pro-lys-thr-arg-arg-glu-ala-glu-asp-leu-gln-val-gly-gln-val-glu-leu-gly-gly-gly-pro-gly-ala-gly-ser-leu-gln-pro-leu-ala-leu-glu-gly-ser-leu-gln-lys-arg-gly-ile-val-glu-gln-cys-cys-thr-ser-ile-cys-ser-leu-tyr-gln-leu-glu-asn-tyr-cys-asn(*).

The asterisk (*) indicates the presence of a termination codon.

The amino acid sequence corresponding to the **B10 mutated** human preproinsulin is:

Met-ala-leu-trp-met-arg-leu-leu-pro-leu-leu-ala-leu-leu-ala-leu-trp-gly-pro-asp-pro-ala-ala-ala-phe-val-asn-gln-his-leu-cys-gly-ser-asp-leu-val-glu-ala-leu-tyr-leu-val-cys-gly-glu-arg-gly-phe-phe-tyr-thr-pro-lys-thr-arg-arg-glu-ala-glu-asp-leu-gln-val-gly-gln-val-glu-leu-gly-gly-gly-pro-gly-ala-gly-ser-leu-gln-pro-leu-ala-leu-glu-gly-ser-leu-gln-lys-arg-gly-ile-val-glu-gln-cys-cys-thr-ser-ile-cys-ser-leu-tyr-gln-leu-glu-asn-tyr-cys-asn(*):

The asterisk (*) indicates the presence of a termination codon. The underlined amino acid residue indicates the position of mutation in comparison to the wild-type human preproinsulin.

The amino acid sequence corresponding to the **furin-compatible, B-C and C-A junction mutated** human preproinsulin is:

Met-ala-leu-trp-met-arg-leu-leu-pro-leu-leu-ala-leu-leu-ala-leu-trp-gly-pro-asp-pro-ala-ala-ala-phe-val-asn-gln-his-leu-cys-gly-ser-his-leu-val-glu-ala-leu-tyr-leu-val-cys-gly-glu-arg-gly-phe-phe-tyr-thr-pro-arg-thr-lys-arg-glu-ala-glu-asp-leu-gln-val-gly-gln-val-glu-leu-gly-gly-gly-pro-gly-ala-gly-ser-leu-gln-pro-leu-ala-leu-glu-gly-ser-arg-gln-lys-arg-gly-ile-val-glu-gln-cys-cys-thr-ser-ile-cys-ser-leu-tyr-gln-leu-glu-asn-tyr-cys-asn-(*).

The asterisk (*) indicates the presence of a termination codon. The sequence of furin tetrabasic cleavage site is: **arg-X-lys (or arg)-arg**. The first tetrabasic sequence, arg-thr-lys-arg, is at the B-C junction of preproinsulin. The second tetrabasic sequence, arg-gln-lys-arg, is at the C-A junction of preproinsulin. The underlined amino acid residues indicate the positions of mutation in comparison to wild-type human preproinsulin.

A.2 DNA sequences of native and mutated preproinsulin cDNA

The nucleotide sequence of the native human preproinsulin cDNA is:

5'ATGGCCCTGTGGATGCGCCTCCTGCCCTGCTGGCGCTGCTGGCCCTCTGGG
GACCTGACCCAGCCGCAGCCTTTGTGAACCAACACCTGTGCGGCTCACACCT
GGTGAAGCTCTCTACCTAGTGTGCGGGGAACGAGGCTTCTTCTACACACCC
AAGACCCGCCGGGAGGCAGAGGACCTGCAGGTGGGGCAGGTGGAGCTGGGC
GGGGGCCCTGGTGCAGGCAGCCTGCAGCCCTTGGCCCTGGAGGGGTCCCTGC
AGAAGCGTGGCATTGTGGAACAATGCTGTACCAGCATCTGCTCCCTCTACCA
GCTGGAGAACTACTGCAACTAG3'.

The nucleotide sequence of the His B10-to-Asp mutated human preproinsulin cDNA is:

5'ATGGCCCTGTGGATGCGCCTCCTGCCCTGCTGGCGCTGCTGGCCCTCTGGG
GACCTGACCCAGCCGCAGCCTTTGTGAACCAACACCTGTGCGGATCCGACCT
GGTGGAAAGCTCTCTACCTAGTGTGCGGGGAACGAGGCTTCTTCTACACACCC
AAGACCCGCCGGGAGGCAGAGGACCTGCAGGTGGGGCAGGTGGAGCTGGGC
GGGGGCCCTGGTGCAGGCAGCCTGCAGCCCTTGGCCCTGGAGGGGTCCCTGC
AGAAGCGTGGCATTGTGGAACAATGCTGTACCAGCATCTGCTCCCTCTACCA
GCTGGAGAACTACTGCAACTAG3'. The underlined codon indicates the position of
mutation in comparison to the wild-type human preproinsulin.

The nucleotide sequence of the furin-compatible, B-C and C-A junction mutated human preproinsulin cDNA is:

5'ATGGCCCTGTGGATGCGCCTCCTGCCCTGCTGGCGCTGCTGGCCCTCTGGG
GACCTGACCCAGCCGCAGCCTTTGTGAACCAACACCTGTGCGGCTCACACCT
GGTGGAAAGCTCTCTACCTAGTGTGCGGGGAACGAGGCTTCTTCTACACACCC
AGGACCAAGCGGGAGGCAGAGGACCTGCAGGTGGGGCAGGTGGAGCTGGGC
GGGGGCCCTGGTGCAGGCAGCCTGCAGCCCTTGGCCCTGGAGGGATCCCGGC
AGAAGCGTGGCATTGTGGAACAATGCTGTACCAGCATCTGCTCCCTCTACCA
GCTGGAGAACTACTGCAACTAG3'. The underlined codons indicate the positions of
mutation in comparison to the wild-type human preproinsulin.

A3. Application of the glucose-responsive Spot 14 (S14) promoter

The S14 promoter has been identified as the carbohydrate-responsive promoter that controls the lipogenic Spot 14 protein in hepatic cells. This promoter's regulatory mechanisms have been characterized and the segment that encodes this promoter has

been cloned [147]. This promoter reacts to glucose in an up-regulated manner with a linear transcription activity in response to extracellular glucose concentrations in primary rat hepatocytes and in HIT hamster insulinomas [148]. In this research, we intended to use the S14 promoter to drive insulin gene expression, thus connecting insulin biosynthesis with glucose-responsiveness in recombinant hepatic cells.

Figure 22A shows the structure of the plasmid, pS14-Renilla, carrying the rat S14 promoter to drive the expression of the reporter, Renilla luciferase (Promega). Figure 22B shows the control plasmid, pSV40-firefly, with a constitutive SV40 promoter to drive the expression of the second reporter, firefly luciferase (Promega). Plasmid pSV40-firefly was co-transfected with the test plasmid, pS14-Renilla, thus monitoring non-specific effects of glucose concentrations on the activity of S14 promoter. Assays of Renilla luciferase and firefly luciferase were performed on the same samples by the dual-luciferase assay system (Promega). Although the glucose responsiveness of S14 promoter has been reported [148-150], our result in Figure 23 cannot reproduce the same activity. In this, HIT cells were co-transfected with the test (S14-driven) and control (SV40-driven) plasmids when cultured in the DMEM or in the RPMI medium then switched to different glucose concentrations. In both types of medium (Figures 23A and 23B), the increased activity of Renilla luciferase in high glucose concentration indicates the elevation of S14-directed transcription. However, during the same period, the activity of the constitutively SV40-driven firefly luciferase also increased, therefore one cannot attribute the elevation of S14-directed transcription to its glucose responsiveness. Instead, the non-specific effect of glucose was too large to differentiate any specific glucose influence on the S14 promoter.

A.4 Dynamics of proinsulin secretion from transcriptionally controlled recombinant myoblasts

In this study, we test the idea of regulating proinsulin secretion via transcription in recombinant myoblasts. It has been known that transcriptionally controlled cells exhibit long time lags between induction and initiation of secretion, as well as between removal of the inducer and termination of the secretory response. Such lags are thought to be inappropriate for achieving glucose homeostasis in higher animals and, eventually, humans. To characterize these time lags and to develop methodologies for their reduction to within acceptable levels, we developed a steroid-inducible gene expression system, using MMTV (mouse mammary tumor virus) promoter to control proinsulin secretion via transcription (Figure 24A). We stably transfected mouse C2C12 myoblasts (ATCC), as a model cell type, with this system. Upon exposure of cells to the agonist dexamethasone, it took approximately 4 hours (lag time + response time) for the proinsulin secretion rate to plateau (Figure 25A); upon removal of the agonist, the proinsulin secretion rate decreased to 25% of the maximum after 8 hours (Figure 25B).

Our hypothesis is that the long time lag in the down-regulation of secretion is due to the mRNA pool in the cells, which continues to be translated after transcription has stopped. Thus, to expedite the down-regulation dynamics, we double-transfected the recombinant C2C12 cells with a plasmid encoding for the constitutive expression of antisense preproinsulin mRNA (Figure 24B). It was found that the antisense expression improved the dynamics of down-regulation and exhibited a fast decline of insulin toward the basal level after removing the agonist (Figure 26A); whereas, antisense interaction

also compromised the specific proinsulin secretion rate (Figure 26B). This finding indicates that an inducible antisense expression system might be helpful in reducing the antisense interference during induction, while generating the sense-antisense interaction when it is needed, i.e., during the down-regulation phase. However, the inducible antisense expression might delay the overall dynamics due to the expected lag time prior to and subsequent to induction of antisense expression. In addition, technically, it would be difficult to implement and maintain two inducible systems at the same time in primary cells for future application.

A.5 From sense-antisense mRNA interaction to nonsense-mediated mRNA decay

Figure 27 is an early experiment that we performed to address a hypothesis, but it led to the discovery of the improvement of secretion dynamics by nonsense-mediated mRNA destabilization. In this experiment, we used the Tet-On expression system, so transcription was down-regulated by removing doxycycline (DOX) from the medium. The figures show the decline of insulin secretion rate, as % of maximum, after removing DOX at time 0.

Our original hypothesis was: Antisense expression can accelerate insulin down-regulation; as the copy number of sense mRNA transcript increases (from one copy to three copies), the sense-antisense interaction becomes stronger and insulin down-regulation becomes faster. As shown in Figure 27A, we found that indeed the down-regulation of insulin secretion was accelerated when the copy number of insulin increased. Constitutively expressed antisense mRNA from CMV promoter had little effect on the one-copy insulin expression system. With the 2- and 3-copy insulin expression systems,

the decline in the presence of antisense was much faster. However, to our surprise, by comparing Figure 27A and 27B, the cause of the faster down-regulation did not come from the expression of antisense, but from the addition of more sense copies of insulin gene to the expression cassette. This led us to realize that the 2- and 3-copy systems have, intrinsically, a faster decline than the one-copy control.

Later, we realized that adding a gene sequence between the translation stop codon and the polyadenylation signal can induce nonsense-mediated preproinsulin mRNA decay. Compared with sense-antisense-mediated mRNA decay, this new method achieves insulin expression and the preproinsulin mRNA destabilization in one transgene expression cassette. Sense-antisense interaction, on the other hand, needs two expression cassettes for each of the sense and antisense expression, which adds complexity in finding suitable expression levels of sense and antisense for maintaining a fast insulin down-regulation while not silencing the insulin gene expression.

In Figure 28A, we used Tet-Off system to evaluate the down-regulation of insulin secretion with different copies (1 to 4) of insulin gene. Transcription was down-regulated by adding DOX at time 0. Compared with the one-copy control, systems with multiple copies of insulin gene all show faster decline of insulin secretion. Figure 28B demonstrates the dynamics of insulin secretion when cells expressing recombinant insulin using the Tet-On system were exposed to a square wave of DOX induction, which mimics glucose stimulating the transcription of insulin gene after a meal. This test indicates that the responses from the 3- and 4-copy systems are similar, and they are faster compared to that of the 2-copy system although not by a lot. Also, the 4-copy system is somewhat more difficult to work with due to its size. This result led us to

decide that, for proof-of-concept, it is sufficient to compare insulin expression between one-copy (control) and three-copy (test) systems. However, Figure 28 shows that multiple-copy systems are all very effective at accelerating the down-regulation of insulin secretion compared with the one-copy control.

In Figure 29, we evaluate the regulation of insulin secretion from transfected HepG2 hepatomas using the tet-on system. This is similar to Figure 7 in CHAPTER 3 except that the cycle is 2 hours of induction followed by 6 hours of basal conditions. The result indicates that insulin secretion from the multiple-copy systems can follow the square waves of DOX induction. On the other hand, the one-copy control failed to do so.

In Figure 30, we connected insulin and luciferase cDNA in another attempt to generate nonsense-mediated mRNA decay. Compared with using multiple copies of insulin gene, this approach has the similarity of creating a distance between the translation stop codon and the polyadenylation signal. The size of luciferase gene is 1650 bp, compared to 360 bp of insulin gene. Upon switching off transcription, Figure 30 shows the down-regulation of preproinsulin-luciferase mRNA (Figure 30A) and the corresponding decline of insulin secretion rate (Figure 30B) are similar to those from the three-copy system as shown previously in Figure 6 (square, CHAPTER 3). This result indicates that the fast down-regulation of insulin secretion is not limited to the systems with multiple-copy of insulin gene. When insulin gene is connected to a different DNA sequence, such as the luciferase gene, a faster down-regulation of insulin expression occurred, compared to the one-copy insulin control (circle, Figure 30).

In summary, these results show that: 1) stopping sense preproinsulin mRNA translation by antisense mRNA is not as effective in down-regulating insulin secretion as

it is to introduce mRNA destabilization by nonsense-mediated decay; 2) using 1, 2, or 3 copies of the insulin gene downstream of the stop codon destabilizes the mRNA and accelerates its degradation following cessation of transcription; however, slightly better results were obtained using the construct with two insulin gene copies after the stop codon (3 copies of insulin gene total); and 3) both the luciferase and insulin genes after the stop codon destabilized the mRNA.

A.6 Real-time PCR technique for preproinsulin mRNA quantification

Reference:

- RNeasy Mini Handbook (QIAGEN)
- Instruction of Reverse Transcription System (Promega A3500)
- User Bulletin Number 2: Relative quantification of gene expression (Applied Biosystems)
- Primer Express software (Applied Biosystems)
- Protocol of TaqMan Universal PAC Master Mix (Applied Biosystems)

Materials:

For RNA isolation and purification: RNeasy Mini Kit (QIAGEN)

- Cells grown in monolayers
- QIAshredder column (QIAGEN cat# 79656) for cell homogenization
- 70% Ethanol
- RNase-free water
- Buffer RLT with 10 μ l/ml β -Mercaptoethanol
- Buffer RW1
- Buffer RPE with ethanol

For reverse transcription to prepare complementary DNA (cDNA) from mRNA: Reverse Transcription System (Promega A3500)

- AMV reverse transcriptase
- Recombinant RNasin Ribonuclease Inhibitor
- Oligo(dT)₁₅ primer
- dNTP mixture
- Reaction 10X Buffer
- MgCl₂, 25mM
- RNase-free water

For TaqMan real-time PCR: developed Applied Biosystems

- ABI PRISM 7700 Sequence Detection System (Applied Biosystems)
- Primer sets for preproinsulin mRNA and internal standard tTA, tetracycline-responsive transcriptional activator (designed by Primer Express software and customized by Applied Biosystems, Table 2)
- Probes labeled with 6FAMTM for preproinsulin cDNA and VICTM for tTA cDNA (designed by Primer Express software and customized by Applied Biosystems, Table 2)
- RNase-free environment: including bench-top, pipettors, tips, microcentrifuge tubes, water.
- TaqMan Universal PCR Master Mix (Applied Biosystems cat# 4304437)
- MicroAmp Optical tubes for PCR reaction (Applied Biosystems cat# N801-0933)
- MicroAmp Optical caps for PCR reaction (Applied Biosystems cat# N801-0935)
- MicroAmp Optical tray retainer (Applied Biosystems cat# N801-0530)
- Support base (Applied Biosystems cat# 4312063)

Procedure #1: Total RNA isolation and purification

1. Lyse cells directly in the culture vessel of a 6-well plate by adding 600 µl of Buffer RLT supplemented with β-Mercaptoethanol.
2. Transfer lysate directly onto a QIAshredder column sitting in a 2-ml collection tube and centrifuge at full speed for 2 min.
3. Add 600 µl of 70% ethanol to the homogenized lysate and mix well by pipetting.
4. Transfer the mixture to an RNeasy column and centrifuge for 15 sec at > 8000 g.

5. Pipet 700 μ l of Buffer RW1 onto the column and centrifuge for 15 sec at > 8000 g.
6. Transfer column into a new collecting tube. Transfer 500 μ l of Buffer RPE onto the column and centrifuge for 15 sec at > 8000 g.
7. Repeat step 6 but centrifuge for 2 min at > 8000 g.
8. Transfer column into a new 1.5-ml collecting tube, and pipet 50 μ l of RNase-free water onto the RNeasy membrane. Centrifuge for 1 min at > 8000 g.
9. Dilute 5 μ l of isolated RNA with 245 μ l of water for quantification.
10. RNA quantification using spectrophotometer at 260 nm: $40 \mu\text{g/ml}$ (conversion factor) $\times A_{260} \times 50$ (dilution factor).
11. Store RNA at -70°C .

Procedure #2: Reverse transcription

1. Prepare a 20- μ l reaction of reverse transcription by adding the following reagents in the order listed.

- MgCl_2 , 25mM	4 μ l
- Reverse Transcription 10X Buffer	2 μ l
- dNTP Mixture, 10mM	2 μ l
- Recombinant RNasin® Ribonuclease Inhibitor	0.5 μ l
- AMV Reverse Transcriptase (High Conc.)	15 u
- Oligo(dT) ₁₅ Primer	0.5 μ g
- RNA sample	1 μ g
- Nuclease-Free Water to a final volume of	20 μ l
2. Incubate the reaction at 42°C for 15 min for first strand cDNA synthesis.
3. Heat-inactivate AMV Reverse Transcriptase at 95°C for 5 min, then incubate at 4°C for 5 min.
4. Store cDNA at -20°C or directly perform real-time PCR.

Procedure #3: TaqMan real-time PCR

1. Use Primer Express software to design of the forward and reverse primer sets and the probes for preproinsulin and tTA cDNAs (Table 2).
2. Order primer sets and probes through Applied Biosystems.

3. Prepare 10X primer and probe solutions.
4. Prepare a 50- μ l reaction of real-time PCR by adding the following reagents.

- TaqMan Universal PCR Master Mix (2X)	25 μ l
- Forward primer	5 μ l (900 nM)
- Reverse primer	5 μ l (900 nM)
- TaqMan probe	5 μ l (250 nM)
- cDNA sample	5 μ l (10-100 ng)
- Water	5 μ l
- Total	50 μ l
5. Preproinsulin cDNA and of the internal reference tTA cDNA were measured, respectively, in separate tubes.
6. Thermal cycle routine: 50°C for 2 min, 95°C for 10 min, and then 40 cycles of 95°C for 15 sec, followed by 60°C for 1 min.
7. A comparative threshold cycle (C_T) method (User Bulletin Number 2; Applied Biosystems) was used to determine relative gene expression. Relative quantification of preproinsulin mRNA was done by normalizing the preproinsulin with the tTA signal and by assigning a value of 100 to the normalized sample level, e.g. at time 0 of a dynamic test of insulin down-regulation.

A.7 Production and purification of recombinant adeno-associated virus serotype 2

Reference:

- Production: AAV Helper-Free System (Stratagene)
- Purification: Auricchio et al. [125]

Materials:

- Low passage HEK293 packaging cell line (ATCC)
- 100-mm tissue culture dishes
- Culture medium for HEK293 cells: DMEM supplemented with 10% FBS and 1% P/S
- FUGENE6 compatible culture medium: HEK293 culture medium without P/S

- FUGENE6 transfection reagent (Roche)
- AAV2 vector plasmid with transgene expression cassette flanked AAV2 inverted terminal repeats (Stratagene)
- Plasmid pAAV-RC (Stratagene)
- Plasmid pHelper (Stratagene)
- Sterile phosphate buffer saline (PBS, Sigma)
- PBS with 0.1 M NaCl
- PBS with 0.4 M NaCl
- DNase I (Roche)
- RNase A (Roche)
- Heparin-agarose suspension (Sigma)
- Deoxycholic acid (Sigma)
- 5- μ m pore size filter (Millipore)
- 0.8- μ m pore size filter (Millipore)
- Glass column (Sigma C4669)
- Luer Luck (Sigma S7396)
- Filtration membrane (Sigma 7271)
- Glycerol (Sigma)

Procedure:

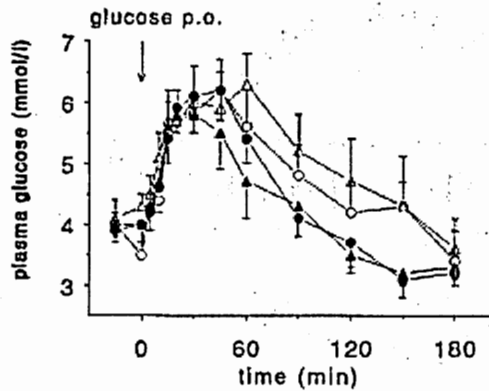
1. For one rAAV2 vector preparation, sixty 100-mm dishes of HEK293 cells split 1:2 were prepared 24 h before transfection.
2. When 70-80% confluency was reached, cells were co-transfected with AAV2 vector plasmid with transgene expression cassette (e.g. pAAV/B10/EGFP), plasmid pAAV-RC and plasmid pHelper using FUGENE6 (Roche) transfection technique.
3. For transfection of each 100-mm dish, 7.5 μ g of overall plasmid amount (2.5 μ g of each plasmid mentioned above) and 15- μ l FUGENE6 reagent were applied.
4. The following procedure is to prepare the transfection mixture for six 100-mm dishes.
 - Prepare a 50-ml conical tube with 3 ml of DMEM.
 - Add 90 μ l of FUGENE 6 reagent to each tube.

- Mix 15 μg of each plasmid (45 μg total) in a 1.5-ml centrifuge tube, then transfer to the DMEM-FUGENE6 cocktail in the 50-mm tube.
 - Incubate for 30 min.
 - Add 30 ml of P/S-free FUGENE6 compatible culture medium to the 50-ml tube, mix, then transfer 5 ml of the mixture to one 100-mm dish.
 - Twelve hours later, add another 5 ml of FUGENE6 compatible culture medium to each dish.
 - Twelve hours later add 10 ml of HEK293 culture medium to each dish.
 - Twenty-four hours later, add 10 ml of culture medium to each dish.
 - Twenty-four hours later, harvest transfected cells.
5. Remove all the culture medium and add 1 ml of DMEM to each dish. Collect HEK293 cells using a cell lifter.
 6. Transfer collected cell suspension to two 50-ml conical tubes (around 30 ml) for 2 rounds of freeze-and-thaw by switching between -70°C ethanol bath and room temperature water bath.
 7. Transfer 2.5 mg of DNase I and RNase A to each tube, then incubate at 37°C for 30 min.
 8. Centrifuge at 3000 rpm (1000 g) at 4 C for 15 min.
 9. The supernatant was transferred to a new tube then add 0.15 g of deoxycholic acid to each tube ($0.15 \text{ g} / 30 \text{ ml} = 0.5\%$) then incubate at 37°C for 30 min.
 10. Filter the solution through 5- μm filters.
 11. Filter the solution through 0.8- μm filters.
 12. Equip a glass column with a Luer lock.
 13. Apply 8 ml of a heparin-agarose suspension to the column and allow the suspension solution to flow through.
 14. Place a filtration membrane on the top of the heparin-agarose bedding.
 15. Equilibrate the heparin-agarose matrix by allowing 25 ml of PBS to flow through the column.
 16. Close the Luer lock, and then apply filtered crude lysate to the column.
 17. Open the Luer lock and control the flow speed around 1 drop/sec.
 18. Wash column twice using 25-ml PBS with 0.1 M NaCl.

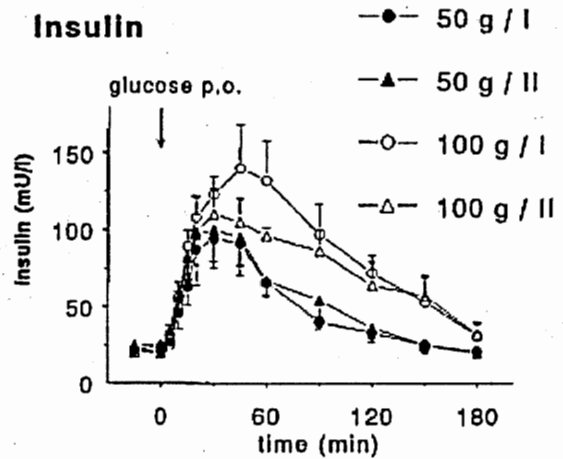
19. Elute virus using 15-ml PBS with 0.4 M NaCl.
20. Discard the first 1 ml flow-through.
21. Collect the rest eluate using a 50-ml conical tube.
22. Add 2.4 ml of glycerol to the viral eluate and mix the solution.
23. Filter through 0.2- μ m membrane.
24. Store the purified viral stock at -70°C .
25. Viral titer can be determined by following the protocol described in AAV Helper-Free System (Stratagene)
26. Purity of viral preparations can be assessed by Coomassie staining of viral loading on SDS gel under reducing conditions.

A.8 Responses of plasma glucose, insulin, GLP-1 and GIP to oral ingestion of glucose in normal human subjects [12]

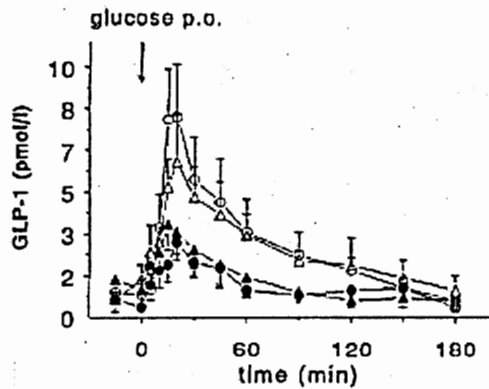
Plasma glucose



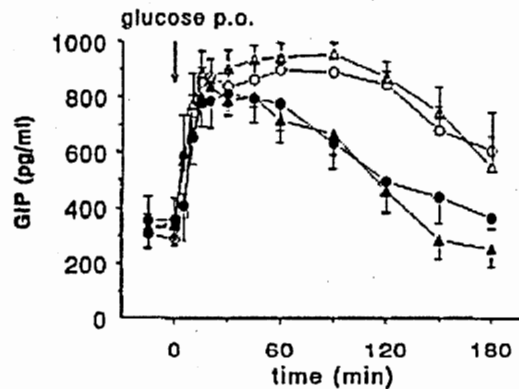
Insulin



GLP-1

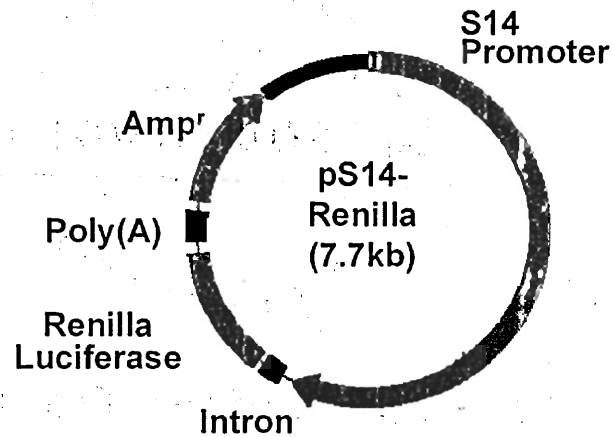


GIP



Arrows indicate the time of oral glucose ingestion (50 or 100 grams). Experiments involved eight volunteers. Mean \pm SEM (standard error of the mean).

(A)



(B)

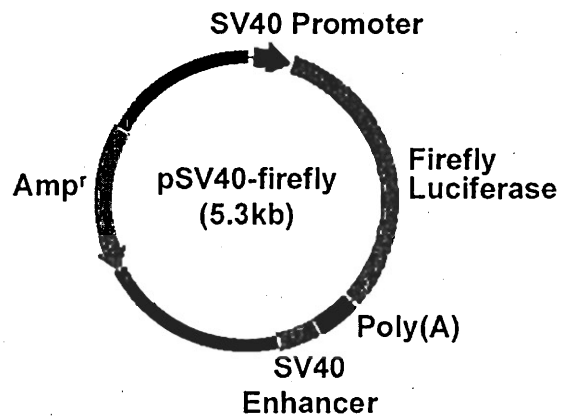


Figure 22. (A) Plasmid structure of the glucose-responsive S14 promoter to direct the reporter, Renilla luciferase, expression (test). (B) Constitutive (SV40-driven) firefly luciferase expression plasmid, as the internal control to monitor non-specific effects of changes in glucose concentrations.

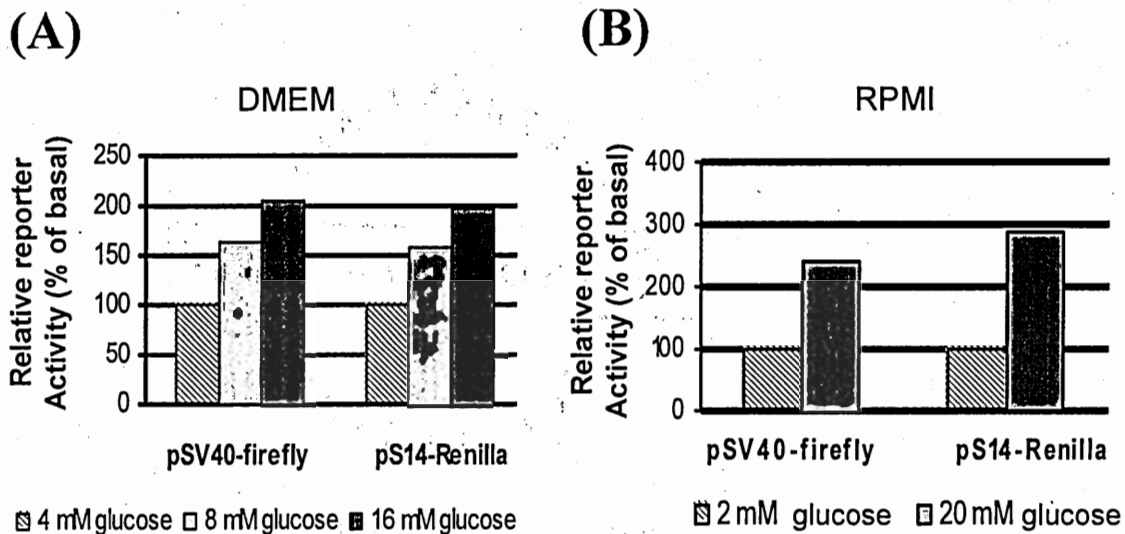
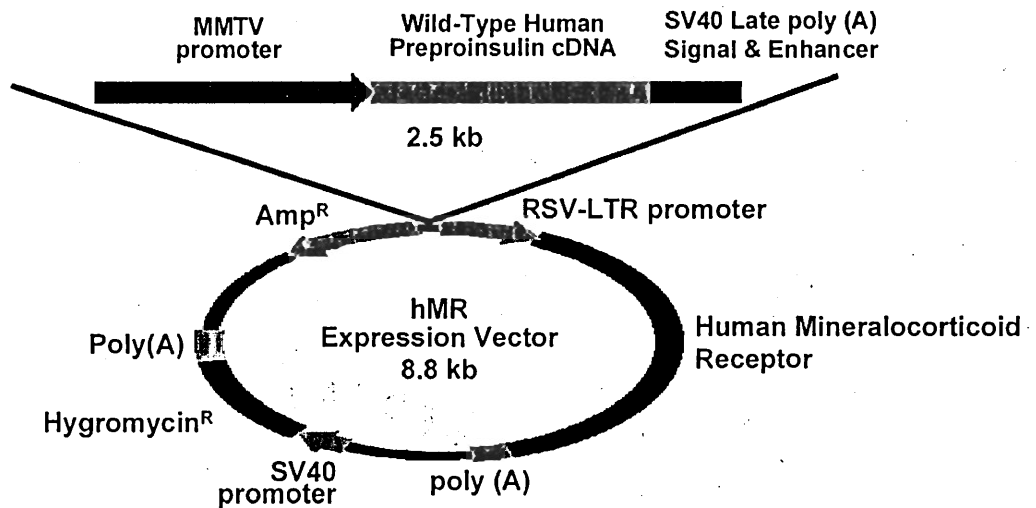


Figure 23. The S14-directed transcription in HIT hamster insulinomas. HIT cells were transiently transfected with the plasmids in Figure 22, and then maintained in low or high glucose concentrations as indicated. Activities of Renilla luciferase (S14-driven, test) and firefly luciferase (SV40-driven, control) were determined by the Promega dual-luciferase assay system. (A) Culture medium: DMEM. (B) Culture medium: RPMI.

(A)



(B)

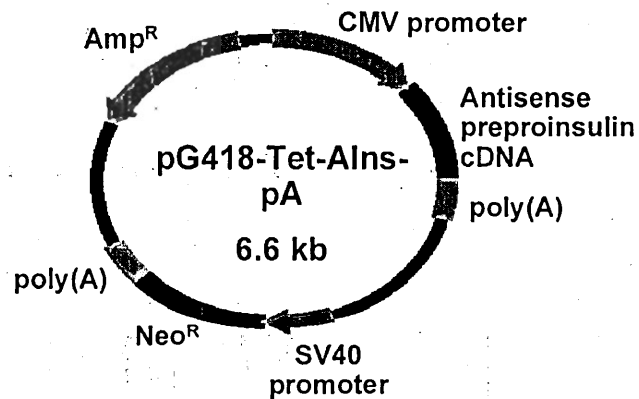


Figure 24. (A) Plasmid structure of the steroid-inducible MMTV promoter to direct the expression of wild-type human insulin gene. A constitutive expression cassette of human mineralocorticoid receptor (MR) is also included. MR binds with steroids such dexamethasone to induce the MMTV promoter. Total size of the plasmid is 11.3 kb. (B) Constitutive (CMV-driven) antisense preproinsulin mRNA expression plasmid.

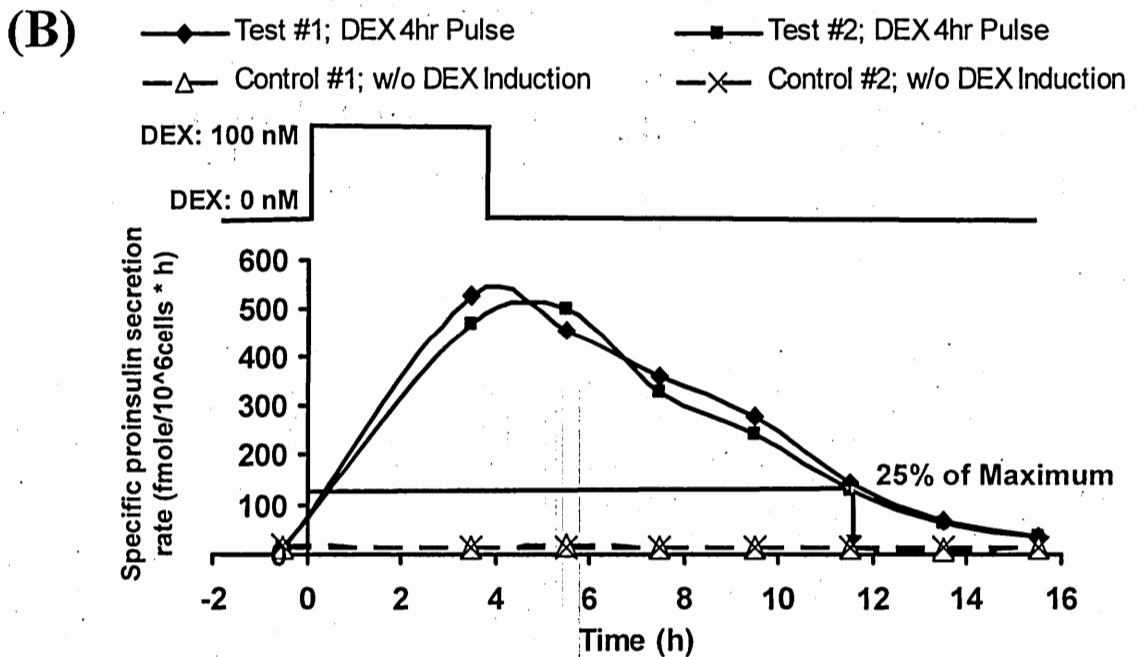
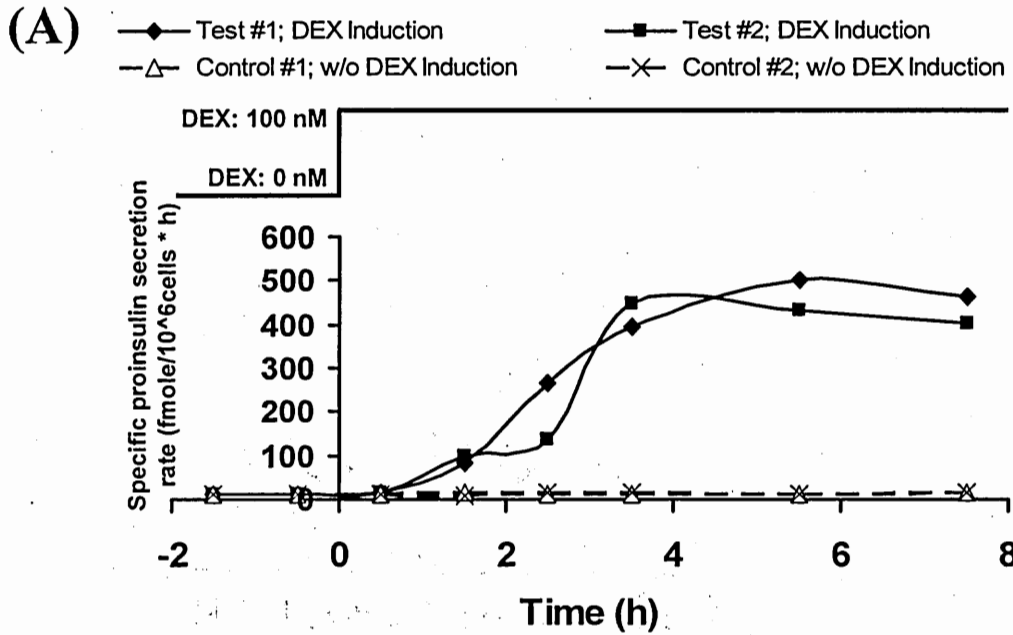
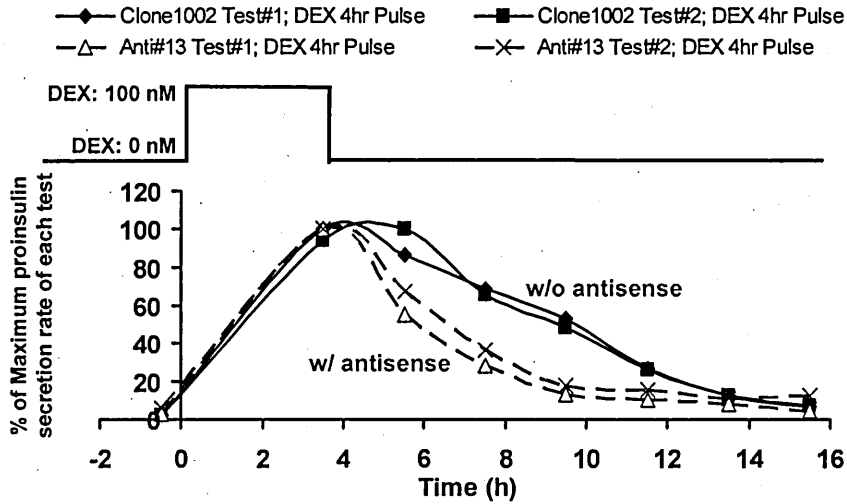


Figure 25. (A) Step induction and (B) pulse induction of proinsulin secretion from recombinant myoblasts with steroid-inducible expression system. DEX: dexamethasone.

(A)



(B)

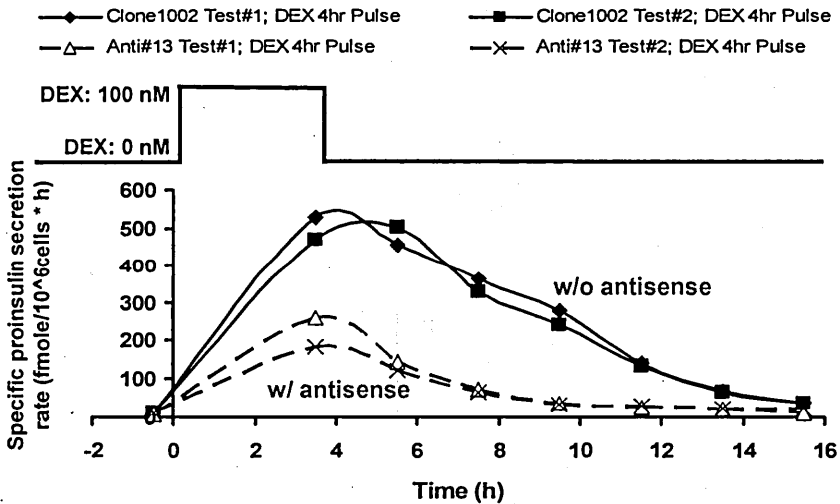


Figure 26. Pulse induction of proinsulin secretion from recombinant myoblasts with constitutive antisense preproinsulin mRNA expression. (A) Y axis: relative proinsulin secretion rate. In each independent test, the insulin secretion rates were normalized by designating the sample with the highest rate as the calibrator and setting it at 100%. (B) Y axis: specific proinsulin secretion rate.

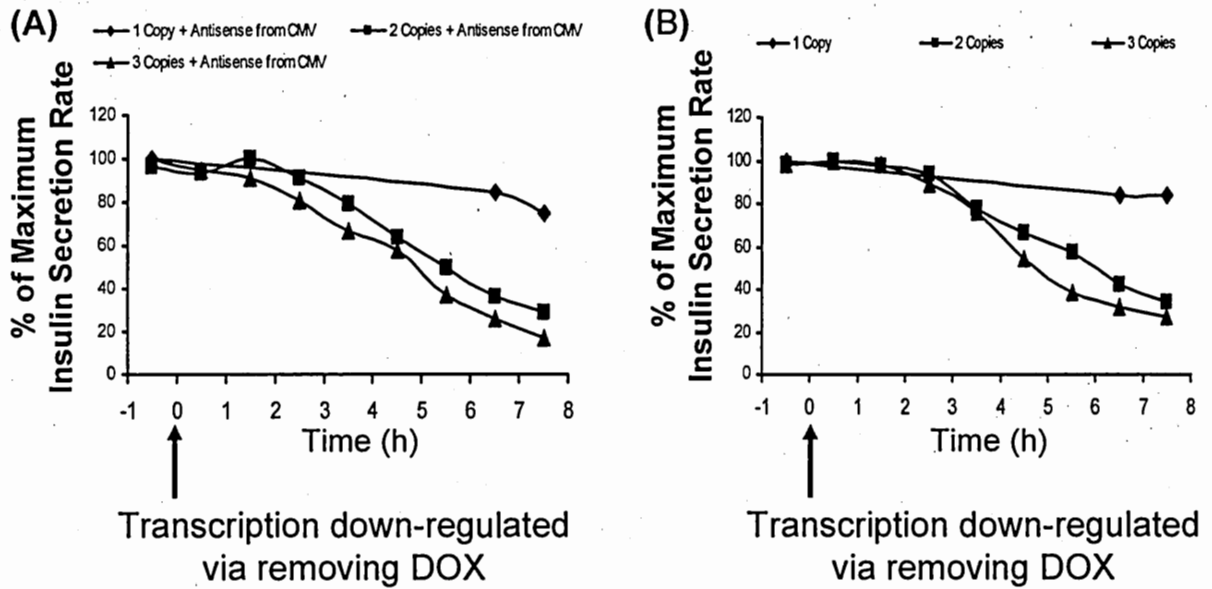


Figure 27. (A) Down-regulation of insulin secretion from transfected HepG2 hepatomas using Tet-On system with different copies of insulin gene and a constitutive expression of antisense preproinsulin mRNA (CMV-driven). (B) Control experiments of (A). In this, no antisense preproinsulin mRNA expression was introduced.

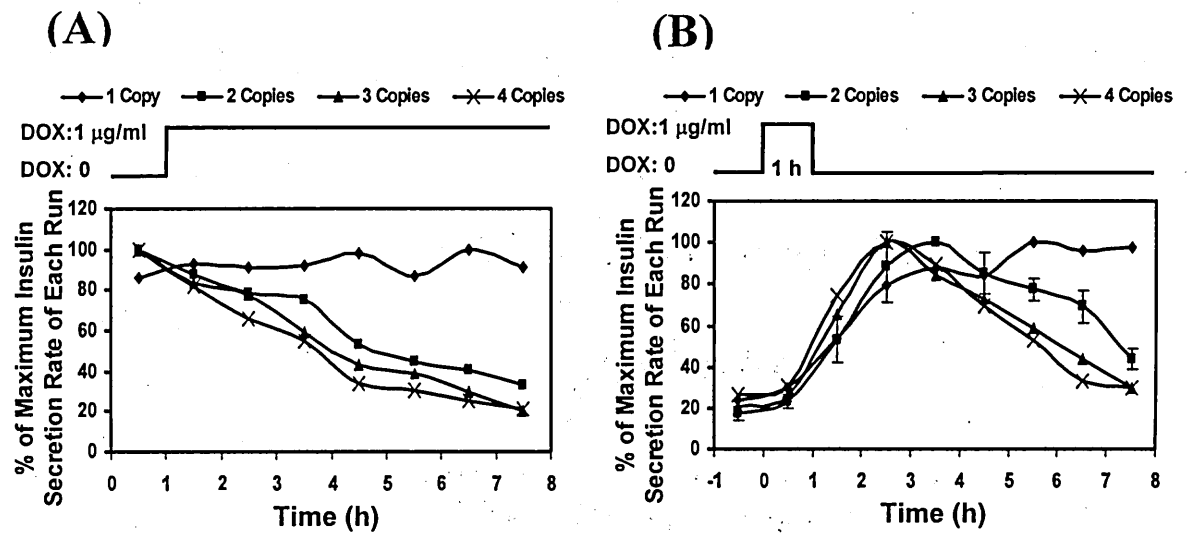


Figure 28. (A) Down-regulation of insulin secretion from transfected HepG2 hepatomas using Tet-Off system with different copies of insulin gene. Step induction of down-regulation by adding doxycycline (DOX). (B) Pulse induction of insulin secretion from transfected HepG2 hepatomas using Tet-On system with different copies of insulin gene.

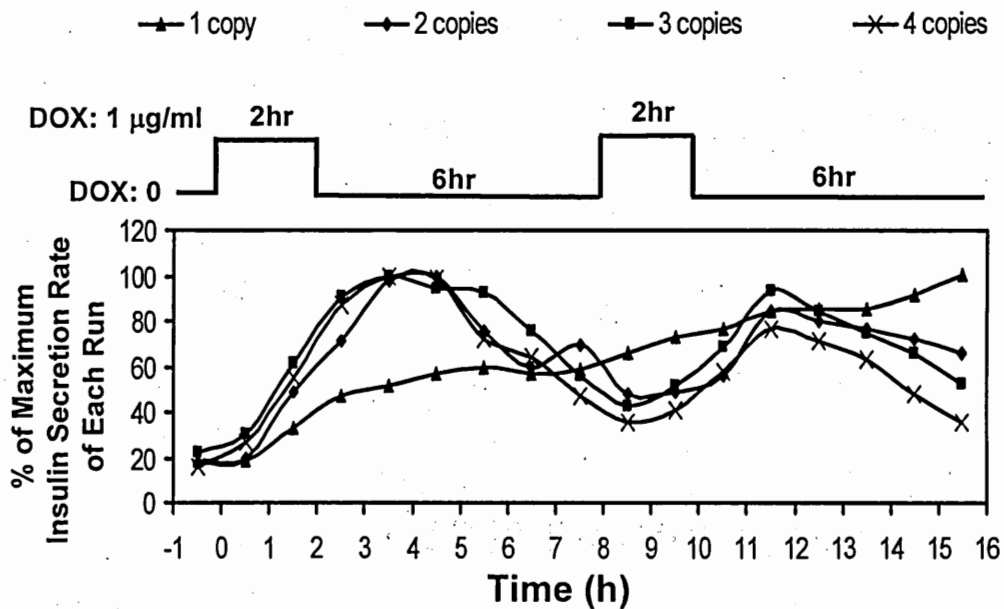


Figure 29. Regulation of insulin secretion from HepG2 hepatomas using Tet-On system with different copies of insulin gene.

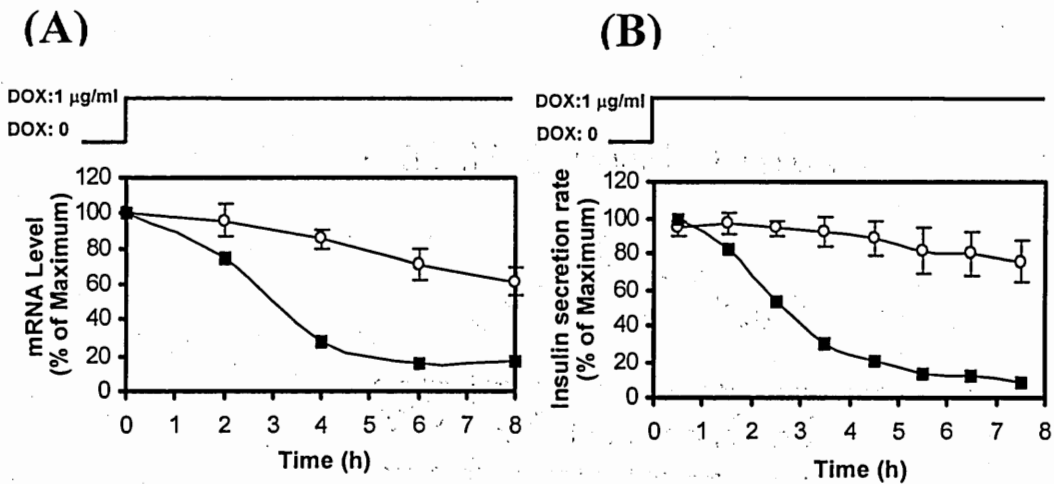


Figure 30. Dynamics of down-regulation of insulin gene expression using Tet-Off system with one-copy control (open circles) and chimeric preproinsulin-luciferase (filled squares) mRNA expression. A: Down-regulation of mRNA. B: Down-regulation of insulin secretion rate. tTA mRNA was used as an internal standard for quantitative mRNA assay. In each independent test, the mRNA was normalized by designating the sample without DOX treatment ($t = 0$) as the calibrator and setting it at 100%; insulin secretion rates were normalized by designating the sample with the highest rate as the calibrator and setting it at 100%.

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