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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6:
G01N 33/50

A2

(11) International Publication Number: WO 99/35495

(43) International Publication Date: 15 July 1999 (15.07.99)

(21) International Application Number: PCT/US99/00551

(22) International Filing Date: 11 January 1999 (11.01.99)

(30) Priority Data:
60/071,193 12 January 1998 (12.01.98) US
60/071,209 12 January 1998 (12.01.98) US
60/072,556 12 January 1998 (12.01.98) US
60/087,821 3 June 1998 (03.06.98) US
60/087,848 3 June 1998 (03.06.98) US

(63) Related by Continuation (CON) or Continuation-in-Part (CIP) to Earlier Applications
US

Filed on
12 January 1998 (12.01.98) 60/071,193 (CIP)
12 January 1998 (12.01.98) 60/071,209 (CIP)
12 January 1998 (12.01.98) 60/072,556 (CIP)
12 January 1998 (12.01.98) 60/087,821 (CIP)
3 June 1998 (03.06.98) 60/087,848 (CIP)

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Published
Without international search report and to be republished upon receipt of that report.

(54) Title: RECOMBINANT CELL LINES FOR DRUG SCREENING

(57) Abstract
The present invention provides methods of screening for modulators of secretory function. In particular, the present invention describes immortalized neuroendocrine secretory cells to screen for novel substances that may be used to regulate secretory function in vitro and in vivo.
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DESCRIPTION

RECOMBINANT CELL LINES FOR DRUG SCREENING

BACKGROUND OF THE INVENTION

The present application is related to co-pending U.S. Patent Applications serial numbers 60/072,556; 60/071,193 and 60/071,209 each of which were filed on January 12, 1998. This application is further related to co-pending U.S. Patent Application serial number 60/087,848 and 60/087,821 both of which were filed on June 3, 1998. The entire text of each of the above-referenced disclosures is specifically incorporated by reference herein without disclaimer.

1. Field of the Invention

The present invention relates generally to the fields of biochemistry and bioengineering of eukaryotic cells. More particularly, it provides compositions and methods for screening for modulators of secretory function.

2. Description of Related Art

Cells of neuroendocrine origin generally have the capacity to synthesize and secrete one or more polypeptide products in a regulated manner. For example, cells of the anterior or intermediate lobes of the pituitary produce growth hormone or proopiomelanocortin (POMC)-derived peptides, such as ACTH and MSH; thyroid C cells secrete calcitonin; and distinct types of pancreatic cells produce and secrete hormones such as glucagon and insulin.

Neuroendocrine cells also exhibit sorting mechanisms whereby a given polypeptide or protein, destined for secretion, is targeted to the regulated secretory pathway or the default constitutive secretory pathway. These cells also have processes for achieving secretory protein maturation, which generally involves protein folding, disulfide bond formation, glycosylation, endoproteolytic processing as well as other types of post-translational modifications. Neuroendocrine cells exhibit controlled release of the secretory protein or polypeptide, most often in response to one or more external signaling molecules, or "secretagogues," and thus have regulatory pathways allowing the cells to secrete a desired product from the secretory storage granules in response to physiological or pharmacological stimuli.
One of the more well known examples of neuroendocrine cells are the β cells of the islets of Langerhans in the pancreas. These cells secrete insulin in response to modulators such as amino acids, glyceraldehyde, free fatty acids, and, most prominently, glucose. The capacity of normal islet β cells to sense a rise in blood glucose concentration and to respond to elevated levels of glucose by secreting insulin is critical to the control of blood glucose levels. Increased insulin secretion in response to a glucose load prevents chronic hyperglycemia in normal individuals by stimulating glucose uptake into peripheral tissues, particularly muscle and adipose tissue. Aberrations in insulin secretion have serious consequences, i.e., diabetes.

Approximately 16 million people in the United States have diabetes, and there has been an increase in the rate of prevalence of this disorder such that the percent of the population with diabetes is projected to increase (ADA, 1996). Some studies project that about 250 million people worldwide will be afflicted by diabetes by the year 2020 (O’Rahilly, 1997). Currently, over 600,000 people are diagnosed with diabetes each year in the United States. The most prevalent forms of this disorder are insulin-dependent diabetes mellitus (IDDM or type I) and noninsulin-dependent diabetes mellitus (NIDDM or type II). The former is typically earlier in onset, accounts for about 10%-15% of total cases, results from the autoimmune destruction of the pancreatic β-cell, and always requires insulin therapy. NIDDM often presents later in life, and progression of the disease is associated with β-cell exhaustion and eventual β-cell failure such that about 60% of people with the disease eventually convert from insulin-independence to an insulin-dependent status. In addition to insulin therapy, NIDDM patients are usually treated with diet and oral agents, particularly in the early phases of the disease. Greater than 90% of NIDDM patients require pharmacological treatment in order to achieve long-term glycemic control (Krall and Beaser, 1989; ADA, 1996).

Insulin was the first therapeutic drug prescribed for the treatment of diabetes. It was introduced in 1922 for the treatment of IDDM and dramatically reduced the mortality rate in this patient population (Joslin Diabetes Manual). Treatment for IDDM largely remains centered around self-injection of insulin once or twice daily. The possibility of islet or pancreas fragment transplantation has been investigated as a means for permanent insulin replacement
(Lacy, 1995). However, this approach has been severely hampered by the difficulties associated with obtaining tissue, as well as the finding that transplanted islets are recognized and destroyed by the same autoimmune mechanism responsible for destruction of the patients’ original islet β cells. Thus, the development of new therapeutic strategies is highly desirable.

In the 1950s, sulfonylurea drugs became available for the treatment of NIDDM. This class of drugs is widely prescribed in the United States and acts directly on the pancreatic β-cell to stimulate insulin secretion. Long-term use of the sulfonylurea drugs often results in a loss of drug efficacy. Usage among patients goes from about 64% in the first five years after diagnosis, to 37% after 20 years of diabetes (Diabetes 1996: vital Statistics).

More recently, Prandin™ (Repaglinide) a novel insulin secretagogue and member of the meglitinide class of drugs, has become available for the treatment of NIDDM. Prandin acts on the same β-cell receptor as the sulfonylureas and stimulates insulin secretion via closure of ATP-dependent potassium channels. However, as the absorption and elimination of Prandin is more rapid than the sulfonylureas, this drug may pose less risk for hypoglycemic episodes (Balfour JA, Faulds D. Repaglinide. Drug Aging, 13(2): 173 180, 1998 and Physicians Desk Reference, Ed 53. 1999). Also, drugs with physiological targets other than the pancreatic β-cell have become available for the treatment of NIDDM (Bressler and Johnson, 1997). Metformin (N,N-dimethylimidocarbonimidic diamide hydrochloride, also known as GLUCOPHAGE®) and troglitazone (a thiazolidinedione, also known as Rezulin) are antihyperglycemic agents that act to decrease glucose production from the liver and to improve peripheral insulin sensitivity (Henry 1997). Acarbose, an oligosaccharide also known as Precose®), exerts its activity in the gastrointestinal tract where it functions as an inhibitor of alpha-glucosidase. Inhibition of this enzyme prevents the conversion of complex carbohydrates to simple, absorbable sugars and thereby functions to decrease postprandial hyperglycemia (Coniff and Krol. 1997). Metformin, troglitazone and acarbose recently have been approved for use in the United States, but it remains to be seen how effective these drugs, or combinations thereof, will be in preventing the short-term and long-term complications of diabetes (Bressler and Johnson, 1997).
Clearly, there is a need for improvement, both in the number of pharmaceutical agents available for the prevention and treatment of diabetes, and in the effectiveness with which such agents prevent or retard the onset of diabetic complications as well as other neuroendocrine based diseases.

**SUMMARY OF THE INVENTION**

This invention describes the genetic tailoring of cell lines and their use in high throughput, biological assays for the identification of novel therapeutic compounds or drug targets. In particular, the use of humanized rodent pancreatic β-cell lines as screening tools for therapeutics in the prevention and/or treatment of diabetes will be described.

Thus, in one aspect, the present invention provides a method of identifying a modulator of secretory function comprising the steps of: (i) providing an immortalized cell having a stable secretory function; (ii) contacting the cell with a candidate substance; (iii) measuring the secretory function of the cell; and (iv) comparing the secretory function of the cell in step (iii) with the secretory function of the cell of step (i), wherein an alteration in the secretory function indicates that the candidate substance is a modulator of the secretory function.

In certain embodiments, the secretory function of the cell comprises the secretion of a polypeptide. The polypeptide may be an amidated polypeptide, a glycosylated polypeptide, a hormone, an enzyme or a growth factor. In other aspects, the secretory function is dependent on a regulator wherein the regulator is selected from the group consisting of calcium ions, cAMP, calmodulin, phosphorylation, dephosphorylation, membrane polarization glucose, ATP, ADP, fatty acids, triglycerides, nitrous oxide (and other free radicals) and NADPH. In other aspects of the present invention, the modulator inhibits the secretion; in yet other embodiments, the modulator stimulates the secretion.

In certain embodiments of the present invention, the cell is encapsulated in a biocompatible matrix. In other embodiments, the cell is in an animal. The cell may be a fetal cell. In other embodiments, the cell is part of a primary cell obtained from human tissue. The cell may, independently be part of an adherent culture or as part of a suspension culture. In
particular embodiments, the cell is an immortalized cell. The cell may be independently, an endocrine cell, and more particularly it may be a neuroendocrine cell. The neuroendocrine cell may be obtained from a human neuroendocrine tumor.

The cell may be an insulinoma cell, a human cell, a non-human cell, a secretory cell, a pancreatic beta cell, a pancreatic alpha cell, a pituitary cell, an adipocyte, a hepatocyte, a muscle cell, a lung cell or a gastrointestinal cell. In other aspects of the invention, the cell is responsive to modulators of secretion. In alternative aspects of the present invention, the cell is non-responsive to modulators of secretion.

The glycosylated polypeptide may be selected from the group consisting of amylin, luteinizing hormone, follicle stimulating hormone and chorionic gonadotrophin. In preferred embodiments, the hormone may be selected from the group consisting of growth hormone, prolactin, placental lactogen, luteinizing hormone, follicle-stimulating hormone, chorionic gonadotropin, thyroid-stimulating hormone, leptin, adrenocorticotropin (ACTH), angiotensin I, angiotensin II, β-endorphin, β-melanocyte stimulating hormone (β-MSH), cholecystokinin, endothelin I, galanin, gastric inhibitory peptide (GIP), glucagon, insulin, lipotropins, neurophysins and somatostatin. The amidated polypeptide may be selected from the group consisting of calcitonin, calcitonin gene related peptide (CGRP), β-calcitonin gene related peptide, hypercalcemia of malignancy factor (1-40) (PTH-rP), parathyroid hormone-related protein (107-139) (PTH-rP), parathyroid hormone-related protein (107-111) (PTH-rP), cholecystokinin (27-33) (CCK), galanin message associated peptide, preprogalanin (65-105), gastrin I, gastrin releasing peptide, glucagon-like peptide (GLP-1), pancreastatin, pancreatic peptide, peptide YY, PHM, secretin, vasoactive intestinal peptide (VIP), oxytocin, vasopressin (AVP), vasotocin, enkephalins, enkephalinamid, metorphinamide (adrenorphin), alpha melanocyte stimulating hormone (alpha-MSH), atrial natriuretic factor (5-28) (ANF), amylin, amyloid P component (SAP-1), corticotropin releasing hormone (CRH), growth hormone releasing factor (GHRH), luteinizing hormone-releasing hormone (LHRH), neuropeptide Y, substance K (neurokinin A), substance P and thyrotropin releasing hormone (TRH). In particularly preferred embodiments, the cell secretes insulin in response to a modulator. The growth factor may be selected from the group consisting of epidermal growth factor, platelet-
derived growth factor, fibroblast growth factor, hepatocyte growth factor and insulin-like growth factor 1. In other embodiments, the polypeptide may be an enzyme that is a secreted enzyme. In particular embodiments, the secreted enzyme may be selected from the group consisting of adenosine deaminase, galactosidase, glucosidase, lecithin:cholesterol acyltransferase (LCAT), factor IX, sphingolipase, lysosomal acid lipase, lipoprotein lipase, hepatic lipase, pancreatic lipase related protein, pancreatic lipase and uronidase. An especially preferred enzyme is LCAT.

In those embodiments wherein the cell is non-responsive to modulators of secretion the cell may be engineered to modify the secretion of the polypeptide in response to the secretagogue. In particular embodiments, the cell may be engineered to secrete an amidated polypeptide, a glycosylated polypeptide, a hormone or a growth factor in response to the secretagogue.

In particular embodiments, the cell is an engineered cell that expresses a recombinant GLUT-2 gene. In other embodiments, the cell is an engineered cell that expresses a recombinant glucokinase (hexokinase IV) gene. In still further embodiments, the cell is an engineered cell that has a reduced hexokinase I activity relative to the cell from which it was prepared.

In particularly preferred aspects of the present invention, the cell is derived from a βTC, RIN, HIT, BHC, CM, TRM, TRM6, AtT20, PC12, BG 49/206, BG40/110, BG-H03, BG 498/45, BG 498/20, NCI-H810 (CRL-5816), BON, NES2Y, NCI-H508 (CLL-253), HEPG2 or HAP5 cell. More particularly, the cell may be selected from the group consisting of βG H01, βG H02, βG H03, βG H04, βG H05, βG H06, βG H07, βG H08, βG H09, βG H10, βG H11, βG H12, βG H13, βG H14, βG H15, βG H16, βG H17, βG H18, βG H19, βG H20, βG H21, βG H22, βG H23 and βG H25.

In certain aspects of the present invention, the cell secretes an endogenous secretory polypeptide. In other aspects, the cell is engineered to increase secretion of an endogenous secretory polypeptide. In still further embodiments, the cell is engineered to modify the
secretion of an endogenous secretory polypeptide in response to the modulator. More particularly, the cell comprises an endogenous gene encoding the polypeptide and in certain aspects, the expression of the gene is inhibited. In still further preferred embodiments, the cell further comprises an exogenous gene that encodes an exogenous secretory polypeptide, the cell secreting the exogenous secretory polypeptide.

It is contemplated that the cell is grown in defined media further supplemented with a growth factor specific for the cell. The cell may be a human pancreatic β-cell and the growth factor is HGF, IGF-1, PDGF, NGF or growth hormone.

In other embodiments, the cell expresses an endogenous receptor. In particular aspects, the cell comprises an endogenous gene encoding the receptor and the expression of the gene is inhibited. In other aspects, the cell may be engineered to modify the expression of an endogenous receptor in response to the modulator. The modulator may be a stimulator of the expression or an inhibitor of the expression.

In those aspects wherein an endogenous gene has been inhibited, the cell may further comprise an exogenous gene that encodes an exogenous receptor, the cell expressing the exogenous receptor. In more particular embodiments, the receptor is selected from the group consisting of α-adrenergic receptor, β-adrenergic receptor, potassium inward rectifying channel, sulphonylurea receptor, GLP-1 receptor, L-Ca^{2+} receptor, voltage-dependant late rectifying channel, growth hormone receptor, luteinizing hormone receptor, corticotrophin receptor, urocortin receptor, glucocorticoid receptor, pancreatic polypeptide receptor, somatostatin receptor, muscarinic receptor, BK channel and leptin receptor.

In particular embodiments, the cell secretory function is responsive to a cell signaling molecule and the cell signaling molecule is a modulator of the secretion. In preferred embodiments, the modulator may be a stimulator or an inhibitor of the secretion. The cell signal may be Ca^{2+} dependent or Ca^{2+} independent.
Also provided by the present invention is a modulator of secretory function identified according a method comprising the steps of: (i) providing an immortalized cell having a stable secretory function; (ii) contacting the cell with a candidate substance; (iii) measuring the secretory function of the cell; and (iv) comparing the secretory function of the cell in step (iii) with the secretory function of the cell of step (i), wherein an alteration in the secretory function indicates that the candidate substance is a modulator the secretory function.

Alternative aspects of the present invention provide a modulator of polypeptide secretion identified according a method comprising the steps of: (i) providing a stable, immortalized cell that secretes a polypeptide; (ii) contacting the cell with a candidate substance; (iii) incubating the cell; (iv) measuring the secretion of the polypeptide; and (v) comparing the secretion of the polypeptide in the cell of step (iii) with the secretion of the polypeptide in the cell of step (i), wherein an alteration in the secretion of the polypeptide indicates that the candidate substance is a modulator the secretion.

In still other aspects, the present invention provides a method of identifying a modulator of insulin secretion comprising the steps of: (i) providing an engineered pancreatic β cell; (ii) contacting the cell with a candidate substance; (iii) measuring the insulin secretion of the cell; and (iv) comparing the insulin secretion of the cell in step (iii) with the insulin secretion of the cell of step (i), wherein an alteration in the insulin secretion indicates that the candidate substance is a modulator the secretion.

Another embodiment of the present invention provides a method of identifying a modulator of insulin secretion comprising the steps of (i) providing an engineered pancreatic β cell; (ii) contacting the cell with a candidate substance; (iii) measuring the intracellular signal of the cell; and (iv) comparing the intracellular signal of the cell in step (iii) with the intracellular signal of the cell of step (i); wherein an alteration in the intracellular signal indicates that the candidate substance is a modulator the insulin secretion. The intracellular signal can include but is not limited to pH, calcium, ATP, ADP, action potentials, membrane polarity, fatty acid pools such as free fatty acids and triglycerides, glycolytic flux, NADPH, NADP, NADH, NAD.
nitrous oxide and other free radicals, DNA fragmentation and other events associated with apoptosis, patterns of gene expression, cAMP, calmodulin, and enzyme activities.

A more specific embodiment of the present invention provides a method of identifying a modulator of insulin secretion comprising the steps of (i) providing an engineered pancreatic β cell; (ii) contacting the cell with a candidate substance; (iii) measuring the intracellular Ca\textsuperscript{2+} of the cell; and (iv) comparing the intracellular Ca\textsuperscript{2+} of the cell in step (iii) with the intracellular Ca\textsuperscript{2+} of the cell of step (i); wherein an alteration in the intracellular Ca\textsuperscript{2+} indicates that the candidate substance is a modulator the insulin secretion.

Another aspect of the present invention provides an engineered human cell line that has a regulated secretory pathway, the cell comprising a transgene encoding a therapeutic polypeptide. In preferred embodiments, the transgene is introduced to the cell by contacting the cell with an expression construct comprising the gene operably linked to a promoter functional in eukaryotic cells. In particularly preferred embodiments, the cell is selected from the group consisting of βTC, RIN, HIT, BHC, CM, TRM, TRM6, AT20, PC12, BG 49/206, BG40/110, BG-H03, BG 498/45, BG 498/20, NCI-H810 (CRL-5816), BON, NES2Y, NCI-H508 (CLL-253), HEPG2 or HAP5 cell. More particularly, the cell may be selected from the group consisting of βG H01, βG H02, βG H03, βG H04, βG H05, βG H06, βG H07, βG H08, βG H09, βG H10, βG H11, βG H12, βG H13, βG H14, βG H15, βG H16, βG H17, βG H18, βG H19, βG H20, βG H21, βG H22, βG H23 and βG H25.

Other objects, features and advantages of the present invention will become apparent from the following detailed description. It should be understood, however, that the detailed description and the specific examples, while indicating preferred embodiments of the invention, are given by way of illustration only, since various changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art from this detailed description.
BRIEF DESCRIPTION OF THE DRAWINGS

The following drawings form part of the present specification and are included to further demonstrate certain aspects of the present invention. The invention may be better understood by reference to one or more of these drawings in combination with the detailed description of specific embodiments presented herein.

FIG. 1. Multiple signaling pathways are involved in the regulation of insulin secretion. Insulin secretion is regulated by fuels and hormones, and is subject to regulation as well by synthetic compounds. The various modulators exert effects via specific cell surface receptors, metabolic pathways, and ion fluxes. Most changes in secretion are mediated through changes in intracellular calcium.

FIG. 2. Schematic summary of how engineered cell lines can facilitate in vitro and in vivo testing of candidate compounds that modulate insulin secretion. Compounds can be screened for their effects on secretory function using primary islets, enriched populations of beta cells, and engineered cell lines. Information from these screens can be used to elucidate potentially novel drug targets and to enrich for compounds that impact secretory function. Encapsulated cells can be transplanted into rodents or other mammals for pre-clinical in vivo testing of candidate compounds.

FIG. 3. Engineered beta-cell lines respond to a variety of secretagogues. βG 49/206 cell lines were plated, cultured for 48 hrs., rinsed and washed two times (20 min. each) in HEPES Buffered Biological Salt Solution (HBBSS). HBBSS supplemented with secretagogue(s) was added to each well and allowed to incubate for 2 hours. Medium was harvested from each well, assayed for insulin, and the amount of insulin secreted per hour, normalized to cell number, was determined. Normalization for cell number was achieved by staining with the neutral red, a viability dye.

FIG. 4. The response of engineered β-cell lines to secretagogues is stable over time and population doublings. βG 49/206 cells were tested for stability of secretagogue responsiveness by monitoring insulin secretion over several population doublings (PD) ranging
from PD12 to PD105. Cells were plated, cultured for 48 hrs, and washed two time (30 min. each) in a HBBSS. Insulin secretion was stimulated with glucose alone (Basal +) or in the absence or presence of glucose (presence indicated by "+") with IBMX. The stimulatory cocktail contains a mixture of secretagogues (BetaGene media supplemented with 10 mM glucose, 10 mM glutamine, leucine, and arginine, 100μM carbachol, and 100μM IBMX).

**FIG. 5.** Secretagogue-stimulated insulin secretion of engineered RIN cell lines is maintained in microbeads. βG 49/206 cells were encapsulated in 50 μl alginate beads, cultured in BetaGene medium for 72 hrs, and tested for secretagogue-responsive insulin secretion. Following washes in HBBSS, cells were stimulated with glucose, glucose plus IBMX, or a Stimulatory Cocktail (BetaGene supplemented with 10 mM glucose, 10 mM glutamine, leucine, and arginine, 100μM carbachol, 100μM IBMX, 0.1% BSA, 20 mM HEPES). As shown, glucose plus IBMX resulted in an 8-fold stimulation in insulin secretion, which is comparable to the fold stimulation observed with adherent cultures.

**FIG. 6.** Engineered RIN cells retain secretory responsiveness in a 96-well format. βG 49/206 cells were plated and assayed in 48-well dishes (100,000/well) as described in the legend to FIG. 3. For 96-well assays, 30,000 βG 49/206 cells were plated and cultured for 48 hrs. in 150 μl of BetaGene Medium/ 2.5% FCS; washed twice, 20 min each, in 200 μl in HBBSS, and cells stimulated with glucose or glucose plus IBMX.

**FIG. 7.** Overexpression of the alpha2-adrenergic receptor in RIN cell lines confers an increased sensitivity to Clonidine in vitro. βG 265/2 and βG 265/4, cell lines that overexpress transgenic alpha2-adrenergic receptor, were compared to the parental cell line (βG 18/3E1) for the capacity of Clonidine to inhibit stimulated insulin secretion. Cell lines were plated, cultured for 48 hrs, and washed two times (30 min. each) in a basal medium (RPMI medium/ without glucose/0.5% BSA/ 20 mM HEPES/ 100 μm diazoxide). Cells were stimulated to secrete insulin with a stimulatory cocktail - amino acids supplemented with 0, 1, 10 or 100 nM Clonidine. 1 nM Clonidine was potently inhibitory of stimulated insulin secretion in both the βG 265/2 and βG 265/4 cell lines resulting in a 60% and 30% reduction,
respectively. The parental βG 18/3E1 cell lines was resistant to the inhibitory effects at all concentrations of Clonidine tested.

**FIG. 8.** Engineered RIN cell lines that express transgenic alpha2-adrenergic receptor are more sensitive than human islets to Clonidine. βG 265/2 cells and human islets were encapsulated in alginate microbeads and stimulated to secrete insulin in 0, 1, 10, 100, or 1000 nM Clonidine. Washes and stimulations were performed as described in the legend to FIG.7. At 10 nM Clonidine, human islets were refractory to Clonidine; whereas, insulin secretion from βG 265/4 cells was inhibited by about 35%.

**FIG. 9.** Overexpression of the alpha2-adrenergic receptor in RIN cell lines confers an increased sensitivity to Clonidine in vivo. βG 265/2 cell lines were encapsulated in alginate beads and injected intraperitoneally (IP) into Zucker diabetic, fatty rats. Following 4-5 days of in vivo growth and normalization of blood glucose, rats were injected IP with Clonidine (50 µg/kg), or Yohimbine (75 µg/kg). 20 minutes post-injection blood samples were taken to determine the levels of human insulin and rat C-peptide in the plasma. Yohimbine had no effect on human insulin or rat C-peptide levels. Clonidine injection resulted in a 50% reduction of human insulin and rat C-peptide in plasma.

**FIG. 10A and FIG. 10B.** Engineered beta-cell lines lose stimulated insulin secretion, but maintain basal insulin secretion in the absence of fetal bovine serum. FIG. 10A. βG 18/3E1 cells were encapsulated in alginate and maintained for one week in culture in BetaGene medium with or without FBS supplement. Beads were washed with basal medium and treated with a cocktail (BetaGene supplemented with 10 mM glucose, 10 mM glutamine, leucine, and arginine, 100µM carbachol, 100µM IBMX, 0.1% BSA, 20 mM HEPES) to stimulate insulin secretion. Cells that had been maintained in FBS-supplemented BetaGene media responded with a 3-4 fold increase in insulin secretion; whereas those cells non-supplemented with FBS failed to stimulate insulin secretion following exposure to the stimulatory cocktail. In contrast to the dramatic differences in secretion, the basal insulin secretion from the two groups is maintained at equivalent levels. FIG. 10B compares the effects of different lots of BetaGene Medium and FBS on cellular growth. As shown, lots 7E183 is equivalent to lot 7H3299 with
respect to support of cellular growth, and 9 days of FBS-depletion diminishes growth of the cells by 10 - 20%.

**FIG. 11A and FIG. 11B. Over-expression of somatostatin receptor (SSTRV) in βG 40/110 confers increased sensitivity to somatostatin (SS-28).** A cell line expressing high levels of the transgenic SSTRV receptor (βG 603/11) was compared to a non-expressing cell line, βG 603/7 (FIG. 11A). As shown, 50 pM SS-28 was potently inhibitory of glucose-stimulated insulin secretion from βG 603/11, but had no effect on βG 603/7 insulin secretion. Secretion studies were performed with HBBSS supplemented with varying concentrations of SS-28 in the absence or presence of 10 mM glucose. As shown in FIG. 11B, 5 nM SS-28 inhibits insulin secretion from βG 603/11 when cells were stimulated with BetaGene Medium in the absence of glucose and in a stimulatory cocktail (BetaGene Media supplemented with 10 mM glucose, 10 mM glutamine, leucine, and arginine, 100µM carbachol, 100µM IBMX, 0.1% BSA, 20 mM HEPES).

**FIG. 12A and FIG. 12B. Efficient processing of overexpressed human proinsulin in engineered human neuroendocrine cells.** Immunoreactive insulin was measured from HPLC-fractionated samples prepared from βG 498/20. Peaks were identified by migration position of standards. FIG. 12A is the analysis of insulin content extracted from the cells, and FIG. 12B is the analysis of insulin secreted into the media.

**FIG. 13A and FIG. 13B. Regulated secretion from engineered human cell lines.** Insulin secretion from βG 498/20 was measured in a two hour static incubation assay at basal conditions (0 mM) or stimulated conditions: 10 mM glucose (10 mM); 10 mM glucose + 100 µM IBMX (IBMX + 10mM); 100 µM carbachol (carb); 100 µM carbachol + 10 mM glucose (carb + 10 mM); 10 nM PMA (PMA); 10 nM PMA + 10 mM glucose (PMA + glucose); RPMI Medium + 100 µM diazoxide + BSA (RPMI + Diaz); or a stimulatory cocktail (RPMI medium supplemented with 10 mM glucose; BSA; 10 mM each arginine, leucine, glutamine; 100 µM carbachol, and 100 µM IBMX). In FIG. 13B, cell line βG 498/45 (created by transfection of BG H03 with a plasmid conferring resistance to neomycin and encoding human insulin) was engineered for increased levels of insulin expression by the introduction of a number of
plasmids, all of which encoded human insulin but varied in the genes encoding antibiotic resistance. The 793, 794, and 796 cell lines are resistant to mycophenolic acid, puromycin, and hygromycin, respectively. The data show the presence of a regulated secretory pathway in the progenitor cell line (498/45) and the maintenance of this capacity through a second round of engineering (793, 794, and 796 cell lines). The increase in stimulated secretion over basal secretion ranges from about 6- to 15-fold among the various clones.

**FIG. 14A, FIG. 14B, and FIG. 14C.** Correction of Diabetes in Rodents. Encapsulated βG 498/20 and βG H03 cells and were implanted into STZ-diabetic, NIH nude rats (FIG. 14A), immune-competent STZ-diabetic Wistar rats (FIG. 14B) or Zucker Diabetic Fatty Rats (ZDF, FIG. 14C). Unengineered, parental cells (βG H03) or low doses of βG 498/20 cells failed to affect hyperglycemia. In contrast, doses of βG 498/20 ranging from 15 to 25 million cells per 100 gm body weight completely corrected hyperglycemia in nude and immune-competent hosts; and in IDDM and NIDDM.

**FIG. 15.** Human C-peptide levels in the serum of rats implanted with βG 498/20 or the parental βG H03 correlate with cell number. As shown, implantation of βG 498/20 cells into STZ-diabetic Wistar rats elevates human C-peptide levels in the serum with the highest dose of cells (25 million/100 gm body weight) producing the highest level of serum human C-peptide levels for the longest period of time. The drop in C-peptide to levels below about 3 ng/ml with both cell doses of βG 498/20 correlate with increases in blood glucose shown in FIG. 14B. βG H03 cells do not produce detectable levels of human C-peptide.

**FIG. 16A and 16B.** Implantation into diabetic rodents with βG 498/20 cells improves glucose tolerance. Following an overnight fast, animals were given a glucose bolus, and blood glucose levels were monitored. As shown both STZ-treated Wistar rats (FIG. 16A) and ZDF rats (FIG. 16B) show a dose dependent improvement in glucose tolerance when implanted with βG 498/20 versus implants with the unengineered parental cell line, βG H03.

**FIG. 17A and 17B.** Cell-based delivery of insulin via encapsulated βG 498/20 cells reduces glycated hemoglobin (GHb) in diabetic rodents. STZ-treated Fisher nudes (FIG.
17A) or STZ-treated Wistar rats (FIG. 17B) that were implanted with βG 498/20 cells experienced about 58% and 33% reduction, respectively, in % glycated hemoglobin as compared to control diabetic animals implanted with the unengineered parental cell line βG H03.

FIG. 18A and FIG. 18B. βG H03 and βG 498/20 cells are resistant to the effects of cytokines. In FIG. 18A, βG H03 and βG 498/20 cells were incubated in BetaGene medium supplemented with various human cytokines as indicated for 48 hours. Tested cytokines had no impact on viability as assessed by comparing cytokine-treated cultures to untreated controls. In FIG. 18B, βG 498/20 cells were tested for the maintenance of secretory function in presence of cytokines (IL-1β 15 ng/ml; IFN 200 units/ml; TNFα and TNFβ each at 10 ng/ml). Insulin secretion was stimulated by incubating the cells in HBBSS containing 0.1% BSA and supplemented with 10 mM glucose, or 10 mM glucose plus either 100 µM carbachol or 10 mM PMA. Two sets of cultures were exposed to cytokines for 24 hours, prior to secretion studies (24h cytokines, and 24h cytokines + HBBSS + cytokines); and two sets of cultures were supplemented with cytokines for the 2 hr secretion period (HBBSS + cytokines, and 24h cytokines + HBBSS + cytokines). The control culture (HBBSS) was not exposed to cytokines. The secretory function of βG 498/20 cells was unaffected by short or long-term exposure to cytokines.

FIG. 19. Engineered βG H04 fail to secrete insulin from the regulated secretory pathway. Transgenic (CMV-insulin/ SV40-Neo), clonal derivatives of βG H04 known to secrete human insulin were tested for the capacity to secrete human insulin from the regulated secretory pathway. There was no difference between basal conditions (HBBSS with no glucose) and stimulated conditions (HBBSS + 25 mM KCl + 2.5 mM Forskolin + 50 µM IBMX) in the βG H04 clones: 707/55, 707/63, 707/76, 707/94, and 707/96. In contrast, in the clonal line derived from βG H03, there was a robust response to the aforementioned secretagogue cocktail, with about a 5-fold difference between basal and stimulated secretion.

FIG. 20. Major components of the counter-regulatory and sympathetic responses to hypoglycemia. Sympathetic activation involves both stimulation of adrenaline secretion
from the adrenal medulla, and increased release of noradrenaline (and adrenaline) from sympathetic nerve endings, which act directly in sympathetically innervated tissues (e.g. the liver and arterioles) and also spill over into the circulation. Vasopressin has weak counter-regulatory effects on its own, but acts synergistically with the other hormones (figure adapted from "Textbook of Diabetes", 2nd. Edn. John C. Pickup and Gareth William Eds., Blackwell Sciences, Publ., 1997).

FIG. 21. General mechanisms for modulating secretion from the regulated secretory pathway. As schematically illustrated, secretion from the regulated secretory pathway can be modulated by the transgenic expression of cellular proteins that act as positive regulators of secretion (oval with a "+") or negative regulators (oval with "-".). Typically such proteins function as receptors at the cell surface. Each class of receptor is subject to activation (ACT.) or inhibition (INH.) of activity by the binding of receptor-specific ligands, and such ligands can be physiological or pharmacological agents. The modulation in receptor activity by ligand binding is translated through intracellular signaling to stimulate or inhibit the secretion of peptide hormones from the regulated secretory pathway.

FIG. 22. A Two-Step method for creating human neuroendocrine cell lines. Primary tissues, such as human islets, or neuroendocrine tumors, such as insulinomas, can be induced to proliferate through transgenic expression of growth-promoting proteins. A preferred protocol for such engineering is to selectively direct gene expression with the use of tissue-specific promoters and to provide transgenes via infection with recombinant adenovirus. Following an induction of proliferation, the cell population of interest is subject to enhanced rates of immortalization via infection with recombinant retroviruses.

FIG. 23. Total insulin release from a human insulinoma. A freshly excised human insulinoma, about 1 cm³, was processed and initially plated into two tissue culture wells, 9.6 cm² each. The cells that survived were subsequently aliquoted into a variety of culture conditions. At the times indicated, tissue culture media samples were obtained from each of the cell samples, and insulin was measured by RIA. The insulin output from the different samples was summed to give total output.
FIG. 24. Maintenance of human islets in BetaGene Medium supplemented with various concentrations of glucose. Islets were cultured in BetaGene Medium with 3.9, 7.8 and 22 mM glucose for 2 weeks. The secretory responses to glucose concentrations of 3.9 mM, 22 mM and 22 mM +50 μM IBMX were then compared. Although lower glucose was less deleterious than the higher concentration, both resulted in impaired secretory response.

FIG. 25. Maintenance of human islets in BetaGene Medium supplemented with various concentrations of fetal bovine serum. The serum requirements of human islets were tested in long term (2 month) cultures supplemented with various amounts of serum, 1%, 3.5%, or 10% FBS and 5% horse serum (ES). In an acute secretion experiment, insulin secretion from islets cultured in 10% FBS exhibited lower response to glucose or to a stronger mixed secretagogue stimulus. The sustained insulin output from human islets with 1% FBS supplementation (in BetaGene Medium) suggested that human islets may also secrete insulin and survive under serum-free conditions.

FIG. 26. Comparison of commonly used medias to BetaGene Medium in the maintenance of human islets. Islets were cultured for 2-3 months with BetaGene Medium, Medium 199, alpha MEM, or CMRL, all with equivalent glucose, and 0.1% BSA. In four independent islet isolations the insulin output was the highest with islets cultured in BetaGene Medium. In contrast, CMRL performed the poorest, essentially with no islet survival past 2 months with all 4 isolations studied.

FIG. 27 Long-term culture of human islets in BetaGene Medium restores and maintains glucose-stimulated insulin secretion. The capacity of BetaGene Medium to sustain the dose-responsive nature of the insulin secretory response was evaluated with continuous cultures. Human islets were stimulated with varied glucose concentrations at intervals to monitor secretory changes that may occur with time. A common finding was an initially poor response (shown at 1 week), with increased function with time of culture in BetaGene Medium (6 weeks and 13 weeks), and a maintained capability to secrete insulin in response to glucose for times >4 months.
FIG. 28A and 28B. Processing of proinsulin to mature insulin is enhanced by culturing human islets in BetaGene Medium. Insulin content was extracted from HI21 and fractionated by HPLC. Initially, 99% of the insulin was unprocessed insulin, with only 29 ng mature insulin/1000 IEQ (FIG. 28A). The mature insulin content was increased 18-fold to 512 ng/1000IEQ after 4 weeks of culture in BetaGene Medium; this represents >90% of the insulin content (FIG. 28B).

FIG. 29A and FIG. 29B. Modified RIP activity in transiently transfected RIN cells. FIG. 29A. A schematic representation of the types of modified RIP promoters. FIG. 29B. Modified RIP promoter – human growth hormone (hGH) constructs were transiently transfected into RIN cells. After 48 to 96 h, hGH protein levels in the medium were determined by a radioimmunoassay. As shown in the figure, the modified RIP promoters, FFE3/-415RIP and FFE6/-415RIP, were approximately 5-fold stronger than the RIP (-415RIP) promoter by itself.

FIG. 30A, FIG. 30B and FIG. 30C. Modified RIP activity in stably transfected RIN cells. The CMV promoter, RIP promoter, and several modified RIP promoters were fused to insulin and were stably transfected into RIN cells. FIG. 30A. Insulin mRNA levels for each promoter construct were determined by Northern blot and quantitated with a phosphoimager. Cyclophilin mRNA levels were also determined by a phosphoimager as a control for Northern blot loading differences. FIG. 30B. Insulin protein levels secreted into the culture medium were determined by a radioimmunoassay. FIG. 30C. Insulin protein levels within the cell were determined by a radioimmunoassay after breaking open the cells by sonication. In all three cases, be it insulin mRNA levels, secreted insulin protein, or insulin protein content inside the cell, the modified RIP promoters were significantly stronger than the RIP promoter by itself. The FFE6 modified RIP promoters approach the activity of the very strong CMV promoter.

FIG. 31. Mitogenic signal pathways in β-cells. Mitogenic pathways are shown for insulin-like growth factor-1 (IGF-1) and for growth hormone (GH). The IGF-1/IGF-1 receptor
complex can signal cell mitogenesis via two pathways but in β cells it does so primarily through the IRS pathway. Mitogenic stimulation of β cells by GH is through the JAK/STAT pathway.

**FIG. 32. IGF-1 stimulation of β cell growth in the presence of increasing glucose concentrations.** IGF-1 (10 nM) was added to INS1 cells incubated at different glucose concentrations. As judged by \(^3\)H-thymidine incorporation glucose alone can initiate INS1 cell growth in a dose-dependent manner reaching a maximum of approximately 10-fold at 18 mM glucose. The effect of glucose on INS1 cell growth is potentiated by IGF-1 reaching a maximum of INS1 cell growth at 15mM glucose.

**FIG. 33. Growth hormone stimulation of β cell growth in the presence of increasing glucose concentrations.** rGH (10nM) was added to INS1 cells incubated at different glucose concentrations. The action of rGH, like that of IGF-1, requires a background of glucose to exert its effects. The rGH has little effect on cell growth until a threshold of 6mM glucose and reaches a maximum at 15 mM glucose where there is an approximately 50-fold increase in \(^3\)H-thymidine incorporation over that at 0 mM glucose.

**FIG. 34. Additive effects of IGF-1 and rGH on β cell growth.** INS1 cells were incubated with either 10 nM IGF-1 alone, 10nM rGH alone, or both 10nM IGF-1 and 10nM rGH at increasing glucose concentrations. As previously shown in Fig. 29 and Fig. 30, both IGF-1 and rGH potentiate the effect of glucose on INS1 cell growth to approximately the same degree. An additive effect on cell growth is observed when both growth factors are added to INS1 cells at the same time.

**FIG. 35. Adenoviral overexpression of IRS-1, IRS-2, and SV40 large T-antigen in INS1 cells.** INS1 cells were infected with either AdV-βGal, AdV-IRS-1, AdV-IRS-2, or AdV-largeT-antigen (Tag) for 1 hour. After 1 hour, the cells were washed and incubated another 24 hours. IGF-1 (10 nM) was added to the INS1 cells in the presence of either 3 mM or 15 mM glucose. Adenoviral-mediated overexpression of IRS-2 in INS1 cells in the presence of 10 nM IGF-1 and 15 mM glucose resulted in an approximately 200-fold increase in \(^3\)H-thymidine
incorporation compared to uninfected cells plus no glucose. AdV-IRS-1 infected cells in the presence of 10 nM IGF-1 and 15 mM glucose showed no increase of \( ^{3}H \)-thymidine incorporation over and above that for uninfected cells or cells infected with AdV-βGal in the presence of 10 nM IGF-1 and 15 mM glucose.

FIG. 36. BetaGene Medium enhances growth of an engineered, human neuroendocrine cell line. The BG785/5 cell line was derived from BGH04 cells which were derived and routinely cultured in RPMI w/FBS. The growth rate of BG785/5 cells in BetaGene and RPMI media, with FBS or SF, is shown. Although cells grown in RPMI w/FBS exhibited a longer lag phase, the growth of cells in BetaGene medium and RPMI w/FBS was similar, all with doubling times of 2 days. However, cells in RPMI w/SF essentially failed to grow, with an apparent doubling time of 26±1 days.

FIG. 37. BetaGene Medium enhances secretory function of an engineered, rodent neuroendocrine cell lines (BG170-hGH). The human growth hormone (hGH) output of cells grown in BetaGene Medium with FBS was approx. 5 times greater than growth hormone output from cells in RPMI w/FBS. Similarly, the hGH output of BetaGene Medium w/SF was more than 5 times that of RPMI w/SF. While BetaGene Medium supplemented with SF sustained hGH output equal to that of RPMI w/FBS, it was not sufficient to support the same secretory function as BetaGene Medium with FBS.

FIG. 38. BetaGene Medium maintains secretory function of BG 18/E1 cell line. The insulin secretory function of BG18/3E1 cells was maintained when cells were cultured in BetaGene Medium supplemented with 5%, 2%, or 1% FBS. There was an impairment of secretory function with cells supplemented with 0.5% FBS or SF during the plateau phase of growth (about day 8 – 9 of culture). The secretory impairment at plateau phase under these conditions may be due to decreased biosynthesis or processing of insulin rather than an impairment of secretion.

FIG. 39. Growth in BetaGene Medium maintains regulated secretion from the BG 18/E1 cell line. BG18/3E1 cells were grown and maintained at plateau phase for 4 days in
BetaGene supplemented with minerals, minerals and amino acids, amino acids, or 2% FBS. The ability to respond to a secretagogue cocktail is shown for various SF- and 2% FBS-supplemented cultures in BetaGene Medium. This demonstrates that the capability of the regulated secretory pathway has been maintained, only the absolute output has been affected in both unstimulated and stimulated states, while the fold response is maintained.

**FIG. 40. BetaGene Medium enhances production of GLP-1 from an engineered, rodent neuroendocrine cell line.** The capability of BetaGene medium to sustain processing and secretion of a peptide that yields proteolytically cleaved and amidated products was evaluated by measuring GLP-1 (amidated and non-amidated) production. Cells, BG191/26, were plated in T25 flasks with BetaGene Medium and then the medium was switched to RPMI, RPMI ("0") with 75 µM ascorbate ("75"), or BetaGene Medium, all with 2% FBS. Both the total GLP-1 and the amidated GLP-1 output/day of cells in BetaGene Medium was essentially double that of cells in RPMI.

**FIG. 41. Ascorbate-2-phosphate supplemented media enhances insulin production of an engineered human neuroendocrine cell line.** A suspension culture of BG498/45 cells (PD33) were plated in varying concentrations of ascorbate or A-2-P. Samples were collected for insulin assay and medium changed after 2 and 5 days of culture. In the initial 2 days of culture ascorbate altered insulin output by reducing insulin about 20%, only at the highest concentration. In the final 3 days cells, high concentrations of ascorbate were cytotoxic, while 400 µM concentrations of both ascorbate and A-2-P enhanced insulin secretion. The highest concentration of A-2-P did not inhibit insulin output.

**FIG. 42. Media supplementation with ascorbate-2-phosphate can effect increased amidation activity with cultured cells.** Production of amidated and nonamidated GLP1 was determined by immunoassay of secreted cell products from cells cultured 1 day in RPMI medium (with 2% FBS) supplemented with varying concentrations of A-2-P. The dose-response shows half-max. and maximal amidation activity with 1 and 10-100 µM of A-2-P. The amount of amidated GLP-1 plateaued from 25-1000 µM. Concentrations of 10 mM consistently (4 separate experiments) resulted in slight decreases in amidated GLP-1, with a
similar tendency to reduce non-amidated GLP-1 output. Supplementation with A-2-P results in a decrease in non-amidated GLP-1, such that amidated/ non-amidated exceeds 100%. Maximal output of amidated GLP-1 with this cell line is \( \Delta 12 \) pmol/million cells-day, representing 5 fold increase over 0 \( \mu \)M A-2-P. This result demonstrates that supplementation with A-2-P can effect increased amidation activity with cultured cells.

**FIG. 43. Optimal Copper Concentration for PAM Activity.** BG191/26 cell monolayers in T25 flasks were changed to RPMI medium ± copper, or BG Medium ± additional copper (the latter medium contains 5 nM copper). Medium samples were collected after 24 h and the GLP-1 species were separated and quantified by HPLC. The results show that supplementing RPMI (which has no copper in its formulation) increases the output of amidated GLP-1. Further supplementation of BG medium with copper to 250 and 500 nM does not increase amidated GLP-1, whereas 1 \( \mu \)M copper tends to decrease amidated GLP-1. These results indicate that 5 nM copper is adequate for PAM activity in cultured neuroendocrine cells.

**FIG. 44. Lack of Cytotoxic Effect of ascorbate-2-phosphate on Primary Human Islets.** Human islets encapsulated in alginate beads were set up in 24 well plates with \( \Delta 50 \) islet equivalents/well and cultured in BetaGene Medium with or without added A-2-P and copper. Secretory function and glucose-sensing was determined by incubating the islets with different concentrations of glucose for 90 minutes (from 2.2 to 22 mM). This glucose dose-response test was performed immediately before adding ascorbate to the cultures and at 2 week intervals. In the first 2 weeks 500 \( \mu \)M A-2-P, and 1 \( \mu \)M copper was supplemented. In the second 2 weeks ascorbate was increased to 2 mM, copper was kept at 1 \( \mu \)M. A-2-P did not impair function as indicated by sensing of glucose, and the maintenance of maximal insulin secretion indicates that there is minimal toxicity of A-2-P for these culture times.

**FIG. 45.** Two sequential rounds of bulk culture growth, bulk cryopreservation, and thaws do not alter secretory function. \( \beta \)G18/3E1 cells which had been grown in bulk cultures were tested for maintained secretory performance. This was determined by assaying insulin secretory response (y-axis) to a secretagogue cocktail ("Swiss") after (x-axis): one bulk culture production and one freeze/thaw (C1 F/T); at harvest after one bulk culture production (C1
PostBulk); at seeding of second bulk culture after one bulk and one freeze/thaw (C1 Seed C2); harvest from second bulk culture (Post C2); and after second bulk culture and second bulk freeze and thaw (C2 F/T). Insulin secretory response from these cells was unaltered by bulk culture and freezing; neither unstimulated (Basal) nor secretagogue-induced ("Stim") secretion was altered.

FIG. 46. Insulin output of cells in defined Betagene Medium. Comparison of insulin output from encapsulated βG18/3E1 cells in defined BetaGene Medium (βGM) with output of cells in BetaGene Medium supplemented with FBS (βBM+) or select media with and without FBS. Aginate-encapsulated βG18/3E1 cells were cultured in 24 well plates in BetaGene Medium without (βGM) or with FBS (βGM+); in MEM without (MEM) or with FBS (MEM+); in a mixture of F12 and MEM without (F12/MEM) or with (F12/MEM+) FBS. Media samples were collected at intervals and assayed for insulin, and growth was determined by assay of viable cell mass terminally. Cell growth of cultures: MEM+ was 50±5% of βGM+ and F12/MEM+, which were equivalent; MEM was <10%, F12/MEM was 50±5%, and βGM was 80±8%. Insulin output of βGM+ was the best, with F12/MEM+ and βGM essentially equivalent.

FIG. 47. Switching cells to Defined BetaGene Medium increases insulin output. A portion of unsupplemented cultures of βG18/3E1 cells of figure 46 were continued an additional 3 days. Half of the cells cultured in F12/MEM (no FBS) were switched to defined βGM for the final 3 days. Switching to defined BetaGene Medium more than doubled insulin output, indicating that BetaGene Medium can compensate for insufficiencies of other defined media.

DESCRIPTION OF ILLUSTRATIVE EMBODIMENTS

Many cells are designed to produce and secrete biologically active molecules in vivo that take effect at locations distal to the secretory cell. Neuroendocrine cells, by definition, have sorting mechanisms, whereby a given polypeptide, protein or hormone destined for secretion, is targeted to the regulated secretory pathway or the default constitutive secretory pathway. Loss or impaired function of neuroendocrine cells is associated with a variety of
human diseases and disorders. For example, the failure of substantia nigra cells to properly produce dopamine results in Parkinson's Disease. The failure of thyroid cells to properly produce thyroid hormones results in athyrotic cretinism, and the loss of adrenal gland cells, with the consequent failure to produce adrenal hormones results in Adison's Disease. Other disorders such as short stature, Paget's Disease, infertility and endometriosis are generally treated by recombinant growth hormone, calcitonin, gonadotropins, and gonadotropin-releasing hormone, respectively. Perhaps one of the most widely known secretory disorder is diabetes, in which the neuroendocrine cells of the Islets of Langherans undergo a loss of function.

In all the cases of secretory cell function disorder, there are significant drawbacks in the currently available treatment regimens, which generally rely on injections and suffer from the additional drawbacks of proper dosing, loss of drug efficacy, drug side effects, and patient compliance. There is a need for both new and effective pharmaceutical agents for the prevention and treatment of neuroendocrine based disease and in the effectiveness with which such agents prevent or retard the onset of complications. The present invention is directed toward addressing these needs.

Secretory cells generally, and in particular neuroendocrine cells, have several endogenous functions that make them uniquely suited for production of a wide range of proteins, including secreted peptide hormones. These specialized functions include the regulated secretory pathway. The regulated secretory pathway embodies the secretory granules of neuroendocrine cells which serve as the site of maturation and storage of a large class of peptide hormones with profound biological functions. Proper biological function of the peptides is due both to their secretion in a regulated and titratable manner and a complex set of post-translational modifications resulting in the final, biologically active product. As a result, these cells can be used as in vitro models of in vivo secretory cell function. Stable cell lines that reflect the in vivo production and secretion of proteins, could be employed in the identification of modulators of said protein secretion.

The present invention is designed to take advantage of the secretory machinery of certain cells for the purpose of screening for modulators of secretory function. A variety of
different modifications may be made to these cells to make them more suitable candidates for drug screening.

The development of therapeutic agents that target the pancreatic β-cell in patients with NIDDM has been hampered by an insufficiency of in vitro assays that are biologically relevant, stable, and allow for high throughput. From the perspective of fidelity to the target biology, the preferred material for in vitro screens for the purposes of identifying therapeutics is functional islets from human cadavers. However, such use of human islets is impractical due to extremely limited amounts of material, age-related difference in β-cell function, and complicated as well by the batch-to-batch variation that has been observed for islet preparations (Jansson et al., 1995; McClenaghan et al., 1996).

One material that could be potentially used in biological screens to identify candidate agents as therapies for NIDDM are rodent β-cell lines. Several such cell lines have been established, maintained for extended periods in vitro, and characterized with regard to the synthesis and regulated secretion of insulin. The cell lines include those established from the pancreatic islets of rat (Chick et al., 1977; Gazdar et al., 1980; McClenaghan et al., 1996) hamster (Ashcroft et al., 1986), and mouse (Miyazaki et al., 1990; Knaack et al., 1994). These cell lines and their clonal derivatives potentially provide an unlimited supply of material; however, they are deficient in other properties important for their use as screening tools for therapeutic agents for diabetes.

All existing β-cell lines are deficient in key functional aspects when compared to islet β-cells. Insulin content is decreased and proinsulin processing is often impaired (Poitout et al., 1996). In β-cell lines the threshold or magnitude of glucose-stimulated insulin secretion (GSIS) and response to agents that can potentiate GSIS are often not representative of primary β-cells (Newgard, Diabetes Rev. 1996). In addition, many β-cell lines express peptides that are not expressed by the primary β-cell such as somatostatin, pancreatic polypeptide, and glucagon (Madsen et al., 1986).
One of the most ubiquitous properties of established β-cell lines is phenotypic instability such that, with extended cultivation, the cells lose those traits that are singularly β-cell in nature. HIT cells, many βTC cell lines, and unengineered RIN cell lines have less insulin content than a normal β-cell (Radvanyi et al., 1993), and this level has been reported to drop if the cells are maintained in culture for multiple population doublings (Clark et al., 1990; Poitout et al., 1996). One study reports that HIT cells lose responsiveness to glucose, arginine, and various secretagogues with serial passages (Zhang et al., 1989). Many βTC cell lines display relatively normal GSIS at early passages, but with continuous propagation, these cells acquire aberrant insulin secretion that is characterized by a hypersensitivity to glucose (Efrat et al., 1988; Efrat et al., 1993; Poitout et al., 1996).

The present invention, however, provides stable cells lines with a phenotypic integrity that allows them to be used as screening tools for the identification of novel substances that can be employed in the modulation of secretory function that is manifest in a number of diseased states including diabetes. These cell lines may be derived from a previously characterized, immortal RIN cell line that has been further engineered with secretory properties. In an alternative embodiment, the present invention provides methods and compositions that will allow one of skill in the art to engineer secretory cells so that they are immortalized. Further, these cells are engineered to ensure that glucose sensing and responsiveness is maintained over a period of time, i.e., indefinitely. The components for such a system, and methods of making and using such cell lines are set forth in detail below.

A. The Use of Engineered β-Cell Lines for Drug Discovery in NIDDM

The onset and progression of NIDDM are marked by discreet stages of β-cell dysfunction and failure with defects in insulin secretion presenting throughout the disease (Porte 1991; Granner and O'Brien 1992; Polonsky 1995; Polonsky, Sturis et al., 1996). The early phases of NIDDM often are characterized by insulin resistance and a compensatory increase in the secretion of insulin. As hyperinsulinemia fails to completely overcome insulin resistance, mild fasting hyperglycemia and impaired glucose tolerance become detectable. With progression of the diabetic state, hypoinsulinemia presents. The first-phase of insulin secretion is short in duration and facilitates immediate glucose disposal. Its loss, typically early
in NIDDM, results in postprandial hyperglycemia. Eventually, the second phase of glucose-stimulated insulin secretion becomes impaired and results in overt NIDDM with fasting hyperglycemia. Other defects in insulin secretion that often are present in NIDDM are abnormal, pulsatile insulin secretion, an increase in the plasma of ratio of proinsulin to insulin, and impairments of various secretagogues to potentiate GSIS (Porte 1991; Granner and O’Brien 1992; Polonsky 1995; Polonsky, Sturis et al., 1996).

It is postulated that the ongoing decline of the β-cell in NIDDM is related to perturbations in glucose homeostasis and fatty acid metabolism, (Porte 1991) or as has more recently been termed "glucolipotoxicity" (DeFronzo,1997). Glucolipotoxicity refers to the detrimental effects that sustained elevations of plasma glucose and free fatty acids have on β-cell metabolism. The resulting disruption of β-cell fuel metabolism leads to faulty insulin secretion and contributes to ongoing β-cell failure. Optimal glucose-sensing and subsequent insulin secretion have been linked to two key β-cell proteins: the glucose transporter, type 2 (GLUT-2) and hexokinase, type IV, (glucokinase). The relatively low affinity of these proteins for glucose allows them to be rate-limiting in the transport and metabolism of glucose and provides the glucose-sensing required for physiologically relevant insulin secretion (Newgard and McGarry 1995; Matschinsky 1996). Cytosolic long chain fatty acyl-CoA esters and free fatty acids are potentiators of GSIS (DeFronzo,1997). Partitioning of fatty acyl-CoA molecules between the cytoplasm and mitochondria is regulated by glucose. Basal glucose levels stimulate transport into the mitochondria and β-oxidation, and elevations of intracellular glucose promote increases in cytoplasmic concentrations of long-chain fatty acyl CoA esters and potentiation of GSIS. A key point for regulating the partitioning of fatty acids is carnitine palmitoyl transferase 1 (CPT-1), the protein that transports fatty acyl-CoA molecules into the mitochondria from the cytoplasm. CPT-1 is inhibited by malonyl CoA, a metabolite that increases with increases in glucose metabolism (DeFronzo, 1997). Thus, the metabolism of glucose in the pancreatic β-cell is tightly coupled to the metabolism of fatty acids, both in normal physiology and in pathogenic states such as diabetes. Whereas short-term elevations in glucose and fatty acids are part of the normal signaling required for fuel homeostasis, chronic exposures are detrimental to β-cell physiology and could contribute to an inhibition of insulin secretion.
Fatty acid metabolism in the β-cell also seems to be regulated by the effects of leptin (Shimabukuro, Koyama et al., 1997; Zhou, Shimabukuro et al., 1997). Leptin is a peptide hormone synthesized in and secreted from adipocytes. It has dramatic effects on body composition by regulating food intake and thermogenesis. Rodent strains with defects either in leptin production or leptin receptors are obese and have high incidence of NIDDM. Increases in plasma leptin levels have been shown to reduce body fat in several obese and non-obese rodent models. Leptin receptors are expressed in tissues throughout the body and have been shown to be present in pancreatic islets. Leptin has been shown to induce enzymes of fatty acid oxidation and deplete triglyceride pools in pancreatic islets (Shimabukuro, Koyama et al., 1997; Zhou, Shimabukuro et al., 1997). It is postulated that leptin functions in normal physiology to protect the β-cell from lipotoxicity and helps to prevent adipogenic diabetes (Shimabukuro, Koyama et al., 1997; Shimabukuro, Koyama et al., 1997; Zhou, Shimabukuro et al., 1997). It is currently unknown if defects in leptin-regulated fatty acid metabolism are causally linked to progressive β-cell failure that is characteristic of NIDDM in humans.

Clearly, β-cell failure characteristic of NIDDM is a complex and multifaceted process. Currently, the only pharmaceutical agents that target the β-cell in NIDDM are compounds that bind the SUR/KIR channels. Such compounds include the widely used sulfonylurea agents and an unrelated compound, Prandin™. These drugs treat the symptom of hyperglycemia by interacting with K⁺-ATP channels to stimulate insulin secretion. However, long-term use of the sulfonylurea drugs often results in a loss of drug efficacy. There are no therapeutic agents available for the following defects that are often seen in diabetes: defects in fatty acid metabolism, oxidative damage to β-cells, loss of potency of glucose and other secretagogues in insulin secretion, loss of receptor-mediated insulin secretion other than SUR/KIR or internal signaling machinery, and progressive β-cell failure.

FIG. 2 illustrates the use of human primary islets or β-cells and engineered cell lines in drug screening and drug discovery programs that target β-cell dysfunction in NIDDM. As shown, human islets can provide a starting point for the discovery of novel targets and validation of candidate targets and candidate compounds. In addition, the use of human islets
throughout a NIDDM drug discovery path could provide a "check" in the identification of hits, optimization of leads, and selection of candidate compounds. In the case of the discovery of novel targets, human islets can be used as a starting point for the synthesis of cDNA libraries so that discreet mRNAs encoding target proteins may be cloned. In a preferred embodiment, the β-cells of the human islet may be enriched to serve as a screening tool or starting material for cDNA synthesis. Enrichment could be achieved through cell sorting or through selective survival of the β-cells of the islet. In the latter case, human islets could be infected with recombinant adenovirus or transfected with DNA plasmids in which expression of neomycin phosphotransferase (NPT) or another enzyme encoding antibiotic resistance is driven by an insulin promoter. Such tissue-specific expression of NPT would provide for the selective survival of the β-cells following exposure of the mixed cell population to G418.

Despite the power and advantages of human islets in NIDDM-drug discovery programs, they are encumbered with multiple short-comings, the most important of which is a severe limitation in supply. The use of engineered cell lines circumvents this potential constriction in NIDDM-drug discovery programs. As shown, engineered cell lines with stable and predictable β-cell phenotypes can be created and used in the screening and enrichment of compounds that alter β-cell secretory function. In a preferred embodiment, following the validation of a target in the β-cell, such a target could be cloned from a human islet or an enriched β-cell population, and subsequently over-expressed in a β-cell line to create a biological model that is suitable for high throughput screening assays. The use of the engineered cell line could extend beyond in vitro screens to use in the creation of a "humanized" animal model. Because engineered RIN cells grow well in rodent models, these cell lines offer the capacity of in vivo testing of candidate compounds for safety and efficacy. In a preferred embodiment, the endogenous β-cells of a rodent would be selectively ablated through the administration of streptozotocin. Encapsulated, engineered cell lines that express the appropriate human target could then be implanted and the in vivo biological effects of candidate compounds on the secretory function of transplant could be assessed.
B. Desired Properties of an Islet β−cell

Thus, in a particular embodiment, the present invention provides a secretory cell that may be employed in the identification of modulators of secretory function. Such compounds will be especially useful in a variety of diseased states in which secretory function has been impaired. Such compounds also will be used against impaired β-cell function and metabolism. An example of such a state would be the loss of β−cell integrity that is manifest in diabetes. Thus, immortalized secretory cells that have a stable neuroendocrine phenotype will be used to identify compounds that will be useful in the regulating this secretory function. There are a number of properties that are desired of a cell line that will be representative of a human islet β−cell. First, it is important to ensure that engineered cell lines have a measurable activity that accurately reflects the regulation and capacity of the secretory pathway. One way to achieve this endpoint is to express a transgenic polypeptide at high levels that is processed and secreted through the regulated secretory pathway. Such a peptide, by definition, will be detectable in the media of the cultured cells, and its secretion will be dependent of the stimulatory or inhibitory signals that are received and processed by the cell.

The regulated pathway of the β−cell encompasses both acute regulation of insulin secretion (i.e. a large increment between the unstimulated and stimulated states) and the complete processing of proinsulin to the mature insulin polypeptide. In islet β−cells, secretory granules allow the storage of insulin as a depot at the plasma membrane that can be released within seconds of arrival of a fuel-derived or hormonal signal, and also serve as the site of conversion of proinsulin to insulin by virtue of their high concentrations of the relevant convertases PC1 (also known as PC3) and PC2. The presence of secretory granules and retention of proinsulin processing capacity represent a major advantage of insulinoma and other neuroendocrine cell lines relative to cells less specialized for secretion of peptide hormones such as hepatoma cells or fibroblasts.

A second, and certainly central parameter, is for the cell to be equipped with a capacity for modulator-sensing and responsiveness. The term "modulator" encompasses stimulators and inhibitors of secretory function. For example, glucose responsiveness has several components that must be considered, including the appropriate threshold for the response (islet β−cells
typically respond to glucose at concentrations in excess of the fasting level of 4-5 mM, rapid response dynamics (β-cells secrete insulin in response to glucose within minutes of its application and turn off insulin secretion nearly as rapidly when glucose is removed) and an appropriate magnitude of response.

Finally, it is imperative that engineered cell lines retain phenotypic and genotypic stability. This includes maintenance of both genes that are inserted or deleted during the course of engineering and key endogenous genes. As discussed below this is a significant limitation on current technology.

RIN 1046-38 cells are derived from a radiation-induced insulinoma but can be shown to be glucose responsive when studied at low passage numbers (Clark et al., 1990). This response is maximal at subphysiological glucose concentrations and is lost entirely when these cells are cultured for more than 40 passages (Clark et al., 1990). Although, RIN 1046-38 cells of low passage number exhibit GSIS, the maximal secretion is at glucose concentrations considerably lower than the threshold for response of normal β-cells (Knaack et al., 1994). These cells also express GLUT-2 and glucokinase, the high Kₘ glucose transporter and glucose phosphorylating enzymes that appear to control glucose flux and GSIS in β-cells (Newgard, 1996). With time in culture, however, RIN 1046-38 cells lose expression of GLUT-2 and glucokinase, become glucose unresponsive, and experience a decline in insulin content (Hughes et al., 1992; Knaack et al., 1994; Ferber et al., 1994). The creation of novel cell lines in which the genes for GLUT-2, glucokinase and human insulin are stably expressed in RIN 1046-38 cells by an "iterative engineering" strategy has been described (Clark et al., 1997). Important characteristics of engineered lines include insulin content that approaches that of normal human islet β-cells, efficient processing of the overexpressed human proinsulin to mature insulin, and stability of expression of the transgenes in vitro and in vivo.

C. Host Cells

A variety of host cells are contemplated for use in assays for identifying modulators of secretory function. For such screening purposes it will be desirable, as stated above, that the polypeptide be released from cells in response to the modulators of the present invention.
These cells may be established cell lines that are engineered to express secretory proteins. Alternatively, a human β–cell line that is immortalized and retains the characteristics of the primary β–cell would be a preferred material to use in assays. The attempts at immortalization of human pancreatic β–cells have resulted in cell lines that do not retain the defining properties of the primary β–cell, such as the capacity to synthesize insulin and secrete it from the regulated secretory pathway. These cells lines and engineering thereof are described in further detail herein below.

Engineering of secretory cells to synthesize secretory proteins for the purposes of the present invention will advantageously make use of many attributes of these cells. Regulated secretory cells present a natural bioreactor containing specialized enzymes involved in the processing and maturation of secreted proteins. These processing enzymes include endoproteases (Steiner et al., 1992) and carboxypeptidases (Fricker, 1988) for the cleavage of prohormones to hormones and PAM, an enzyme catalyzing the amidation of a number of peptide hormones (Eipper et al., 1992a). Similarly, maturation and folding of peptide hormones is performed in a controlled, stepwise manner with defined parameters including pH, calcium and redox states.

Complete processing requires sufficient levels of the processing enzymes as well as sufficient retention of the maturing peptides. In this way, physiological signals leading to the release of the contents of the secretory granules ensures release of fully processed, active proteins. This is important for both maximum production for in vitro purposes and for the possible use of cells for in vivo purposes.

All cells secrete proteins through a constitutive, non-regulated secretory pathway. A subset of cells are able to secrete proteins through a specialized regulated secretory pathway. Proteins destined for secretion by either mechanism are targeted to the endoplasmic reticulum and pass through the golgi apparatus. Constitutively secreted proteins pass directly from the golgi to the plasma membrane in vesicles, fusing and releasing the contents constitutively without the need for external stimuli. In cells with a regulated pathway, proteins leave the golgi and concentrate in storage vesicles or secretory granules. Release of the proteins from secretory
granules is regulated, requiring external stimuli. This external stimulus, defined as a
secretagogue, can vary depending on cell type, optimal concentration of secretagogue, and
dynamics of secretion. Proteins can be stored in secretory granules in their final processed
form. In this way a large intracellular pool of mature secretory product exists which can be
released quickly upon secretagogue stimulation.

A cell specialized for secreting proteins via a regulated pathway also can secrete
proteins via the constitutive secretory pathway. Many cell types secrete proteins by the
constitutive pathway with little or no secretion through a regulated pathway. As used herein,
"secretory cell" defines cells specialized for regulated secretion, and excludes cells that are not
specialized for regulated secretion. The regulated secretory pathway is found in secretory cell
types such as endocrine, exocrine, neuronal, some gastrointestinal tract cells and other cells of
the diffuse endocrine system.

The origin of the starting cells for use in the present invention thus includes human
tissues and tumors of neuroendocrine lineages that have a well defined regulated secretory
pathway. Cells with defined conditions for culturing ex vivo with some replicative capacity
also are preferred. Pancreatic β-cells, pancreatic α-cells and pituitary cells are preferred for use
in the present invention, with β-cells being more preferred. Examples of such cells are shown
in Table 1 (Pearse and Takor, 1979; Nylen and Becker, 1995).

The neuroendocrine cells of the invention preferably will secrete one or more of the
endogenous secretory polypeptides listed herein in Table 1. Stable β-cells that secrete insulin
will be preferred in certain aspects of the invention, with cells that secrete correctly processed
human insulin being more preferred. The stable β-cells of the invention also may be
advantageously used to secrete endogenous human amylin. The other preferred cell types of
the invention, pituitary cells, may be used advantageously to secrete endogenous human growth
hormone, ACTH or MSH.

In addition to pancreatic β cells, pancreatic α-cells, and pituitary cells, further cells
within Table 1 that are more preferred for use in the present invention include thyroid C cells,
which secrete endogenous human calcitonin; intestinal endocrine cells, which secrete endogenous human GLP-1 and GIP; and pancreatic α cells, which secrete endogenous human glucagon. Particularly preferred cells are shown in Table 2.

The term "regulated secretory pathway" means that the rate of secretion of an endogenous polypeptide can be stimulated by external stimuli, commonly referred to as secretagogues. Thus, as used herein a secretagogue is a substance that stimulates the secretion of a polypeptide. Secretagogues can be physiological in nature, e.g., glucose, amino acids, or hormones, or pharmacological, e.g., IBMX, forskolin, or sulfonylureas. Polypeptides destined for the regulated secretory pathway are stored in intracellular storage vesicles known as secretory granules.

Glucose is the most important stimulator of insulin secretion, not only because of its potent direct effects, but also because it is permissive for the stimulatory action of a wide array of other secretagogues. While there is good evidence to suggest that glucose exerts its effect through its own metabolism, resulting in the creation of signals that appear to work through modulation of ion channel activities and influx of extracellular Ca²⁺, the exact nature of the metabolic coupling factors remains unknown. The magnitude of the insulin secretory response appears to be related to the rate of β-cell glucose metabolism, and both parameters are sharply increased in response to modest increments in extracellular glucose concentrations within the physiological range of 4 to 8 mM. β-cells are equipped with the glucose transporter GLUT-2 and the glucose phosphorylating enzyme glucokinase which have kinetic properties, particularly a relatively low affinity for glucose, that are ideal for modulation of glucose responsiveness at the relatively high concentrations of the sugar encountered in the circulation (Newgard and McGarry 1995).

Although glucose is widely regarded as the predominant signal for insulin release in the postprandial phase, changes in the levels of other metabolic fuels, "incretin" hormones, and neurotransmitters provide important amplification and modulation of the glucose signal (Cryer. 1992). Nutritional signals also are derived from amino acids and lipids that each serve as potentiators of glucose-stimulated insulin secretion. Many secretagogues effect a response in the β-cell via interaction with specific receptors. Glucagon-like peptide 1 (GLP-1) is an
example of such a secretagogue. This peptide hormone binds its receptor and potentiates insulin secretion in the presence of glucose. Activin A has been reported to stimulate insulin secretion in rat pancreatic islets and HIT cell lines. This stimulation is receptor-mediated and Ca^{2+}-dependent (Shibata et al., 1996). Leptin receptors have been reported to be expressed in β-cells (Kieffer et al., 1996); however, reports of the effects of leptin on insulin secretion are varied (Emilsson et al., 1997; Tanizawa et al., 1997). Recent studies suggest that leptin directly affects the triglyceride pools in the β-cell (Shimabukuro et al., 1997; Zhou et al., 1997); thus with long-term exposure leptin indirectly affects insulin secretion by impacting the incretin effects that fatty acids have on GSIS.

Epinephrine and its analogues, such as Clonidine, act to inhibit insulin secretion via signaling through the alpha 2 adrenergic receptor. Likewise, somatostatin and pancreatic polypeptide VIP, PACAP, GIP, acetylcholine, cholecystokinin also act via specific receptors to inhibit insulin secretion (Lambert and Atkins 1989).

β cells also respond to a number of non-physiological compounds that act via endogenous receptors or signaling machinery (Clark et al., 1990). Carbachol stimulates insulin secretion via activation of muscarinic receptors. Inhibition of phosphodiesterase via administration of IBMX allows for the accumulation of cyclic AMP, a potentiator of insulin secretion. Stimulation of kinase activity, such as the phorbol ester activation of protein kinase C, will stimulate insulin secretion if cells are exposed the kinase-activator for short periods of times. Diazoxide inhibits insulin secretion by interacting with and opening the potassium-ATP channel. Sulfonylurea drugs such as glibenclamide and tolbutamide maintain the K^+·ATP channel in a closed state, allowing for membrane depolarization, increased calcium influx, and increased insulin secretion (Bressler and Johnson, 1997). Other kinds of K^+ channels, such as large-conductance Ca^{2+}-dependent K^+ (BK) channels and late rectifying voltage dependant channels, have been reported to be expressed in pancreatic β-cells and participate in regulating membrane polarity and secretion (Dukes and Philipson, 1996; Kalman et al., 1998). Compounds that open or block these channels are currently under development and may be useful pharmacological agents (Olesen et al., 1994; Strobaek et al., 1996).
PRANDIN™ (repaglinide) is an oral blood glucose-lowering drug of the meglitinide class used in the management of type 2 diabetes mellitus (also known as non-insulin dependent diabetes mellitus or NIDDM). Repaglinide, S(+)-2-ethoxy-4(2((3-methyl-1-(2-(1-piperidinyl)phenyl)-butyl) amino)-2-oxoethyl) benzoic acid, is chemically unrelated to the oral sulfonylurea insulin secretagogues. Repaglinide lowers blood glucose levels by stimulating the release of insulin from the pancreas. This action is dependent upon functioning β-cells in the pancreatic islets. Insulin release is glucose-dependent and diminishes at low glucose concentrations.

Repaglinide acts by closing ATP-dependent potassium channels in the β-cell membrane by binding at characterizable sites. This potassium channel blockade depolarizes the β-cell, which leads to an opening of calcium channels. The resulting increased calcium influx induces insulin secretion. The ion channel mechanism is highly tissue selective with low affinity for heart and skeletal muscle. In patients with type 1 diabetes, administration of Prandin™ improves glycemic control, as reflected by HbA₁C and fasting glucose levels. This is associated with a reduction in the diabetic complications retinopathy, neuropathy, and nephropathy. Although controlling the blood glucose in type 2 diabetes has not been established to be effective in preventing the long-term cardiovascular and neural complications of diabetes, improved glycemic control is an important goal in patients with non-insulin-dependent disease because it is presumed that the mechanisms by which glucose causes complications is the same in both forms of diabetes.

1. Glucose Responsive Cells

For some peptide hormones or factors, it may be desirable to cause the polypeptide to be released from cells in response to changes in the circulating glucose concentration. The most obvious example of a secretory cell type that is regulated in this fashion is the β-cell of the pancreatic islets of Langerhans, which releases insulin and amylin in response to changes in the blood glucose concentration. Engineering of primary β-cells for production of products other than insulin is not practical. Instead, a preferred vehicle may be one of the several cell lines derived from islet β-cells that have emerged over the past two decades. While early lines were derived from radiation- or virus-induced tumors (Gazdar et al., 1980, Santerre et al., 1981), more recent work has centered on the application of transgenic technology (Efrat et al., 1988,
Miyazaki et al., 1990). A general approach taken with the latter technique is to express an oncogene, most often SV40 T-antigen, under control of the insulin promoter in transgenic animals, thereby generating β-cell tumors that can be used for propagating insulinoma cell lines (Efrat et al., 1988. Miyazaki et al., 1990). While insulinoma lines provide an advantage in that they can be grown in essentially unlimited quantity at relatively low cost, most exhibit differences in their glucose-stimulated insulin secretory response relative to normal islets. These differences can be quite profound, such as in the case of RINm5F cells, which were derived from a radiation-induced insulinoma and which in their current form are completely lacking in any acute glucose-induced insulin secretion response (Halban et al., 1983). RIN 1046-38 cells are also derived from a radiation-induced insulinoma but can be shown to be glucose responsive when studied at low passage numbers (Clark et al., 1990). This response is maximal at subphysiological glucose concentrations and is lost entirely when these cells are cultured for more than 40 passages (Clark et al., 1990). GLUT-2 and glucokinase are expressed in low passage RIN 1046-38 cells but are gradually diminished with time in culture in synchrony with the loss of glucose-stimulated insulin release (Ferber et al., 1994). Restoration of GLUT-2 and glucokinase expression in RIN 1046-38 cells by stable transfection restores glucose-stimulated insulin secretion (Ferber et al., 1994), and the use of these genes as a general tool for engineering of glucose sensing has been described in a previously issued patent (Newgard, U.S. Patent 5,427,940). RIN 1046-38 cells transfected with the GLUT-2 gene alone are maximally glucose responsive at low concentrations of the sugar (approximately 50 μM), but the threshold for response can be shifted by preincubating the cells with 2-deoxyglucose, which when converted to 2-deoxyglucose-6-phosphate inside the cell serves as an inhibitor of low K_m hexokinase, but not glucokinase activity (Ferber et al., 1994).

Recently, Asafari et al. reported on the isolation of a new insulinoma cell line called INS-1 that retains many of the characteristics of the differentiated β-cell, most notably a relatively high insulin content and a glucose-stimulated insulin secretion response that occurs over the physiological range (Asafari et al., 1992). This line was isolated by propagating cells freshly dispersed from an X-ray induced insulinoma tumor in media containing 2-mercaptoethanol. Consistent with the finding of physiological glucose responsiveness, a recent report indicates that INS-1 cells express GLUT-2 and glucokinase as their predominant glucose
transporter and glucose phosphorylating enzyme, respectively (Marie et al., 1993). INS-1 cells grow very slowly and require 2-mercaptoethanol. It remains to be determined whether glucose responsiveness and expression of GLUT-2 and glucokinase are retained with prolonged culturing of these cells, or in vivo.

Cell lines derived by transgenic expression of T-antigen in β-cells (generally termed β TC cells) also exhibit variable phenotypes (Efrat et al., 1988, Miyazaki et al., 1990, Whitesell et al., 1991 and Efrat et al., 1993). Some lines have little glucose-stimulated insulin release or exhibit maximal responses at subphysiological glucose concentrations (Efrat et al., 1988, Miyazaki et al., 1990, Whitesell et al., 1991), while others respond to glucose concentrations over the physiological range (Miyazaki et al., 1990 and Efrat et al., 1993). It appears that the near-normal responsiveness of the latter cell lines is not permanent, since further time in culture results in a shift in glucose dose response such that the cells secrete insulin at subphysiological glucose concentrations (Efrat et al., 1993). In some cases, these changes have been correlated with changes in the expression of glucose transporters and glucose-phosphorylating enzymes. Miyazaki et al., isolated two classes of clones from transgenic animals expressing an insulin promoter/T-antigen construct. Glucose-unresponsive lines such as MIN-7 were found to express GLUT-1 rather than GLUT-2 as their major glucose transporter isoform, while MIN-6 cells were found to express GLUT-2 and to exhibit normal glucose-stimulated insulin secretion (Miyazaki et al., 1990). More recently, Efrat and coworkers demonstrated that their cell line βTC-6, which exhibits a glucose-stimulated insulin secretion response that resembles that of the islet in magnitude and concentration dependence, expressed GLUT-2 and contained a glucokinase:hexokinase activity ratio similar to that of the normal islet (Efrat et al., 1993). With time in culture, glucose-stimulated insulin release became maximal at low, subphysiological glucose concentrations. GLUT-2 expression did not change with time in culture, and glucokinase activity actually increased slightly, but the major change was a large (approximately 6-fold) increase in hexokinase expression (Efrat et al., 1993). Furthermore, overexpression of hexokinase I, but not GLUT-1, in well-differentiated MIN-6 cells results in both increased glucose metabolism and insulin release at subphysiological glucose concentrations. Similar results have been obtained upon overexpression of hexokinase I in normal rat islets (Becker et al., 1994b). These results are all consistent with the observations of
Ferber et al., described above in showing that a high hexokinase:glucokinase ratio will cause insulin-secreting cells to respond to glucose concentrations less than those required to stimulate the normal β-cell.

2. Non-glucose Responsive Cells

An alternative host to insulinoma cell lines are non-islet cell lines of neuroendocrine origin that are engineered for insulin expression. The foremost example of this is the AtT-20 cell, which is derived from ACTH secreting cells of the anterior pituitary. A decade ago, Moore et al. demonstrated that stable transfection of AtT-20 cells with a construct in which a viral promoter is used to direct expression of the human proinsulin cDNA resulted in cell lines that secreted the correctly processed and mature insulin polypeptide (Moore et al., 1983). Insulin secretion from such lines (generally termed AtT-20ins) can be stimulated by agents such as forskolin or dibutyryl cAMP, with the major secreted product in the form of mature insulin. This suggests that these cells contain a regulated secretory pathway that is similar to that operative in the islet β-cell (Moore et al., 1983; Gross et al., 1989). More recently, it has become clear that the endopeptidases that process proinsulin to insulin in the islet β-cell, termed PC2 and PC3, are also expressed in AtT-20ins cells (Smeekens et al., 1990, Hakes et al., 1991). AtT-20ins cells do not respond to glucose as a secretagogue (Hughes et al., 1991). Interestingly, AtT-20 cells express the glucokinase gene (Hughes et al., 1991, Liang et al., 1991) and at least in some lines, low levels of glucokinase activity (Hughes et al., 1991 and 1992, Quaade et al., 1991), but are completely lacking in GLUT-2 expression (Hughes et al., 1991 and 1992). Stable transfection of these cells with GLUT-2, but not the related transporter GLUT-1, confers glucose-stimulated insulin secretion, albeit with maximal responsiveness at subphysiological glucose levels, probably because of a non-optimal hexokinase:glucokinase ratio (Hughes et al., 1992, 1993).

The studies with AtT-20ins cells are important because they demonstrate that neuroendocrine cell lines that normally lack glucose-stimulated peptide release may be engineered for this function. Other cell lines that are characterized as neuroendocrine, but lacking in endogenous glucose response include PC12, a neuronal cell line (ATCC CRL 1721) and GH3, an anterior pituitary cell line that secretes growth hormone (ATCC CCL82.1). It is
not possible to determine whether such cell lines will gain glucose responsiveness by engineering similar to that described for the AtT-20ins cell system without performing the experiments. However, these lines do exhibit other properties important for this invention such as a regulated secretory pathway, expression of endopeptidases required for processing of prohormones to their mature hormone products, and post-translational modification enzymes. Some or all neuroendocrine lines also will be useful for glucose-regulated product delivery, using the methods described in U.S. Patent 5,427,940 to generate such responsiveness.

3. Stable Human Secretory Cells

In particular embodiments, the present invention uses stable human secretory cells by transforming a non-stable secretory cell such that it is immortalized and retains its phenotype through numerous cell culture passages. The final attributes of such cell lines of the present invention are functionally defined as having maintained a regulated secretory pathway, being stable to \textit{in vitro} culture and, preferably, as being amenable to further engineering. The present section describes the production of these cells for use in the screening assays of the present invention.

The human secretory or neuroendocrine cell will be "culturable," \textit{i.e.}, it will be capable of growing \textit{in vitro} and producing the desired endogenous secretory polypeptide with a demonstrated regulated secretory pathway. A "stable, transformed" human regulated secretory cell in the context of the present invention will be a cell that exhibits \textit{in vitro} growth for at least twenty population doublings. The resultant human regulated secretory cell also will maintain the required differentiated phenotype after transformation, \textit{i.e.}, it will exhibit the phenotypic properties of a demonstrable regulated secretory pathway, secretory storage granules, the capacity for peptide processing, and will produce the selected endogenous secretory polypeptide.

In particular embodiments, the stable human secretory cell is a $\beta$-cell. The human $\beta$-cells of the present invention will exhibit the capacity to grow \textit{in vitro}, with a minimum of at least about 20 population doublings, or preferably, of about 30, about 40, about 50, about 60, about 70, or about 80 population doublings. Even more preferably, the resultant human $\beta$-cells
of the invention will have even further increments of population doublings up to and including a completely transformed state wherein the cells grow in perpetuity.

The human β-cells of the present invention also will exhibit the capacity to produce biologically active human insulin. The insulin produced may be comprised entirely of mature insulin; or entirely of the biological precursor of mature insulin, termed proinsulin; or of all possible mixtures of proinsulin, insulin, and processing intermediates that are produced in the course of conversion of proinsulin to insulin. While the preferred embodiment of the present invention are cells that produce primarily or exclusively mature insulin, cells that produce proinsulin will also be useful in various embodiments. Such cells are useful per se, particularly as any form of insulin can be obtained in vitro, purified and converted to mature insulin. Furthermore, insulin is an exemplary secretory protein, the stable human neuroendocrine cell line may be engineered to express a variety of secretory proteins for the purposes of identifying specific modulators of secretory function.

Cells that produce primarily or exclusively immature insulin also are useful in that the capacity to produce mature insulin can be re-engineered into the cells themselves, in which instances the stable cells can then be used in vivo. By way of example only, proteases known as PC2 and PC3 that are responsible for the conversion of proinsulin to insulin can be introduced into the stable human β cells by genetic engineering methods, thereby enhancing the efficiency of conversion of proinsulin to insulin.

The stable human β cells of the present invention generally will exhibit a minimal insulin content of about 5 ng/million cells, but may contain as much as, or even more insulin than, normal isolated human islets, which have approximately 1-10 µg/million cells. It will be understood that the cells of the present invention may contain any amount of insulin within the above-specified ranges, such as about 10 ng insulin/million cells, about 50 ng, about 100 ng, about 200 ng, about 500 ng, about 1000 ng (1 µg), about 2 µg, about 5 µg, about 10 µg, about 20 µg, about 50 µg, about 75 µg, up to and including about 100 µg insulin/million cells. It will be understood that any and all integers within these ranges will define an insulin content that may be present within the stable human β cells of the invention.
In further preferred embodiments, the human β cells of the present invention may be defined as cells having an insulin content of between about 10%, about 20%, about 30%, about 40%, about 50%, about 60%, about 70%, about 80%, about 90%, up to and including about 100% of normal human islet content, which is about 1-10 μg/million cells.

The human β cells of the present invention will preferably exhibit enhanced insulin secretion when exposed to one or more secretagogues selected from IBMX, carbachol, amino acids, and glucose, or when exposed to a secretory "cocktail" of such compounds. The human β cells will more preferably exhibit enhanced insulin secretion when exposed to glucose, and will most preferably exhibit enhanced insulin secretion when exposed to 10 mM glucose.

The increase in insulin secretion in response to a non-glucose secretagogue or cocktail thereof should be at least about 1.1 times or about 1.5 times that observed in cells incubated in the absence of the secretagogue or secretory cocktail. However, in preferred embodiments, the increase in insulin secretion in response to a non-glucose secretagogue or cocktail thereof should be at least about double that observed in cells incubated in the absence of the secretagogue or secretory cocktail. In more preferred embodiments, a higher increase will be observed, up to and including a 3-fold, 5-fold, 10-fold, 20-fold, 50-fold, 100-fold, 200-fold, 300-fold, 500-fold, 750-fold or even about a 1000-fold enhancement.

The human β-cells of the present invention preferably will exhibit a glucose-stimulated insulin secretion (GSIS) response. This increase in secretion should be at least about 1.1 times or about 1.5 times that observed in cells incubated in the absence of glucose. More preferred are increases in secretion of at least about double that observed in cells incubated in the absence of glucose, with even more preferred increases being higher, up to and including a 3-fold, 5-fold, 10-fold, 20-fold, 50-fold, 100-fold, 200-fold, 300-fold, 500-fold, 750-fold or even about a 1000-fold enhancement. including all increments therebetween.

In preferred embodiments, glucose responsive insulin secretion will be observed in the range of 1.0 to 20 mM glucose. GSIS response will occur more preferably with a threshold for
response of 3-5 mM glucose, with maximal secretion stimulated by 10-20 mM glucose, as occurs in normal human islets. Cell lines with glucose dose responses occurring over a higher or lower range also will have significant utility, given that any regulated insulin production will be useful.

Many secretory cells have a GSIS response not originally in the range observed for normal human islets still will be useful as such cells will be amenable to genetic engineering methods, as embodied in U.S. Patent 5,427,940, and as further disclosed herein, in order to alter the glucose dose response. These methods are contemplated for use in applications with stable human cells to achieve the desired glucose concentration dependence. Furthermore, as stated, human β cells that are completely lacking glucose responsiveness also are included within the invention, since the known genetic engineering methods (U.S. Patent 5,427,940) can be used to confer glucose sensing in neuroendocrine cell lines previously lacking a glucose response.

a. Starting Cells

Primary human neuroendocrine secretory cells are immortalized as described in further detail elsewhere in the specification. The present section is directed to describing the starting cells that may be further engineered for the drug screening purposes of the present invention.

Fetal Cells. Human fetal panaces at 18 to 24 gestational weeks can be obtained through nonprofit organ procurement centers, with patient consent for tissue donation being obtained. Dissection of specific organs from the fetuses is often done at the procurement centers. Isolation of fetal pancreases and islets is performed by established techniques (Otonkoski et al., 1993; incorporated herein by reference).

Cells from Primary Human Tissues. Human organs can be obtained from autopsies through nonprofit organ procurement centers. High quality human islets are available, for example, from Dr. Camillo Ricordi of the University of Miami Medical Center, an islet transplant surgeon who supplies human islets to scientists throughout the United States. Automated methods for isolation of human pancreatic islets have been established (Ricordi et al., 1988; incorporated herein by reference).
**Cells from Resected Neuroendocrine Tumors.** Explanted tumor samples from surgically resected tumors are another preferred starting material. More preferred are insulinomas and pituitary tumors. Two exemplary insulinomas have been reported (Gueli et al., 1987; Cavallo et al., 1992). Although none of the described human "insulinomas" actually have the properties required to be properly described as stable human β cells, the techniques of the present invention are still suitable for use with such cell populations as starting materials in order to procure a pure population of stable, human insulin-producing cells from the mixture of cells currently available.

**Human Neuroendocrine Cell Lines.** It will be understood that tumor cell lines and insulinomas arising from explants of resected neuroendocrine tumors are not necessarily, by definition, stable cells; some such cells maintain a differentiated phenotype for two, four or about six months at the maximum. However, such cells are intended for use as starting materials in the present invention.

**Other Neuroendocrine Cell Types.** Table 1 shown below (Pearse and Takor, 1979; Nylen and Becker, 1995), while not a complete list, is exemplary of the types of cells contemplated for use in the present invention. β cells, α-cells and pituitary cells are preferred for use in the present invention, with β cells being more preferred. Additional cell types useful in the present invention will be readily known to those of skill in the art.
<table>
<thead>
<tr>
<th>Neuroendocrine cell</th>
<th>Endogenous hormone</th>
<th>Tissue specific promoter</th>
<th>Context specific promoter</th>
<th>Associated Tumors</th>
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<td>Hypothalamic/pituitary cells</td>
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<td></td>
<td></td>
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<tr>
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<td>POMC (V01510, K02406)</td>
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<td>Neuroendocrine cell</td>
<td>Endogenous hormone</td>
<td>Tissue specific promoter</td>
<td>Context specific promoter</td>
<td>Associated Tumors</td>
</tr>
<tr>
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<td>Gastric D cells</td>
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<tr>
<td>Enteroneuroendocrine cell</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>types</td>
<td></td>
<td></td>
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<tr>
<td>1. cells</td>
<td>Glucagon Family peptides</td>
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<tr>
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<td>Peptide YY</td>
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<td>D cells</td>
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<td>Somatostatin (J00306)</td>
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<td>Secretin</td>
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<td>Associated Tumors</td>
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<tr>
<td>K cells</td>
<td>GIP</td>
<td>GIP (M31674)</td>
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<tr>
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<td>VIP</td>
<td></td>
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<td>Motilin (X15392, Y07505, M30277)</td>
<td>Substance P (M68906)</td>
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<tr>
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<td>Glucagon (X03991), Corticotropin releasing hormone (X55962)</td>
<td>Glucagon (X03991),</td>
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<td>PP cells</td>
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<tr>
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<td>Gastrinoma</td>
</tr>
<tr>
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<td>Tissue specific promoter</td>
<td>Context specific promoter</td>
<td>Associated Tumors</td>
</tr>
<tr>
<td>------------------------------------</td>
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<td>Substance P (M68906)</td>
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<td>VIP (M33027, M37460)</td>
<td>VIPomas</td>
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<td>Supraoptic and paraventricular nuclei</td>
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<td>Sympathetic ganglion</td>
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<tr>
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<td></td>
<td>VIP (M33027, M37460)</td>
<td>VIPomas</td>
</tr>
</tbody>
</table>
In addition to β cells, pituitary cells are preferred for use with this invention. In general, pituitary cells may allow for higher efficiency of transformation as culture conditions have been reported for promoting the proliferation of rodent pituitary cells in vitro (Nicol et al., 1990). The inventors contemplate establishing similar conditions for human pituitary cells which will allow for retroviral infection and provide a means for efficiently introducing transforming genes.

Cells from the intermediate lobe may have an advantage for use in cell-based therapies of IDDM as there is a suggestion that this cell type can survive and sustain secretory function in autoimmune disease. These cells would therefore be useful in providing an indication of the effects of the modulators in vivo, as these cells would be less prone to attack from the host. The POMC promoter was used to drive expression of insulin in the cells of the intermediate lobe of transgenic nonobese diabetic (NOD) mice. Such cells were resistant to autoimmune-dependent destruction even when implanted next to islets in which β cells were destroyed during the course of the disease (Lipes et al., 1996).
<table>
<thead>
<tr>
<th>βG ID</th>
<th>NCI/ATCC</th>
<th>Origin</th>
<th>NEprofile</th>
<th>SELEC AB&lt;sup&gt;SR&lt;/sup&gt;</th>
<th>TG&lt;sup&gt;7/c&lt;/sup&gt;</th>
<th>NuTum Grow&lt;sup&gt;v&lt;/sup&gt;</th>
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<th>GHT device</th>
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<td>-</td>
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<td>Origin</td>
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<td>SELEC (\text{AB}^{s/r})</td>
<td>TG(^{+/-})</td>
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<td>AlgBeads</td>
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<td>H460/HTB-177</td>
<td>Lung</td>
<td>PAM+, SYN-, PC1/PC3-, PC2-, VIM+, CPE-</td>
<td>G^R, H^R, O^R</td>
<td></td>
<td>NT</td>
<td>NT</td>
<td></td>
</tr>
<tr>
<td>H21</td>
<td>NA/CRL-2195</td>
<td>Lung</td>
<td>PAM+, SYN+, PC1/PC3+, PC2+, VIM-, CPE+</td>
<td>G^S, H^S, O^S</td>
<td>I-/+</td>
<td>+</td>
<td>+</td>
<td>G^+</td>
</tr>
<tr>
<td>H22</td>
<td>H2098/Gazdar</td>
<td>Insulinoma</td>
<td>PAM+, SYN+, PC1/PC3+, PC2+, VIM-, CPE+</td>
<td>G^S, H^S, O^S</td>
<td></td>
<td>NT</td>
<td>NT</td>
<td></td>
</tr>
<tr>
<td>βG ID</td>
<td>NCI/ATCC</td>
<td>Origin</td>
<td>NEprofile PEP(^{1/})</td>
<td>SELEC AB(^{S/R})</td>
<td>TG(^{1/})</td>
<td>Nutum Grow(^{+/−})</td>
<td>AlgBeads</td>
<td>GHT device</td>
</tr>
<tr>
<td>-------</td>
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<td>------------------------</td>
<td>---------------------</td>
<td>-------------</td>
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<td>----------</td>
<td>------------</td>
</tr>
<tr>
<td>H23</td>
<td>NA/NA (BON)</td>
<td>Pancreatic</td>
<td>PAM+, SYN+, PC1/PC3+, PC2- VIM+, CPE+</td>
<td>G(^{2}), H(^{1}), O(^{3})</td>
<td>NP(^{+}), I(^{1}), G(^{+})</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>H25</td>
<td>NA (HepG)/HB-8065</td>
<td>Hepatocytes</td>
<td>PAM+, SYN-, PC1/PC3-, PC2-, VIM-, CPE+/−</td>
<td>G(^{5}), H(^{NT}), O(^{NT})</td>
<td>N(^{+})</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>293</td>
<td>Kidney</td>
<td></td>
<td>PAM+, SYN-, PC1/PC3-, PC2-, VIM+, CPE-</td>
<td>G(^{8}), H(^{5}), O(^{5})</td>
<td>NP(^{+}), I(^{1}), G(^{+}), GH(^{+}), G2+</td>
<td>+</td>
<td>+</td>
<td>−</td>
</tr>
</tbody>
</table>

**KEY:** NCI, National Cancer Institute; ATCC, American Type Culture Collection; NE, neuroendocrine; PAM, peptidylglycine alpha-amidating monoxygenase; SYN, synaptophysin; PC, proconvertase; VIM, vimentin; AB, antibiotic; S/R, sensitive/resistant; G, G418; H, hygromycin; O, ouabain; P, puromycin; B, blastcidin; Hd, histidinol; Mca, mycophenolic acid; Z, Zeocin; TG, transgene expression +/−; NP, neomycin phosphotransferase; I, insulin; G, glucagon/glycentin; GH, growth hormone; NT, not tested.
Table 2 describes the properties of certain cell lines that are exemplary starting cells for use in the instant application. βG H03 cells are derived from a human non-small cell lung carcinoma (ATCC Number: CRL-5816). These cells have a neuroendocrine phenotype and can be grown in a monolayer. This line was derived by Gazdar and associates from a lung tissue obtained from a patient prior to therapy. H03 cells as obtained from the ATCC are not able to synthesize the peptide neuromedin B (NMB) or the gastrin releasing peptide (GRP).

Other lung carcinoma cells include cells designated herein as βG H04, βG H05, βG H07, βG H09, βG H19, βG H20 and βG H21. These, as well as additional cells lines derived from other sources, are described in further detail herein below. These cell lines are only exemplary starting cells for use in the present application, given the teachings provided herein, one of ordinary skill in the art will be able to identify additional cells with characteristics that would make them appealing as cells to be engineered for use in the present invention.

H01 cells (ATCC Number: CCL-251) also may be used in the present invention. These cells are human colorectal carcinoma cells having an epithelial morphology. These cells grow in floating aggregates of round cells. A characteristic that makes these cells useful in the context of the present invention is that they contain cytoplasmic dense core granules characteristic of endocrine secretion.

βG H02 cells are obtained from the ATCC (CRL-1803) are derived from a thyroid medullary carcinoma. Their morphology is epithelial and are known to produce high levels of calcitonin and carcinoembryonic antigen (CEA). Chromosomal analysis of the cell line and tumors induced in nude mice reveal an aneuploid human karyotype with several marker chromosomes.

βG H04 cells are obtained from the ATCC (CRL-5803) are lung carcinoma cells and have a neuroendocrine phenotype. The cells have a homozygous partial deletion of the p53 protein, and lack expression of p53 protein. The cells are able to synthesize the peptide NMB at 0.1 pmol/mg protein, but not the gastrin releasing peptide (GRP).
Another lung carcinoma cell that may prove a useful host cell in the context of the recent invention is designated βG H05 (ATCC Number: CRL-5808). This is a classic small cell lung cancer cell line with an epithelial morphology. This line was derived from cells recovered from pleural effusion taken from a patient after chemotherapy. NCI-H378 expresses elevated levels of the 4 biochemical markers of SCLC: neuron specific enolase, the brain isoenzyme of creatine kinase, L-dopa carboxylase and bombesin-like immunoreactivity. The cells express the c-kit gene as well as the L-myc gene, and L-myc is amplified. The cells express easily detectable levels of p53 mRNA compared to levels found in normal lung.

Also useful is βG H06, (ATCC Number: CRL-5815), having an epithelial morphology, these cells produce neuromedin B (NMB). This line was derived from tissue taken prior to therapy. This is the best differentiated of the available bronchial carcinoid lines. The cells express easily detectable levels of p53 mRNA compared to levels found in normal lung. The cells are able to synthesize the peptide NMB (at 0.1 pmol/mg protein), but not the gastrin releasing peptide (GRP). The cell line secretes a parathyroid hormone-like protein which is calcium stimulated through a protein kinase C pathway. Further, growth of NCI-H727 cells is inhibited by epidermal growth factor (EGF) receptor monoclonal antibodies.

Another classic small cell lung cancer cell line is βG H07 (ATCC Number: CRL-5804). This line was derived from cells recovered from pleural effusion obtained from a patient prior to therapy, and expresses elevated levels of the 4 biochemical markers of SCLC: neuron-specific enolase, the brain isoenzyme of creatine kinase, L-dopa carboxylase and bombesin-like immunoreactivity. Only trace amounts of the retinoblastoma susceptibility gene (RB) mRNA, were detected. RB protein was not detected. The cells express the c-kit gene as well as the N-myc gene. N-myc is not amplified. The cells are not able to synthesize the peptide neuromedin B (NMB) or the gastrin-releasing peptide (GRP). They express easily detectable levels of p53 mRNA compared to levels found in normal lung. These cells are useful for transfection studies.

βG H08 are carcinoma cells isolated from a stage 3A squamous cell lymph node carcinoma (ATCC Number: CRL-5867). βG H09 are derived from an atypical lung carcinoid and are available form the ATCC (CRL-5838). βG H10 cell line is a commercially available
cell line derive from lung carcinoma (ATCC Number CCL-185). Another similar cell line is ATCC number CCL-185.1 derived from CCL-185 which was initiated through explant culture of lung carcinomatous tissue. CCL-185.1 are adapted to growth in serum-free medium.

βG H11 cells may be obtained from ATCC (number HTB-9) and are derived from a bladder carcinoma. βG H13 (ATCC Number: CRL-2139) are from a primitive neuroectodermal brain tumor. The cells express CCK specific mRNA and synthesize considerable quantities of variably processed CCK prohormone.

ATCC Number: CCL-249 are designated herein as βG H14 and are derived from a colon adenocarcinoma. This is one of 14 colorectal carcinoma cell lines derived from a well differentiated sigmoid tumor from a patient prior to therapy. The line was initially grown in medium with fetal bovine serum, but was later adapted to growth in the chemically defined medium ACL-4. Floating aggregates produce tubuloglandular structures lined by columnar epithelia.

βG H15 are from a colorectal carcinoma (ATCC Number: CCL-253) and have an epithelial phenotype. This line was derived from a metastasis to the abdominal wall obtained from a patient after treatment with 5-fluorouracil.

βG H16 are the same as the commercially available cell line of ATCC Number: CRL-5974. These are gastric carcinoma cells that express the surface glycoproteins carcinoembryonic antigen (CEA) and TAG 72 and the muscarinic cholinergic and vasoactive intestinal peptide (VIP) receptors but lack gastrin receptors.

ATCC Number: HTB-10 are the cells referred to herein as βG H18, these cells are derived from a neuroblastoma cell line and is one of two cell lines (see also ATCC HTB-11) of neurogenic origin.
βG H19 or ATCC Number: HTB-184 are small cell lung carcinoma cells of an extrapulmonary origin and are from an adrenal metastasis in an adult. The cells produce easily detectable p53 mRNA at levels comparable to those in normal lung tissue.

βG H20 (ATCC Number: HTB-177) are a large cell carcinoma cell line derived from the pleural fluid of a patient with large cell cancer of the lung. The cells stain positively for keratin and vimentin but are negative for neurofilament triplet protein. The line expresses some properties of neuroendocrine cells, is relatively chemosensitive and can be cloned in soft agar (with or without serum).

βG H21 (ATCC Number: CRL-2195) is yet another small cell lung carcinoma cell that may be useful as a starting cell in the present invention. It can grow as suspension and loosely adherent culture and is a biochemically stable continuously cultured cell line which has retained important features of SCLC. The line was derived from a non-encapsulated primary lung tumor from the apical portion of the upper lobe of the left lung. This cell line is an unusual undifferentiated large cell variant of small cell lung carcinoma. It has the morphology of a variant, but the biochemical properties of a classic SCLC. Electron microscopy revealed the presence of gland formation and intracytoplasmic lamellar bodies. The cells have neuroendocrine markers L-dopa decarboxylase and dense core secretory granules.

βG H23 is a long-term tissue culture cell line derived from a metastatic human carcinoid tumor of the pancreas (Evers et al., 1991; Parekh et al., 1994). This cell line is also known as BON (Evers et al., 1991). Tumors derived from this cell line are histologically identical to the original tumor. The cells have significant amounts of neurotensin, pancreastatin, and serotonin (5-HT) are demonstrated in the cells by radioimmunoassay (RIA) and the presence of chromogranin A, bombesin, and 5-HT is confirmed by immunocytochemistry. Further, the cells possess neurosecretory granules and functional receptors for acetylcholine, 5-HT, isoproterenol, and somatostatin. BON cells possess a specific transport system for uptake of 5-HT from the medium; this uptake system may be a route for regulation of autocrine effects of 5-HT on carcinoid cells (Parekh et al., 1994). This unique human carcinoid tumor cell line should provides an exemplary starting material for the bioengineering described herein and will be
useful in that they possess intracellular mechanisms ideally adapted for secretagogue action in the release of amines and peptides.

Yet another starting cell that may be useful in the present invention is designated βG H25 (ATCC Number: HB-8065) derived from a hepatoblastoma. This cell line produces alpha-fetoprotein (α fetoprotein); albumin; alpha2 macroglobulin (α-2-macroglobulin); alpha1 antitrypsin (α-1-antitrypsin); transferrin; alpha1 antichymotrypsin;(α-1-antichymotrypsin); haptoglobin; ceruloplasmin; plasminogen and demonstrates decreased expression of apoA-I mRNA and increased expression of catalase mRNA in response to gramicidone (oxidative stress) complement (C4); C3 activator; fibrinogen; alpha1 acid glycoprotein (α-1 acid glycoprotein); alpha2 HS glycoprotein (α-2-HS-glycoprotein); beta lipoprotein (β-lipoprotein); retinol binding protein.

4. Regulation of the Growth of Stable Cells

There is an advantage to developing human cell lines that do not ultimately express the transforming constructs. The transforming genetic construct may, therefore, be functionally and/or physically separated from the cell after transformation. Advantages include generation of cell lines that do not constitutively express oncogenes which can act as tumor antigens in vivo, control of growth of the resulting tumor lines for stable in vivo use and possibly the control of the differentiated state of the resultant cell line.

a. Functional Separation

Temperature-Sensitive Regulation of Oncogene Expression. The use of temperature sensitive oncogenes allows for turning the growth promoting activity on and off. In general, oncogenes that are active at lower than physiological temperatures (i.e., 32°C to 34°C) and off at physiological or higher temperatures (37°C to 39°C) are preferred. Using this approach, stable cell lines can be expanded, and further genetic modifications can be made and characterized in vitro at the low, permissive temperatures. When placed in vivo, these same cell lines will be exposed to the non-permissive temperature, and will not grow. As an example of an oncogene with these traits, a temperature sensitive version of the SV40 virus was isolated.
and shown to have a mutation in the coding region of the large T antigen gene (Bourre and Sarasin. 1983).

**Conditional Expression.** Promoters capable of driving expression of heterologous genes in response to an exogenously added compound allow for conditional expression of oncogenes inserted under the control of the promoter. The addition of the promoting agent then allows stable cell lines to be expanded and transformed. When placed *in vivo*, expression of the oncogene is turned off, unless the activating factor is provided.

Examples of such systems include the lac repressor system (Fieck *et al.*, 1992; Wyborski and Short, 1991; each incorporated herein by reference) and tetracycline regulatory system (U.S. Patent 5,464,758; Gossen and Bujard, 1992; Gossen *et al.*, 1995; each incorporated herein by reference).

**b. Physical Separation**

The present invention contemplates the use of the *Cre/Lox* site-specific recombination system (Sauer, 1993, available through Gibco/BRL, Inc., Gaithersburg, Md.) to rescue specific genes out of a genome. Briefly, the system involves the use of a bacterial nucleotide sequence known as a *LoxP* site, which is recognized by the bacterial *Cre* protein. The *Cre* protein catalyzes a site-specific recombination event. This event is bidirectional, *i.e.*, *Cre* will catalyze the insertion of sequences at a *LoxP* site or excise sequences that lie between two *LoxP* sites. Thus, if a construct for insertion also has flanking *LoxP* sites, introduction of the *Cre* protein, or a polynucleotide encoding the *Cre* protein, into the cell will catalyze the removal of the construct DNA. This technology is enabled in U.S. Patent No. 4,959,317, which is hereby incorporated by reference in its entirety.

The present invention also contemplates the use of recombination activating genes (RAG) 1 and 2 to rescue specific genes from the genome of transformed cell lines. RAG-1 (GenBank accession number M29475) and RAG-2 (GenBank accession numbers M64796 and M33828) recognize specific recombination signal sequences (RSSs) and catalyze V(D)J recombination required for the assembly of immunoglobulin and T cell receptor genes (Schatz
et al., 1989; Oettinger et al., 1990; Cumo and Oettinger, 1994). Transgenic expression of RAG-1 and RAG-2 proteins in non-lymphoid cells supports V(D)J recombination of reporter substrates (Oettinger et al., 1990). For use in the present invention, the transforming construct of interest is engineered to contain flanking RSSs. Following transformation, the transforming construct that is internal to the RSSs can be deleted from the genome by the transient expression of RAG-1 and RAG-2 in the transformed cell.

D. Methods of Blocking Endogenous Polypeptides

In certain embodiments, it may be necessary to block expression of an endogenous gene product as an initial modification of host cells according to the present invention. The targeted endogenous gene encodes a protein normally secreted by the host cell. Blocking expression of this endogenous gene product, while engineering high level expression of genes of interest, represents a unique way of usurping secretory function cells for exogenous protein production.

Cells generated by this two-step process express heterologous proteins, including a variety of natural or engineered proteins (fusions, chimeras, protein fragments, etc.). Cell lines developed in this way are uniquely suited for in vitro assays for the identification of modulators of protein secretion as well as in vivo cell-based delivery or in vitro large-scale production of defined peptide hormones with little or no contaminating or unwanted endogenous protein production.

A number of basic approaches are contemplated for blocking of expression of an endogenous gene in host cells. First, constructs are designed to homologously recombine into particular endogenous gene loci, rendering the endogenous gene nonfunctional. Second, constructs are designed to randomly integrate throughout the genome, resulting in loss of expression of the endogenous gene. Third, constructs are designed to introduce nucleic acids complementary to a target endogenous gene. Expression of RNAs corresponding to these complementary nucleic acids will interfere with the transcription and/or translation of the target sequences. Fourth, constructs are designed to introduce nucleic acids encoding ribozymes - RNA-cleaving enzymes - that will specifically cleave a target mRNA corresponding to the endogenous gene. Fifth, endogenous gene can be rendered dysfunctional by genomic site
directed mutagenesis. Each of these methods for blocking protein production are well known to those of skill in the art. By way of example, WO publication numbers WO 97/26334 (published July 24, 1997) and WO 97/26321 (published July 24, 1997) describe these methodologies and are specifically incorporated herein by reference.

E. Iterative Engineering to Create Novel Cell Lines with Stable Expression

As an alternative to the transgenic expression of T-antigen in islet β-cells to produce β-cell lines as screening tool, the present inventors contemplate the use of the tools of molecular biology to engineer cell lines with properties that approximate those of the normal β-cell (Hughes et al., 1992; Ferber et al., 1994).

The cell line chosen for these studies, RIN 1046-38, loses glucose responsiveness as well as GLUT-2 and glucokinase expression with time in culture (Clark et al., 1990; Ferber et al., 1994). Stable transfection of RIN 1046-38 cells of intermediate, but not high passage numbers with GLUT-2 reconstitutes GSIS and induces a 4-fold increase in glucokinase activity relative to untransfected control cells (Ferber et al., 1994). While these studies represent an important start point, major issues must be dealt with before the cells can be perceived as having any therapeutic value. Among the fundamental deficiencies in RIN 1046-38 cells is that the insulin content of RIN 1046-38 cells is less than one-tenth of the normal human islet β-cell (Clark et al., 1990). This notwithstanding, RIN 1046-38 cells express rat rather than human insulin. Another problem is that the up-regulation of glucokinase activity in response to GLUT-2 transfection is transient, and the cells lose glucose responsiveness over time (Ferber et al., 1994). And finally, the maximal increase in insulin secretion in response to glucose is only 3-fold, and occurs at subphysiological glucose concentrations (50-100 µM) (Ferber et al., 1994). The inventors address these deficiencies by molecular engineering, which will require the introduction of several genes (GLUT-2, glucokinase, human insulin) into a single cell line, as well as a reduction in expression of other undesired genes that are normally expressed by these cells (hexokinase I, rat insulin). As a major step towards this goal, the inventors have used a process of "iterative engineering" to create novel RIN cell lines with stable expression of human insulin, GLUT-2 and glucokinase. The results of these investigations are described in further detail herein below.
In many situations, multiple rounds of iterative engineering will be undertaken in generating the final cell lines. The events that may be conducted as separate construction events include blocking expression of endogenous gene products by molecular methods (including targeting of both copies of the endogenous gene), introducing a heterologous gene, and further modification of the host cell to achieve high level expression. The particular difficulty in performing multiple steps like this is the need for distinct selectable markers. This is a limitation in that only a few selectable markers are available for use in mammalian cells and not all of these work sufficiently well for the purposes of this invention.

The present invention therefore contemplates the use of the Cre/Lox site-specific recombination system (Sauer, 1993, available through Gibco/BRL, Inc., Gaithersburg, Md.) to rescue specific genes out of a genome, most notably drug selection markers. It is claimed as a way of increasing the number of rounds of engineering. Briefly, the system involves the use of a bacterial nucleotide sequence known as a LoxP site, which is recognized by the bacterial Cre protein. The Cre protein catalyzes a site-specific recombination event. This event is bidirectional, i.e., Cre will catalyze the insertion of sequences at a LoxP site or excise sequences that lie between two LoxP sites. Thus, if a construct containing a selectable marker also has LoxP sites flanking the selectable marker, introduction of the Cre protein, or a polynucleotide encoding the Cre protein, into the cell will catalyze the removal of the selectable marker. If successfully accomplished, this will make the selectable marker again available for use in further genetic engineering of the cell. This technology is explained in detail in U.S. Patent No. 4,959,317, which is hereby incorporated by reference in its entirety.

In certain embodiments, in order to increase the output of an endogenous peptide or even of a heterologous peptide, the present invention contemplates the supplemental expression or overexpression of proteins involved in maintaining the specialized phenotype of host cells, especially their secretory capacity. Such proteins may be used to supplement the cell’s natural enzymes. In such cases engineering the overexpression of a cell type-specific transcription factor, such as the Insulin Promoter Factor 1 (IPF1) found in pancreatic β-cells (Ohlsson et al., 1993), is particularly contemplated.
Insulin promoter factor 1 (IPF-1; also referred to as STF-1, IDX-1, PDX-1 and βTF-1) is a homeodomain-containing transcription factor proposed to play an important role in both pancreatic development and insulin gene expression in mature β-cells (Ohlsson et al., 1993, Leonard et al., 1993, Miller et al., 1994, Kruse et al., 1993). In embryos, IPF-1 is expressed prior to islet cell hormone gene expression and is restricted to positions within the primitive foregut where pancreas will later form. Indeed, mice in which the IPF-1 gene is disrupted by targeted knockout do not form a pancreas (Jonsson et al., 1994). Later in pancreatic development, as the different cell types of the pancreas start to emerge, IPF-1 expression becomes restricted predominantly to β-cells. IPF-1 binds to TAAT consensus motifs contained within the FLAT E and P1 elements of the insulin enhancer/promoter, whereupon, it interacts with other transcription factors to activate insulin gene transcription (Peers et al., 1994).

Although IPF-1 will generally be present in the resultant stable human β-cells of the present invention, the overexpression of IPF-1 in human β-cell lines may be used to serve two purposes. First, it will increase transgene expression under the control of the insulin enhancer/promoter. Second, as IPF-1 appears to be critically involved in β-cell maturation, stable overexpression of IPF-1 in the β-cell lines should encourage these cells to maintain the differentiated function of a normal human β-cell.

1. Proteins

A variety of different proteins can be expressed according to the present invention. Proteins can be grouped generally into two categories - secreted and non-secreted. Discussions of each are detailed below. There are some general properties of proteins that are worthy of discussion at this juncture.

First, it is contemplated that many proteins will not have a single sequence but, rather, will exists in many forms. These forms may represent allelic variation or, rather, mutant forms of a given protein. Second, it is contemplated that various proteins may be expressed advantageously as "fusion" proteins. Fusions are generated by linking together the coding regions for two proteins, or parts of two proteins. This generates a new, single coding region
that gives rise to the fusion protein. Fusions may be useful in producing secreted forms of proteins that are not normally secreted or producing molecules that are immunologically tagged. Tagged proteins may be more easily purified or monitored using antibodies to the tag. A third variation contemplated by the present invention involves the expression of protein fragments. It may not be necessary to express an entire protein and, in some cases, it may be desirable to express a particular functional domain, for example, where the protein fragment remains functional but is more stable, or less antigenic, or both.

a. Secreted Proteins

Expression of several proteins that are normally secreted can be engineered into neuroendocrine cells. The cDNA's encoding a number of useful human proteins are available. Examples would include soluble CD-4, Factor VIII, Factor IX, von Willebrand Factor, TPA, urokinase, hirudin, interferons, TNF, interleukins, hematopoietic growth factors, antibodies, albumin, leptin, transferrin and nerve growth factors.

Peptide Hormones. Peptide hormones claimed herein for engineering in neuroendocrine cells are grouped into three classes with specific examples given for each. These classes are defined by the complexity of their post-translational processing. Class I is represented by Growth Hormone, Prolactin and Parathyroid hormone. A more extensive list of human peptides that are included in Class I is given in Table 3. These require relatively limited proteolytic processing followed by storage and stimulated release from secretory granules. Class II is represented by Insulin and Glucagon. A more extensive list of human peptide hormones that are included in Class II are given in Table 4. Further proteolytic processing is required, with both endoproteases and carboxypeptidases processing of larger precursor molecules occurring in the secretory granules. Class III is represented by Amylin, Glucagon-like Peptide I and Calcitonin. Again, a more extensive list of Class III human peptide hormones is given in Table 5. In addition to the proteolytic processing found in the Class II peptides, amidation of the C-terminus is required for proper biological function. Examples of engineering expression of all three of these classes of peptide hormones in a neuroendocrine cell are found in this specification.
### TABLE 3
**CLASS I HUMAN PEPTIDE HORMONES**

- Growth Hormone
- Prolactin
- Placental Lactogen
- Luteinizing Hormone
- Follicle-stimulating Hormone
- Chorionic Gonadotropin
- Thyroid-stimulating Hormone
- Leptin
- Relaxin

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### TABLE 4
**HUMAN PEPTIDE HORMONES PROCESSED BY ENDOPROTEASES FROM LARGER PRECURSORS**

- Adrenocorticotropic (ACTH)
- Angiotensin I and II
- \(\beta\)-endorphin
- \(\beta\)-Melanocyte Stimulating Hormone (\(\beta\)-MSH)
- Cholecystokinin
- Endothelin I
- Galanin
- Gastric Inhibitory Peptide (GIP)
- Glucagon
- Insulin
- Lipotropins
- Neurophysins
- Somatostatin
| TABLE 5 |
| AMIDATED HUMAN PEPTIDE HORMONES |

Calcium Metabolism Peptides:
- Calcitonin
- Calcitonin Gene related Peptide (CGRP)
- β-Calcitonin Gene Related Peptide
- Hypercalcemia of Malignancy Factor (1-40) (PTH-rP)
- Parathyroid Hormone-related protein (107-139) (PTH-rP)
- Parathyroid Hormone-related protein (107-111) (PTH-rP)

Gastrointestinal Peptides:
- Cholecystokinin (27-33) (CCK)
- Galanin Message Associated Peptide, Preprogalanin (65-105)
- Gastrin I
- Gastrin Releasing Peptide
- Glucagon-like Peptide (GLP-1)
- Pancreastatin
- Pancreatic Peptide
- Peptide YY
- PHM
- Secretin
- Vasoactive Intestinal Peptide (VIP)

Pituitary Peptides:
- Oxytocin
- Vasopressin (AVP)
- Vasotocin

Enkephalins:
- Enkephalinamide
- Metorphinamide (Adrenorphin)

Alpha Melanocyte Stimulating Hormone (alpha-MSH)

Atrial Natriuretic Factor (5-28) (ANF)

Amylin
Table 5 - Continued

Amyloid P Component (SAP-1)
Corticotropin Releasing Hormone (CRH)
Growth Hormone Releasing Factor (GHRH)
Luteinizing Hormone-Releasing Hormone (LHRH)
Neuropeptide Y
Substance K (Neurokinin A)
Substance P
Thyrotropin Releasing Hormone (TRH)

b. Non-Secreted Proteins

Expression of non-secreted proteins can be engineered into neuroendocrine cells. Two general classes of such proteins can be defined. The first are proteins that, once expressed in cells, stay associated with the cells in a variety of destinations. These destinations include the cytoplasm, nucleus, mitochondria, endoplasmic reticulum, golgi, membrane of secretory granules and plasma membrane. Non-secreted proteins are both soluble and membrane associated. The second class of proteins are ones that are normally associated with the cell, but have been modified such that they are now secreted by the cell. Modifications would include site-directed mutagenesis or expression of truncations of engineered proteins resulting in their secretion as well as creating novel fusion proteins that result in secretion of a normally non-secreted protein.

Cells engineered to produce such proteins will be used for in vitro and in vivo screening for modulators of protein production and secretion. This will entail purification of the secreted protein from the conditioned media from cells secreting the engineered protein. In vivo, cell-based screening methods would either be based on secretion of the engineered protein or beneficial effects of the cells expressing a non-secreted protein.

The cDNA's encoding a number of therapeutically useful human proteins are available. These include cell surface receptors, transporters and channels such as GLUT2, CFTR, leptin receptor, sulfonylurea receptor, β-cell inward rectifying channels, α2-adrenergic receptor,
pancreatic polypeptide receptor, somatostatin receptor, glucocorticoid receptor, potassium inward rectifying channel, GLP-1 receptor and muscarinic receptor etc. Other proteins include protein processing enzymes such as PC2 and PC3, and PAM, transcription factors such as IPF1, and metabolic enzymes such as adenosine deaminase, phenylalanine hydroxylase, glucocerebrosidase.

Engineering mutated, truncated or fusion proteins into neuroendocrine cells also is contemplated. Examples of each type of engineering resulting in secretion of a protein are given (Ferber et al., 1991; Mains et al., 1995). Reviews on the use of such proteins for studying the regulated secretion pathway are also cited (Burgess and Kelly, 1987; Chavez et al., 1994).

**Leptin - Engineering Leptin Expression in Cells.** In another embodiment of the present invention, the engineered cells may express and overexpress the obesity-associated protein known as leptin. Leptin is a peptide hormone that controls body composition and is believed to do so, at least in part, via interaction with hypothalamic receptors that regulate food intake and body weight. The various isoforms of leptin receptor (Ob-R), including the long isoform (OB-Rb), are widely expressed in various tissues, suggesting that leptin may play an important role in actions on extraneural tissues as well.

Additional evidence that leptin has non-neural function comes from a report that extraordinary changes in body fat are seen in rats made chronically hyperleptinemic by treatment with an adenovirus vector expressing the leptin cDNA. Chen et al., *Proc. Nat'l Acad. Sci. USA* 93:14795 (1996). In this report, rats lost all discernible body fat within 7 days of adenovirus infusion, while animals that were "pair-fed" at the same low rate of food intake as the hyperleptinemic animals retain more of their body fat. The magnitude and rapidity of the lipid depletion suggested the possibility of a direct "hormone-to-cell" action by leptin, in addition to effects cause through the sympathetic nervous system.

Chen et al. (1996) also examined the effects of leptin overexpression on plasma glucose, insulin, plasma triglycerides and free fatty acid levels. While glucose did not change, both
plasma triglycerides and free fatty acids dropped by about 50% in adenoviral-leptin treated animals, when compared to controls (Ad-β-gal or saline). These studies now have been confirmed and extended with respect to phospholipids. No clear cut changes in phospholipid concentration was observed. However, using an in vitro system, it was established that reductions in triglyceride levels could be achieved in the absence of sympathetic nervous system effects. Studies performed to determine what pathways are involved in the triglyceride depletion indicated that leptin-induced triglyceride depletion involves a novel mechanisms by which triglyceride disappears through enhanced intracellular triglyceride metabolism, rather than through more traditional free fatty acid export pathways.

Insulin levels in adenovirus-leptin infected rats dropped even more dramatically than the fatty acids, being only about 1/3 of the amount seen in controls. As stated above, the glucose levels of these animals was normal, however. These findings are consistent with enhanced insulin sensitivity in treated animals. Pancreata were isolated from hyperleptinemic rats and examined for β-cell function and morphology. The most striking finding was the complete absence of insulin secretion in response to either glucose or arginine. The morphology appeared normal, and it was determined that insulin secretion could be reestablished following perfusion of pancreatic tissue in the presence of free fatty acids, thereby establishing an important role for these molecules in β-cell function. These studies also indicate that leptin-mediated reduction of elevated tissue lipid levels will improve β-cell function, reduce insulin resistance and help restore abnormal glucose homeostasis in obese individuals.

A further connection between diabetes and leptin comes from studies with genetically obese ZDF rats, which contain mutant OB-R genes. The islets of these animals become overloaded with fat at the time that hyperglycemia begins. Because maneuvers that reduce islet fat content prevent diabetes in ZDF rats, it has been proposed that the accumulation of triglycerides in islets plays a causal role in β-cell dysfunction. Thus, the predisposition to diabetes in homozygous ZDF rats may reflect the fact that their tissue have been completely "un leptinized" throughout their life and therefore have accumulated high levels of TG. In normal rats, this accumulation is prevented by the action of leptin. It is expected that any
therapy that reduces triglycerides in islets and in the target tissues of insulin will improve β-cell function and reduce insulin resistance.

In hyperleptinemic rats, every tissue that was examined was lipopenic. Thus, it is speculated that normal non-adipocytes carry a minute quantity of triglyceride, perhaps to serve as a reserve source of fuel in adipocytes that are depleted of fat by starvation and become unable to meet the fuel needs of certain tissues. It is suspected that this triglyceride storage function is closely regulated by leptin. In the obese ZDF rats, this regulatory control is absent, and these putative intracellular triglycerides reserves soar to levels of over 1000-times that of hyperleptinemic rats.

In light of these observations, the present application therefore encompasses various engineered cells which express leptin in amounts in excess of normal. The methods by which leptin genes may be manipulated and introduced are much the same as for other genes included herein, such as amylin. A preferred embodiment would involve the use of a viral vector to deliver a leptin-encoding gene, for example, an adenoviral vector. This approach may be exploited in at least two ways. First, in the engineering of cells to produce certain polypeptides in vitro, it may be desirable to express high levels of leptin in order to down-regulate various cellular functions, including synthesis of certain proteins. Similarly, leptin overexpression may synergize with cellular functions, resulting in the increased expression of an endogenous or exogenous polypeptide of interest.

Second, it may be desirable to use a leptin-overexpressing cell, or a leptin expression construct, such as a leptin-expressing adenovirus, in an in vivo context. This includes various "combination" approaches to the treatment of disease states such as obesity, hyperlipidemia and diabetes. For example, leptin expressing cell lines may provide for prolonged expression of leptin in vivo and for high level expression. Preliminary results indicate that injection of recombinantly produced leptin is less efficacious at achieving weight loss and reduction of lipids. Induction of hyperleptinemia using cells lines or expression constructs also may find use in reducing fat content in livestock just prior to slaughter. Moreover, because leptin-induced weight loss may act through different mechanisms than those currently employed, it
may be possible to avoid related side effects such as diet-induced ketosis, heart attack and other diet-related symptoms. These regimens may involve combinations of other engineered cells, cells engineered with leptin and at least one other gene or genetic construct (knock-out, antisense, ribozyme, etc.), combination gene therapy or combination with a drug. The methods of delivering such pharmaceutical preparations are described elsewhere in this document.

**Enzymes.** In still further embodiments, of the present invention, the engineered cells may express and/or overexpress certain enzymes of therapeutic value. Such enzymes include by are not limited to adenosine deaminase (*e.g.* Genbank Accession Nos. P55265; U18121; U73107; Z97053; P00813; U75503; DUHUA), galactosidase (*e.g.* Genbank Accession Nos. P54803; P51569; P23780; D00039), glucosidase (*e.g.* Genbank Accession Nos. P29064 (α-glucosidase), P26208 (β-glucosidase), lecithin:cholesterol acyltransferase (LCAT, *e.g.* Genbank Accession Nos. 729921 (baboon), P04180 (human), XXHUN (human LCAT precursor), X04981), factor IX (*e.g.* Genbank Accession Nos. P00740 (human) K02402 (human) P00741 (bovine) and A22493), sphingolipase, lysosomal acid lipase (*e.g.* Genbank Accession Nos. P38571; S41408), lipoprotein lipase (*e.g.* Genbank Accession No. P06858), hepatic lipase (*e.g.* Genbank Accession Nos. AF037404; P11150; P07098), pancreatic lipase related protein (*e.g.* Genbank Accession Nos. P54315; P54317) pancreatic lipase (P16233) and uronidase.

2. **Processing Enzymes**

The present invention also contemplates augmenting or increasing the capabilities of cells to produce biologically active polypeptides. This can be accomplished, in some instances, by overexpressing the proteins involved in protein processing, such as the endoproteases PC2 and PC3 (Steiner *et al.*, 1992) or the peptide amidating enzyme. PAM (Eipper *et al.*, 1992a) in the case of amidated peptide hormones.

Expression of proteins involved in maintaining the specialized phenotype of host cells, especially their secretory capacity, is important. Engineering the overexpression of a cell type-specific transcription factor such as the Insulin Promoter Factor 1 (IPF1) found in pancreatic β-cells (Ohlsson *et al.*, 1993) could increase or stabilize the capabilities of engineered neuroendocrine cells. Insulin promoter factor 1 (IPF-1: also referred to as STF-1, IDX-1, PDX-
l and βTF-1) is a homeodomain-containing transcription factor proposed to play an important role in both pancreatic development and insulin gene expression in mature β-cells (Ohlsson et al., 1993, Leonard et al., 1993, Miller et al., 1994, Kruse et al., 1993). In embryos, IPF-1 is expressed prior to islet cell hormone gene expression and is restricted to positions within the primitive foregut where pancreas will later form. Indeed, mice in which the IPF-1 gene is disrupted by targeted knockout do not form a pancreas (Jonsson et al., 1994). Later in pancreatic development, as the different cell types of the pancreas start to emerge, IPF-1 expression becomes restricted predominantly to β-cells. IPF-1 binds to TAAT consensus motifs contained within the FLAT E and P1 elements of the insulin enhancer/promoter, whereupon, it interacts with other transcription factors to activate insulin gene transcription (Peers et al., 1994).

Stable overexpression of IPF-1 in neuroendocrine β-cell lines will serve two purposes. First, it will increase transgene expression under the control of the insulin enhancer/promoter. Second, because IPF-1 appears to be critically involved in β-cell maturation, stable overexpression of IPF-1 in β-cell lines should cause these mostly dedifferentiated β-cells to regain the more differentiated function of a normal animal β-cell. If so, then these redifferentiated β-cell lines could potentially function as a more effective neuroendocrine cell type for cell-based delivery of fully processed, bioactive peptide hormones.

Also, further engineering of cells to generate a more physiologically-relevant regulated secretory response is claimed. Examples would include engineering the ratios of glucokinase to hexokinase in rat insulinoma cells that also overexpress the Type II glucose transporter (GLUT-2) such that a physiologically-relevant glucose-stimulated secretion of peptide hormones is achieved. Other examples include engineering overexpression of other signaling proteins known to play a role in the regulated secretory response of neuroendocrine cells. These include cell surface proteins such as the β-cell-specific inwardly rectifying potassium channel (BIR; Inagaki et al., 1995), involved in release of the secretory granule contents upon glucose stimulation, the sulfonylurea receptor (SUR), and other ATP sensitive channels. Other cell surface signaling receptors which help potentiate the glucose-stimulated degranulation of β-cells including the glucagon-like peptide 1 receptor (Thorens, 1992) and the glucose-dependent
insulinotropic polypeptide receptor (also known as gastric inhibitory peptide receptor) (Usdin, 1993) can be engineered into neuroendocrine cells. These β-cell-specific signaling receptors, as well as GLUT-2 and glucokinase, are involved in secretory granule release in response to glucose. In this way, glucose stimulated release of any heterologous peptide targeted to the secretory granule can be engineered. Alternatively, other cell surface signaling proteins involved in non-glucose-stimulated release of secretory granule contents can be engineered into neuroendocrine cells. Examples would include releasing factor receptors such as Growth Hormone Releasing Factor Receptor (Lin et al., 1992) and Somatostatin or Growth Hormone Releasing Hormone Receptor (Mayo, 1992).

3. Modified Secretory Response

The present invention further includes embodiments where the resultant stable neuroendocrine cells are further engineered to modify the secretion of the endogenous secretory polypeptide in response to one or more secretagogues.

The engineering of the resultant stable cells to generate a more physiologically-relevant regulated secretory response includes engineering the expression or overexpression of signaling proteins known to play a role in the regulated secretory response of neuroendocrine cells. These include cell surface proteins such as the β-cell-specific inwardly rectifying potassium channel (β cell inward rectifier, BIR; Inagaki et al., 1995), involved in release of the secretory granule contents upon glucose stimulation, the sulfonylurea receptor (SUR), and ATP sensitive channel. Other heterologous releasing factor receptors may be used in these aspects of the invention, as may adrenergic receptors and the like.

Other cell surface signaling receptors which assist with potentiating the glucose-stimulated degranulation of β-cells include the glucagon-like peptide 1 receptor (Thorens, 1992) and the glucose-dependent insulinotropic polypeptide receptor (also known as gastric inhibitory peptide receptor) (Usdin et al., 1993), which can also be engineered into neuroendocrine cells according to the present invention. These β-cell-specific signaling receptors, as well as GLUT-2 and glucokinase (see below), are involved in secretory granule
release in response to glucose. In this way, glucose stimulated release of a peptides targeted to
the secretory granule can be reengineered or enhanced.

In still further embodiments, other cell surface signaling proteins involved in non-
glucose-stimulated release of secretory granule contents can be engineered into the stable
neuroendocrine cells of the invention. Examples include releasing factor receptors such as
Growth Hormone Releasing Factor Receptor (Lin et al., 1992) and Somatostatin or Growth
Hormone Releasing Hormone Receptor (Mayo, 1992).

4. Cell Signaling Machinery

The pancreatic β-cell is continually exposed to a complex mixture of molecules that
modulate insulin synthesis, storage, and exocytosis. The information in this mixture is
translated to regulatory signals by three distinct mechanisms: (1) transport into the cell and
metabolism of fuels, (2) ion fluxes, relative to extracellular and intracellular ion pools, and (3)
hormonal signals that are mediated via receptors (reviewed in Komatsu et al., 1997). The
transport and metabolism of glucose is the dominant signal that regulates insulin secretion. A
large portion of the glucose effect is mediated by K⁺-ATP channels, depends on membrane
polarity, and regulates the influx of extracellular calcium through L-type Ca²⁺ channels. Amino
acids are another fuel that participate in insulin secretion via the regulation of the K⁺-ATP
channel.

Glucose metabolism also affects intracellular Ca²⁺ stores by mechanisms that are
independent of K⁺-ATP channels. This portion of glucose-regulated insulin secretion is
augmented by many other molecules involved in glycemic control such as fatty acids and
muscarinic receptor ligands. Binding to the muscarinic receptor by acetylcholine results in the
activation of phospholipases, enzymes that catalyze the conversion of phosphoinositides to
inositol triphosphates (IP3) and diacylglycerol (DAG). Increased IP3 levels stimulate the
release of Ca²⁺ from intracellular stores and contribute to signals for exocytosis of insulin. A
central theme in Ca²⁺-induced secretion is the activation of Ca²⁺/calmodulin-dependent kinases
that link Ca²⁺ levels to exocytosis (Ashcroft, 1994).
There is also evidence that glucose regulates insulin secretion by events that are both 
K⁺-ATP channel-independent and Ca²⁺-independent. This form of regulation applies most 
often to the augmenting effects observed for hormones that bind receptors such as GLP-1, GIP, 
pituitary adenylate cyclase activating peptide (PACAP), and vasoactive intestinal peptide 
(VIP). Receptors for these peptide hormones are typically coupled to GTP-binding proteins 
that regulate the membrane bound form of adenylate cyclase. Stimulation of the receptors 
results in increases in cyclic AMP levels and increases in the activity of protein kinase A, a 
potentiatior of insulin secretion. Other secretory kinase effects may also be independent of the 
K⁺-ATP channels and intracellular Ca²⁺. Protein kinase C is stimulated by DAG and functions 
to augment glucose-stimulated insulin secretion. (Komatsu et al., 1997).

Prentki has proposed a model for glucose metabolism that takes into account many 
aspects of stimulated insulin secretion (Prentki, 1994). It categorizes stimulatory events as 
glycolytic and post-glycolytic and supports the view that the glucose-mediated regulation of 
insulin secretion cannot be fully explained by the effects of the K⁺-ATP channels and increases 
in intracellular Ca²⁺. In the model proposed, pyruvate is a key intermediate metabolite and its 
fates provide two distinct mechanisms to stimulate secretion. Pyruvate dehydrogenase, which 
is stimulated by an increase in the ATP/ADP ratios resulting from glycolysis, moves the 
metabolism of glucose toward the citric acid cycle by the conversion of pyruvate to acetyl CoA. 
Carbon fluxes through the citric acid cycle boost the ATP/ADP ratios even higher, and 
stimulate the closure of K⁺-ATP channels and the concomitant increases in intracellular Ca²⁺.

Pyruvate also is a key metabolite in anaplerosis, the replenishment of citric acid cycle 
intermediates. This arm of pyruvate metabolism is initiated by the activity of pyruvate 
carboxylase, an enzyme that catalyzes the conversion of pyruvate to citrate. When citrate is 
abundant it can be transported from the mitochondria into the cytoplasm and converted to 
malonyl CoA, a molecule that provides a link between glucose metabolism and fatty acid 
metabolism. Increases in malonyl CoA promote the accumulation of fatty acid intermediates, 
potentiators of insulin secretion that appear to be independent of Ca²⁺ (Prentki, 1994).
F. Transforming Genetic Constructs

The present invention further provides methods for preparing immortalized stable human neuroendocrine cells or secretory cells that have maintained their regulated secretory pathway. The methods generally comprise providing to a human neuroendocrine or regulated secretory cell an effective amount of a transforming genetic construct that comprises an operative transforming unit under the transcriptional control of a promoter specific for the target neuroendocrine cell.

In preferred aspects of the preparative methods, the target human neuroendocrine cells will be provided with the transforming genetic construct by infection with a recombinant virus, most preferably an adenovirus, that comprises the transforming construct. The methods described herein may involve the use of two, three or more distinct transforming genetic constructs. In certain aspects the use of defined media, or the use of defined media supplemented with one or more growth factors specific for the target neuroendocrine cells is contemplated. Also contemplated is the use of one or more promoters that have enhanced transcriptional activity, such as promoters comprising multimerized promoter elements, the additional provision of a growth factor receptor gene to the target cell and/or the use of transforming genetic constructs that involve elements for effecting controlled or regulated expression or subsequent excision. The present section relates to the transforming genes and genetic constructs.

1. Genes

Exemplary transforming genes and constructs are listed herein in Table 6. Any one or more of the genes listed therein may be used in the context of the present invention. Where two or more transforming genes are provided to a human neuroendocrine cell, it may be preferable to provide genes from different functional categories, such as those that perturb signal transduction, affect cell cycle, alter nuclear transcription, alter telomere structure or function, inhibit apoptosis, or that exert pleiotropic activities. It will be understood that the genes listed in Table 6 are only exemplary of the types of oncogenes, mutated tumor suppressors and other transforming genetic constructs and elements that may be used in this invention. Further transforming genes and constructs will be known to those of ordinary skill in the art.
<table>
<thead>
<tr>
<th>I. ONCOGENES</th>
<th>MODE OF ACTION</th>
</tr>
</thead>
<tbody>
<tr>
<td>tyrosine kinases, both membrane-associated and</td>
<td>perturb signal transduction</td>
</tr>
<tr>
<td>cytoplasmic forms, such as Src family, Jak/Stats,</td>
<td></td>
</tr>
<tr>
<td>Ros, Neu, Fms, Ret, abl. Met</td>
<td></td>
</tr>
<tr>
<td>serine/threonine kinases: Mos, Raf, protein</td>
<td></td>
</tr>
<tr>
<td>kinase C, PIM-1</td>
<td></td>
</tr>
<tr>
<td>growth factor and receptors: platelet derived</td>
<td></td>
</tr>
<tr>
<td>growth factor (PDGF), insulin-like growth factor</td>
<td></td>
</tr>
<tr>
<td>(IGF-1), insulin receptor substrate (IRS-1 and</td>
<td></td>
</tr>
<tr>
<td>IRS-2), Erb family, epidermal growth factor</td>
<td></td>
</tr>
<tr>
<td>factor (EGF), growth hormone, hepatocyte</td>
<td></td>
</tr>
<tr>
<td>growth factor (HGF) basic fibroblast growth</td>
<td></td>
</tr>
<tr>
<td>factor (bFGF) small GTPases (G) proteins</td>
<td></td>
</tr>
<tr>
<td>including the ras family, rab family, and Gsα</td>
<td></td>
</tr>
<tr>
<td>receptor-type tyrosine phosphatase IA-2</td>
<td></td>
</tr>
<tr>
<td>cyclin-dependent protein kinases (cdk), classes</td>
<td>affect cell cycle</td>
</tr>
<tr>
<td>A - E; members of the cyclin family such as</td>
<td></td>
</tr>
<tr>
<td>cyclin D</td>
<td></td>
</tr>
<tr>
<td>Myc family members including c-myc, N-myc, and</td>
<td>alter nuclear transcription</td>
</tr>
<tr>
<td>L-myc; Rel family members including NF-kappaB;</td>
<td></td>
</tr>
<tr>
<td>c-Myb, Ap-1, fos, jun, insulinoma associated</td>
<td></td>
</tr>
<tr>
<td>cDNA (IA-1), ErbB-1, PAX gene family</td>
<td></td>
</tr>
<tr>
<td>telomerase</td>
<td>lengthens telomeres of</td>
</tr>
<tr>
<td>bcl-2 and family members including Bcl-xl, Mcl-1</td>
<td>inhibit apoptosis</td>
</tr>
<tr>
<td>Bak, A1, A20</td>
<td></td>
</tr>
<tr>
<td>inhibitors of interleukin-1b-converting</td>
<td></td>
</tr>
<tr>
<td>enzyme and family members</td>
<td></td>
</tr>
</tbody>
</table>
Table 6 - Continued

<table>
<thead>
<tr>
<th>I. ONGOGENES</th>
<th>MODE OF ACTION</th>
</tr>
</thead>
<tbody>
<tr>
<td>viral proteins such as SV40 and polyoma large T antigens,</td>
<td>pleiotropic activities</td>
</tr>
<tr>
<td>SV40 temperature sensitive large T antigen, adenovirus E1A</td>
<td></td>
</tr>
<tr>
<td>and E1B, papilomavirus E6 and E7</td>
<td></td>
</tr>
</tbody>
</table>

II. MUTANT TUMOR SUPPRESSORS

| p53, retinoblastoma gene (Rb), Wilm’s tumor (WT1), bax                | failure to promote     |
| alpha, interleukin-1b-converting enzyme and family, MEN-1              | apoptosis              |
| gene (chromosome 11q13), neurofibromatosis, type 1 (NF1),              |                         |
| cdk inhibitor p16, colorectal cancer gene (DCC), familial              |                         |
| adenomatosis polyposis gene (FAP), multiple tumor                      |                         |
| suppressor gene (MTS-1), BRCA1, BRCA2                                 |                         |

In certain embodiments, the immortalizing genetic construct will comprise a gene or cDNA that is responsible for the perturbation of signal transduction. Representative members of this class are genes or cDNAs encoding tyrosine kinases, serine/threonine kinases, growth factors and receptors, small GTPases, and receptor-type tyrosine phosphatase 1A-2. Exemplary of the members preferred for use in the present invention is neu (also known as her2 or erbB-2; GenBank accession numbers M11730, X03363, U02326 and S57296). neu was discovered as an oncogene in breast cancer, but it is also found in other forms of cancer. neu appears to be a member of the receptor tyrosine kinase family. Also preferred is hepatocyte growth factor receptor (HGFR, also known as scatter factor receptor; GenBank accession number U11813). This is an example of a receptor, either endogenously present or expressed from a recombinant adenovirus, that is used to stimulate proliferation of a target cell population. Other preferred members are insulin-like growth factor 1 receptor (GenBank accession number X04434 and M24599), and GTPase Gs<sub>a</sub> (GenBank accession numbers X56009, X04409). Gs<sub>a</sub> is associated with pituitary tumors that secrete growth hormone, but not other neuroendocrine or endocrine tumors.
In alternative embodiments, the immortalization genetic construct may be a factor that affects the cell cycle. Exemplary of this type of factor is cyclin D1 (also known as PRAD or bcl-1; GenBank accession numbers M64349 and M73554). This is associated as an oncogene primarily with parathyroid tumors. Other factors that may comprise the genetic immortalization construct include those gene that alter nuclear transcription c-myc (GenBank accession numbers J00120, K01980, M23541, V00501, X00364). Inhibitors of apoptosis are also preferred for use is bcl-2 (distinct from bcl-1, cyclin D1; GenBank accession numbers M14745, X06487). Overexpression of this oncogene was first discovered in T cell lymphomas. bcl-2 functions as an oncogene by binding and inactivating Bax, a protein in the apoptotic pathway. In other aspects the genetic constructs comprise molecules with pleiotropic activities. preferred from this class is SV40 large T antigen (TAG; GenBank accession number J02400). Also preferred is temperature sensitive large T antigen.

Other genes that will be useful in immortalizing the neuroendocrine cells are constructs that result in the failure to promote apoptosis. Preferred in this category are p53 and the retinoblastoma gene. Most forms of cancer have reports of p53 mutations. Inactivation of p53 results in a failure to promote apoptosis. With this failure, cancer cells progress in tumorogenesis rather than be destined for cell death. A short list of cancers and mutations found in p53 is: ovarian (GenBank accession numbers S53545, S62213, S62216); liver (GenBank accession numbers S62711, S62713, S62714, S67715, S72716); gastric (GenBank accession numbers S63157); colon (GenBank accession numbers S63610); bladder (GenBank accession numbers S85568, S85570, S85691); lung (GenBank accession numbers S41969, S41977); and glioma (GenBank accession numbers S85807, S85712, S85713).

In still further preferred embodiments, the invention contemplates the use of growth factor receptor genes and growth factor genes as the transforming elements. As an example of this embodiment, human β cells are infected with a recombinant adenovirus that provides overexpression of growth hormone receptor (GenBank Accession Nos. J04811 and X06562) controlled by the modRIP (or modHIP) promoter. β cells cultured in a defined medium would then be stimulated to proliferate by the addition of growth hormone to the medium. The replicating population of β cells are then transformed by retroviral constructs that will result in
stable expression of growth hormone receptor or an alternate transforming gene. The use of other growth promoting genes such as IGF-1 receptor (and its ligand in the medium) and or the signaling substrate of growth factor receptors (such as IRS-2 in the case of IGF-1 receptor) could similarly be used to achieve growth and transformation.

In still further preferred embodiments, the invention contemplates the use of several transforming gene constructs in combination. As an example of this embodiment, the transforming genetic construct may include more than one operative transforming unit, or more than one construct can be supplied.

2. Constitutive Promoters

Throughout this application, the term "expression construct" is meant to include any type of genetic construct containing a nucleic acid coding for a gene product in which part or all of the nucleic acid encoding sequence is capable of being transcribed. The transcript may be translated into a protein, but it need not be. In certain embodiments, expression includes both transcription of a gene and translation of mRNA into a gene product. In other embodiments, expression only includes transcription of the nucleic acid encoding a gene of interest.

In preferred embodiments, the nucleic acid encoding a gene product is under transcriptional control of a promoter. A "promoter" refers to a DNA sequence recognized by the synthetic machinery of the cell, or introduced synthetic machinery, required to initiate the specific transcription of a gene. The phrase "under transcriptional control" means that the promoter is in the correct location and orientation in relation to the nucleic acid to control RNA polymerase initiation and expression of the gene.

The term promoter will be used here to refer to a group of transcriptional control modules that are clustered around the initiation site for RNA polymerase II. Much of the thinking about how promoters are organized derives from analyses of several viral promoters, including those for the HSV thymidine kinase (tk) and SV40 early transcription units. These studies, augmented by more recent work, have shown that promoters are composed of discrete functional modules, each consisting of approximately 7-20 bp of DNA, and containing one or more recognition sites for transcriptional activator or repressor proteins.
At least one module in each promoter functions to position the start site for RNA synthesis. The best known example of this is the TATA box, but in some promoters lacking a TATA box, such as the promoter for the mammalian terminal deoxynucleotidyl transferase gene and the promoter for the SV40 late genes, a discrete element overlying the start site itself helps to fix the place of initiation.

Additional promoter elements regulate the frequency of transcriptional initiation. Typically, these are located in the region 30-110 bp upstream of the start site, although a number of promoters have recently been shown to contain functional elements downstream of the start site as well. The spacing between promoter elements frequently is flexible, so that promoter function is preserved when elements are inverted or moved relative to one another. In the tk promoter, the spacing between promoter elements can be increased to 50 bp apart before activity begins to decline. Depending on the promoter, it appears that individual elements can function either co-operatively or independently to activate transcription.

The particular promoter that is employed to control the expression of a nucleic acid encoding a particular gene is not believed to be important, so long as it is capable of expressing the nucleic acid in the targeted cell. Thus, where a human cell is targeted, it is preferable to position the nucleic acid coding region adjacent to and under the control of a promoter that is capable of being expressed in a human cell. Generally speaking, such a promoter might include either a human or viral promoter.

In various embodiments, the human cytomegalovirus (CMV) immediate early gene promoter, the SV40 early promoter, the Rous sarcoma virus long terminal repeat, rat insulin promoter and glyceraldehyde-3-phosphate dehydrogenase can be used to obtain high-level expression of the gene of interest. The use of other viral or mammalian cellular or bacterial phage promoters which are well-known in the art to achieve expression of a gene of interest is contemplated as well, provided that the levels of expression are sufficient for a given purpose.
By employing a promoter with well-known properties, the level and pattern of expression of the gene product following transfection can be optimized. Further, selection of a promoter that is regulated in response to specific physiologic signals can permit inducible expression of the gene product. Tables 6 and 7 list several elements/promoters which may be employed, in the context of the present invention, to regulate the expression of the gene of interest. This list is not intended to be exhaustive of all the possible elements involved in the promotion of gene expression but, merely, to be exemplary thereof.

Enhancers were originally detected as genetic elements that increased transcription from a promoter located at a distant position on the same molecule of DNA. This ability to act over a large distance had little precedent in classic studies of prokaryotic transcriptional regulation. Subsequent work showed that regions of DNA with enhancer activity are organized much like promoters. That is, they are composed of many individual elements, each of which binds to one or more transcriptional proteins.

The basic distinction between enhancers and promoters is operational. An enhancer region as a whole must be able to stimulate transcription at a distance; this need not be true of a promoter region or its component elements. On the other hand, a promoter must have one or more elements that direct initiation of RNA synthesis at a particular site and in a particular orientation, whereas enhancers lack these specificities. Promoters and enhancers are often overlapping and contiguous, often seeming to have a very similar modular organization.

Below is a list of viral promoters, cellular promoters/enhancers and inducible promoters/enhancers that could be used in combination with the nucleic acid encoding a gene of interest in an expression construct (Table 7 and Table 8). Additionally, any promoter/enhancer combination (as per the Eukaryotic Promoter Data Base EPDB) could also be used to drive expression of the gene. Eukaryotic cells can support cytoplasmic transcription from certain bacterial promoters if the appropriate bacterial polymerase is provided, either as part of the delivery complex or as an additional genetic expression construct.
<table>
<thead>
<tr>
<th>ENHANCER</th>
</tr>
</thead>
<tbody>
<tr>
<td>Immunoglobulin Heavy Chain</td>
</tr>
<tr>
<td>Immunoglobulin Light Chain</td>
</tr>
<tr>
<td>T-Cell Receptor</td>
</tr>
<tr>
<td>HLA DQ $\alpha$ and DQ $\beta$</td>
</tr>
<tr>
<td>$\beta$-Interferon</td>
</tr>
<tr>
<td>Interleukin-2</td>
</tr>
<tr>
<td>Interleukin-2 Receptor</td>
</tr>
<tr>
<td>MHC Class II 5</td>
</tr>
<tr>
<td>MHC Class II HLA-DR$\alpha$</td>
</tr>
<tr>
<td>$\beta$-Actin</td>
</tr>
<tr>
<td>Muscle Creatine Kinase</td>
</tr>
<tr>
<td>Prealbumin (Transthyretin)</td>
</tr>
<tr>
<td>Elastase $I$</td>
</tr>
<tr>
<td>Metallothionein</td>
</tr>
<tr>
<td>Collagenase</td>
</tr>
<tr>
<td>Albumin Gene</td>
</tr>
<tr>
<td>$\alpha$-Fetoprotein</td>
</tr>
<tr>
<td>$\tau$-Globin</td>
</tr>
<tr>
<td>$\beta$-Globin</td>
</tr>
<tr>
<td>e-fos</td>
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<td>c-HA-ras</td>
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<tr>
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<td>α1-Antitrypsin</td>
</tr>
<tr>
<td>H2B (TH2B) Histone</td>
</tr>
<tr>
<td>Mouse or Type I Collagen</td>
</tr>
<tr>
<td>Glucose-Regulated Proteins (GRP94 and GRP78)</td>
</tr>
<tr>
<td>Rat Growth Hormone</td>
</tr>
<tr>
<td>Human Serum Amyloid A (SAA)</td>
</tr>
<tr>
<td>Troponin I (TN I)</td>
</tr>
<tr>
<td>Platelet-Derived Growth Factor</td>
</tr>
<tr>
<td>Duchenne Muscular Dystrophy</td>
</tr>
<tr>
<td>SV40</td>
</tr>
<tr>
<td>Polyoma</td>
</tr>
<tr>
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</tr>
<tr>
<td>Papilloma Virus</td>
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<td>Human Immunodeficiency Virus</td>
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<tr>
<td>Cytomegalovirus</td>
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<td>Gibbon Ape Leukemia Virus</td>
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### TABLE 8

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<tr>
<td>MT II</td>
<td>Phorbol Ester (TPA)</td>
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<tr>
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<td>Heavy metals</td>
</tr>
<tr>
<td>MMTV (mouse mammary tumor virus)</td>
<td>Glucocorticoids</td>
</tr>
<tr>
<td>β-Interferon</td>
<td>poly(rl)X</td>
</tr>
<tr>
<td></td>
<td>poly(rc)</td>
</tr>
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<td>Adenovirus 5 E2</td>
<td>Ela</td>
</tr>
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<td>c-jun</td>
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<td>Murine MX Gene</td>
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</tr>
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<td>GRP78 Gene</td>
<td>A23187</td>
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<tr>
<td>α-2-Macroglobulin</td>
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<tr>
<td>Vimentin</td>
<td>Serum</td>
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<td>MHC Class I Gene H-2kB</td>
<td>Interferon</td>
</tr>
<tr>
<td>HSP70</td>
<td>Ela, SV40 Large T Antigen</td>
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<td>Proliferin</td>
<td>Phorbol Ester-TPA</td>
</tr>
<tr>
<td>Tumor Necrosis Factor</td>
<td>FMA</td>
</tr>
<tr>
<td>Thyroid Stimulating Hormone α Gene</td>
<td>Thyroid Hormone</td>
</tr>
<tr>
<td>Insulin E Box</td>
<td>Glucose</td>
</tr>
</tbody>
</table>

3. **Secretory Cell-Specific Promoters**

In certain aspects of the present invention, the expression of the transforming genetic construct is under the control of a promoter. The promoter is required to express the transforming genetic construct to a degree sufficient to effect transformation of a target cell type amongst a population of different cell types such that the transformed target cell results in the generation of a stable human regulated secretory cell.
Promoters can be classified into two groups, ubiquitous and tissue- or cell-specific. Ubiquitous promoters activate transcription in all or most tissues and cell types. Examples of ubiquitous promoters are cellular promoters like the histone promoters, promoters for many metabolic enzyme genes such as hexokinase I and glyceraldehyde-3-phosphate dehydrogenase, and many viral promoters such as CMVp and the Rous sarcoma virus promoter (RSVp).

Tissue- or cell-specific promoters activate transcription in a restricted set of tissues or cell types or, in some cases, only in a single cell type of a particular tissue. Examples of stringent cell-specific promoters are the insulin gene promoters which are expressed in only a single cell type (pancreatic β cells) while remaining silent in all other cell types, and the immunoglobulin gene promoters which are expressed only in cell types of the immune system.

Various exemplary promoters are shown above in Table 1 (Pearse and Takor, 1979; Nylen and Becker, 1995). Although not a complete list, these promoters are exemplary of the types of promoters contemplated for use in the present invention. Additional promoters useful in the present invention will be readily known to those of skill in the art.

The promoter may be "context specific" in that it will be expressed only in the desired cell type and not in other cell types that are likely to be present in the population of target cells, e.g., it will be expressed in β cells, but not in α or δ cells, when introduced into intact human islets. In this scenario, an insulin promoter targets the expression of a linked transforming oncogene selectively to β cells of a human islet preparation even though many other contaminating cell types exist in the preparation.

As the present invention is applicable to the generation of stably transformed neuroendocrine secretory cell lines other than β cells, other context specific promoters may be employed. For example, the cell-specific prolactin gene promoter can be used to express a linked transforming oncogene selectively to lactotrophs surrounded by all the other cell types present in a pituitary cell preparation.
a. **β Cell-Specific Promoters.**

It has been documented that the two rat insulin gene promoters, RIP1 (GenBank accession number J00747) and RIP2 (GenBank accession number J00748), as well as the human insulin promoter (HIP; GenBank accession number V00565), direct stringent cell-specific expression of the insulin gene in rodent β cell insulinoma lines (German et al. 1990), primary islet cells (Melloul et al. 1993), and in β cells of transgenic mice (Efrat et al. 1988).

As the sequence and position of the functional promoter elements are well conserved between HIP, RIP1 and RIP2, the transcription factors that interact with these elements are likely to be conserved across species. In fact, HIP can direct cell-specific expression of linked genes in rodent β cell lines and rat primary islets, albeit, at a somewhat lower level than that observed for RIP1 (Melloul et al. 1993).

The inventors postulate that RIP1 and RIP2 should function effectively in human β cells. However, due to the lack of any human insulinoma cell lines and to the difficulty of obtaining human primary islets, there has been a dearth of analysis of the human or rat insulin promoters in human β cells.

Melloul et al., (1993) demonstrated that the isolated 50-bp RIP1 FAR/FLAT minienhancer (FF), an essential promoter element for RIP1 activity, could express a linked reporter gene in both adult rat and human islet cells. Furthermore, FF activity could be substantially induced by increased concentrations of glucose in both species of adult islets. Additional results from gel-shift studies strongly suggested that the same or similar β cell-specific transcription factor(s) from both adult rat and human islet cell nuclear extracts bound to conserved sequences contained within both the RIP1 FF and the analogous region of HIP.

b. **Further Neuroendocrine Cell-Specific Promoters.**

Representative are the glucagon promoter, GenBank accession number X03991; growth hormone promoter, GenBank accession numbers J03071 and K00470; POMC gene promoter, GenBank accession numbers V01510 and K02406; calcitonin promoter, GenBank accession number X15943; and the GIP gene promoter, GenBank accession number M31674.
c. Modified Promoters.

Promoters can be modified in a number of ways to increase their transcriptional activity. Multiple copies of a given promoter can be linked in tandem, mutations which increase activity may be introduced, single or multiple copies of individual promoter elements may be attached, parts of unrelated promoters may be fused together, or some combination of all of the above can be employed to generate highly active promoters. All such methods are contemplated for use in connection with the present invention.

German et al., (1992) mutated three nucleotides in the transcriptionally important FLAT E box of the rat insulin I gene promoter (RIP), resulting in a three- to four-fold increase in transcriptional activity of the mutated RIP compared to that of a nonmutated RIP as assayed in transiently transfected HIT cells. Also, the introduction of multiple copies of a promoter element from the E. coli tetracycline resistance operon promoter were introduced into the CMV promoter, significantly increasing the activity of this already very potent promoter (Liang et al., 1996). Additionally, part of the CMV promoter, which has high but short-lived transcriptional activity in dog myoblasts, was linked to the muscle-specific creatine kinase promoter (MCKp), which has weak but sustained expression in dog myoblasts, resulting in a hybrid promoter that sustained high-level expression for extended periods in dog myoblasts.

d. Multimerized Promoters.

Several modified rat insulin promoters (modRIP) containing multimerized enhancer elements have been engineered. The currently preferred modRIP contains six multimerized repeats of a 50 base pair region of the cis acting enhancer of RIP, placed upstream of an intact copy of RIP.

These novel promoters have been shown to direct expression of transgenes in stably engineered β cell lines at levels above those attained with unmodified insulin promoters and, in some cases, approaching the levels achieved with the Cytomegalovirus promoter (CMVp). CMVp is one of the strongest activating promoters known, but in a very non-tissue specific manner. Therefore, the present modified rat insulin promoters can be used to direct the tissue
specific expression of transforming genes at levels presently achievable only with the non-
specific CMVp.

4. Other Regulatory Elements

Where a cDNA insert is employed, one will typically desire to include a
polyadenylation signal to effect proper polyadenylation of the gene transcript. The nature of
the polyadenylation signal is not believed to be crucial to the successful practice of the
invention, and any such sequence may be employed. Also contemplated as an element of the
expression cassette is a terminator. These elements can serve to enhance message levels and to
minimize read through from the cassette into other sequences.

5. Selectable Markers

In certain embodiments of the invention, the delivery of a nucleic acid in a cell may be
identified *in vitro* or *in vivo* by including a marker in the expression construct. The marker
would result in an identifiable change to the transfected cell permitting easy identification of
expression. Usually the inclusion of a drug selection marker aids in cloning and in the selection
of transformants, for example, neomycin, puromycin, hygromycin, DHFR, GPT, zeocin and
histidinol. Alternatively, enzymes such as herpes simplex virus thymidine kinase (*tk*)
(eukaryotic) or chloramphenicol acetyltransferase (CAT) (prokaryotic) may be employed.
Immunologic markers also can be employed. The selectable marker employed is not believed
to be important, so long as it is capable of being expressed simultaneously with the nucleic acid
encoding a gene product. Further examples of selectable markers are well known to one of skill
in the art.

6. Multigene constructs and IRES

In certain embodiments of the invention, the use of internal ribosome binding sites
(IRES) elements are used to create multigene, or polycistronic, messages. IRES elements are
able to bypass the ribosome scanning model of 5' methylated Cap dependent translation and
begin translation at internal sites (Pelletier and Sonenberg, 1988). IRES elements from two
members of the picanovirus family (polio and encephalomyocarditis) have been described
(Pelletier and Sonenberg, 1988), as well an IRES from a mammalian message (Macejak and
Sarnow, 1991). IRES elements can be linked to heterologous open reading frames. Multiple open reading frames can be transcribed together, each separated by an IRES, creating polycistronic messages. By virtue of the IRES element, each open reading frame is accessible to ribosomes for efficient translation. Multiple genes can be efficiently expressed using a single promoter/enhancer to transcribe a single message.

Any heterologous open reading frame can be linked to IRES elements. This includes genes for secreted proteins, multi-subunit proteins, encoded by independent genes, intracellular or membrane-bound proteins and selectable markers. In this way, expression of several proteins can be simultaneously engineered into a cell with a single construct and a single selectable marker.

**G. Secretory Cell Culture**

The neuroendocrine cell and the stable neuroendocrine cells of the present invention will be grown in cell culture. The present section describes the methodology related to growth of cells in culture.

**1. Culture Conditions**

Primary cells are expanded by established culture conditions. For example, β cells can be cultured and even induced to divide as described (Clark and Chick, 1990; Beattie et al., 1991; Hayek et al., 1995; each incorporated herein by reference).

Human islets isolated by an automated method (Ricordi et al., 1988) are maintained in culture and transformed by the inventive engineered expression of proteins that promote accelerated cell replication. The transformation methods of the invention preferably involve the use of specific culture conditions designed to preferentially promote neuroendocrine cell growth, which are used in conjunction with the stable activation of cell specific gene promoter controlled protein expression.

The culture conditions are achieved by manipulating the following cell culture variables: media growth/survival factors (such as IGF-1, growth hormone, prolactin, PDGF,
hepatocyte growth factor, and transferrin), media differentiation factors (such as TGF-β), media lipids, media metabolites (such as glucose, pyruvate, galactose, and amino acids), media serum (percentage serum, serum fraction, species of serum), gaseous exchange (ratio atmospheric O₂:CO₂, and media volume), physical form of the islets prior to plating (whole, dispersed, or cell sorted islet cells), and extracellular substrate for cellular attachment (such as laminin, collagen, matrigel, and HTB-9 bladder carcinoma derived matrix).

Fibroblast growth/survival in culture is eliminated by culturing the islets in defined media using factors (such as IGF-1, cysteine, and growth hormone) to selectively confer a growth/survival advantage to β cells in preference to fibroblasts. Establishment of fibroblast free cultures will allow prolonged maintenance of human islet β cells in culture for subsequent infection with adenovirus expression vectors in cases where β cells are in a non-proliferative state, or retrovirus expression vectors for β cells which have been induced to proliferate by oncogene expression. Fibroblasts may even be killed by fibroblast-directed toxins.

2. Defined Media

Media comprising one or more growth factors that stimulate the growth of the target neuroendocrine cell and do not substantially stimulate growth of distinct cells in the cell population; i.e., act to induce preferential growth of the target cells rather than faster-growing, more hardy cells in the population, as may be used to deplete fibroblasts. Examples include defined serum free conditions used for β cells (Clark et al., 1990; incorporated herein by reference), or inclusion of growth or differentiation factors known to allow preferential growth of β cells (WO 95/29989; incorporated herein by reference).

In particular embodiment, the inventors have developed a media composition that will be particularly useful in the growth and propagation of the cells of the present invention. The rational behind the development of "BetaGene" medium had its beginnings with the observation that in bioreactor high density cultures of β-cell line RIN-38, ethanolamine was a rapidly consumed component of the growth medium. An equimolar mixture of ethanolamine-phosphoethanolamine was found to protect RIN-38 β-cells from linoleic acid toxicity (approximately 30 μg/ml in serum-free medium).
Subsequently, it was found that bioreactor cultures of this cell line could be maintained for weeks in the absence of serum when the medium was supplemented with a mixture of ethanolamine-phosphoethanolamine, bovine serum albumin, and transferrin. These indications for critical lipid components ensured that other lipid components as lipoic acid, inositol (both indicated as protective in vivo, in the literature), cholesterol, TWEEN 80 and putrescine would be included in the subsequent medium formulations.

A second finding was critical to the determination of the optimal formulation for β-cells. This was the development of a rapid screening method for evaluating the best commercial medium formulation. The method entails encapsulating βG18/3E1 cells- a rodent β-cell line engineered to secrete human insulin- in 1.5% alginate beads. β-cells encapsulated in beads are very amenable to serumless culture, and beads were cultured in different media ±FBS for 3-6 days and insulin secretion was monitored to estimate growth and function.

The serumless cultures then were returned to the same base medium supplemented with FBS, with continued insulin monitoring. The media screened were those most commonly used for culture of primary islets in the literature. Performance of the different media were indicated by the rate and magnitude of functional loss, as well as the rate of recovery and completeness of recovery after return to FBS supplementation. One medium CMRL1066 was clearly inferior, while M199, and a-MEM were fairly equivalent. Media such as F12 and RPMI were not readily evaluated by this approach, due to the low calcium concentration of these media and resultant deterioration of the Ca-alginate hydrogel. The latter were then evaluated as equal mixtures with M199 and MEM. An M199-F12 mixture was determined to be the best performing formulation tested, while an MEM-F12 mixture could be used with at least short term equivalency. Many components of the βG Medium are at concentrations that would be found in mixed formulations (others reflect our optimization).

Cells in alginate beads have been used routinely to screen medium components. This approach has simplified and greatly accelerated screening studies. The use of cells in beads has been refined to include acutely stimulated insulin secretion. This has led to the identification of
culture supplements that are critical to maintaining secretory function in β-cells / β-cell lines in the absence of serum. (Additional lipids minor, BSA & transferrin major).

The knowledge that normal β-cells have high ascorbate concentrations, and that PAM, the enzyme responsible for amidation of such islet peptides as pancreatic polypeptide and amylin, requires ascorbate, and Cu, led to the inclusion of these components. However, ascorbate is quite unstable in medium at 37°C, therefore, a stabilized form of ascorbate was screened for dose-dependent deleterious effects on growth and insulin secretion. None was encountered over a wide range of concentrations from 10^-6 to 10^-3 M. An intermediate concentration of stabilized ascorbate (ascorbate-phosphate) was tested for its effect on amidation, using cells engineered to express GLP-1 or amylin. The intermediate concentration (50-100 μM) was found to greatly improve amidation, both in flask and high density scale-up cultures, and was thus identified as the concentration for βG Medium.

Bicarbonate was increased in the formulation to provide better pH control for scale-up cultures (such as the CellCube™). Zinc was supplemented because primary beta cells have high concentrations of zinc and several islet enzymes bind Zn, and insulin crystal is coordinate with Zn. Finally, glucose concentrations are known to be critical for β-cell culture. One objective of the medium development was to derive a formulation that would optimally support primary pancreatic islets as well as β-cell lines. As a result, human islets were used to determine a glucose concentration that could support survival and function of human islets in culture. Glucose in the range of 7 mM (6-8 mM) provided long term survival (months) of human islets, with maintenance of glucose sensing, as indicated by dose-response studies of glucose-induced insulin secretion, and by maintained (and in islets of two donors restored insulin processing).
### TABLE 9
THE FOLLOWING SPECIFICATION PROVIDES AN EXEMPLARY COMPOSITION OF BETAGENE MEDIUM

<table>
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<td>g/L</td>
<td>mmol/L</td>
<td>g/L</td>
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<td>0.280615</td>
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<td>0.2107</td>
<td>0.998578</td>
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<td>Arginine•H₂O</td>
<td>0.05</td>
<td>0.333333</td>
<td>d-biotin</td>
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<td>Aspartate</td>
<td>0.03</td>
<td>0.225564</td>
<td>Ergocalciferol</td>
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<tr>
<td>Cysteine•HCl•H₂O</td>
<td>0.03512</td>
<td>0.199545</td>
<td>D-Ca Pantothenate</td>
</tr>
<tr>
<td>Cystine•2HCl</td>
<td>0.01564</td>
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<td>Glutamic acid•2H₂O</td>
<td>0.075</td>
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<td>0.684932</td>
<td>myo-inositol</td>
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<td>Glycine</td>
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### Table 9 - Continued

<table>
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<th>Formulation</th>
<th>Component</th>
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<tr>
<td><strong>Amino acids</strong></td>
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<td><strong>Vitamins</strong></td>
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<td>Valine</td>
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<td></td>
</tr>
<tr>
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<td>-</td>
<td></td>
</tr>
<tr>
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<td>-</td>
<td></td>
</tr>
<tr>
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<td><strong>Salts</strong></td>
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<td>Glucose</td>
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<td>CaCl$_2$</td>
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<td>1.050541</td>
<td></td>
</tr>
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<td>Glutathione (reduced)</td>
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<td>8.14332 E-05</td>
<td></td>
</tr>
<tr>
<td>CuSO$_4$•5H$_2$O</td>
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<td>0.000010</td>
<td></td>
</tr>
<tr>
<td>Guanine•HCl</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Fe(NO$_3$)$_3$•9H$_2$O</td>
<td>-</td>
<td>Hypoxanthine</td>
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</tr>
<tr>
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</tr>
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<tr>
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<td>NaCl</td>
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<td>Putrescine•2HCl</td>
</tr>
<tr>
<td>NaHCO$_3$</td>
<td>2.2</td>
<td>26.190476</td>
<td>Ribose</td>
</tr>
<tr>
<td>NaH$_2$PO$_4$•H$_2$O</td>
<td>0.07</td>
<td>0.507246</td>
<td>Sodium Acetate</td>
</tr>
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<td>Na$_2$PO$_4$</td>
<td>0.07102</td>
<td>0.500141</td>
<td>Sodium Pyruvate</td>
</tr>
<tr>
<td>ZnSO$_4$•7H$_2$O</td>
<td>0.000863</td>
<td>0.002997</td>
<td>Thymidine</td>
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<tr>
<td>Manganese</td>
<td></td>
<td>TWEEN 80</td>
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</tr>
<tr>
<td>Xanthine Na</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Uracil</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>


3. **Proliferation**

Cells may be induced to proliferate by initial infection with adenovirus or adeno-associated virus (AAV) comprising a gene that induces cellular proliferation, the gene being under the control of a promoter specific for the regulated secretory cell. The cells may be induced to proliferate by growth on a stimulatory cell matrix (Hayek et al., 1995).

4. **In vivo Passage**

A potential concern is that the studies of Hayek and associates (WO 95/29989) have indicated that as human islet cell growth is stimulated, insulin content can fall rapidly. If this same phenomenon occurs as β cell proliferation is stimulated by methods of the present invention, expression of the insulin promoter driven oncogene or of the endogenous insulin gene may also decline. The use of modified RIP promoters with enhanced activity may overcome this concern.

Alternatively, previous investigators have shown that the fall in insulin content experienced in replicating human islet cells can be partially restored by transplantation of the cells in athymic rodents (Neisor et al., 1979; Beattie et al., 1995). Therefore, to complete the transformation process, it may be necessary to expose the cells to the *in vivo* environment.

Cell transplantation studies in nude rats are straightforward and *in vivo* passage can readily be included as a component of human β cell line generation. The transformed human cells may be placed *in vivo*, e.g., under kidney capsule of the nude rat, to allow outgrowth of transformed cells. In addition to promoting maintenance of the tissue specific expression of the oncogene in the primary cells, the lack of an immune response in the nude rat is known to allow long term survival and expression of recombinant adenovirus infected cells (Dai et al., 1995; Yang et al., 1994b).

**H. DNA Delivery**

In order for the neuroendocrine cell to be immortalized by the genetic construct or to stably express the secretory polypeptide to interest, the nucleic acid encoding the genes may be stably integrated into the genome of the cell. In yet further embodiments, the nucleic acid may
be stably maintained in the cell as a separate, episomal segment of DNA. Such nucleic acid segments or "episomes" encode sequences sufficient to permit maintenance and replication independent of, or in synchronization with the host cell cycle. How the expression construct is delivered to a cell and where in the cell the nucleic acid remains is dependent on the type of expression construct employed. All expression constructs and delivery methods are contemplated for use in the context of the present invention, although certain methods are preferred, as outlined below.

1. Transfection

In order to effect expression of a gene construct, the expression construct must be delivered into a cell. As described below, the preferred mechanism for delivery is via viral infection, where the expression construct is encapsidated in an infectious viral particle. However, several non-viral methods for the transfer of expression constructs into cultured mammalian cells also are contemplated by the present invention. In one embodiment of the present invention, the expression construct may consist only of naked recombinant DNA or plasmids. Transfer of the construct may be performed by any of the methods mentioned which physically or chemically permeabilize the cell membrane.

a. Liposome-Mediated Transfection.

In a particular embodiment of the invention, the expression construct may be entrapped in a liposome. Liposomes are vesicular structures characterized by a phospholipid bilayer membrane and an inner aqueous medium. Multilamellar liposomes have multiple lipid layers separated by aqueous medium. They form spontaneously when phospholipids are suspended in an excess of aqueous solution. The lipid components undergo self-rearrangement before the formation of closed structures and entrap water and dissolved solutes between the lipid bilayers (Ghosh and Bachhawat, 1991). Also contemplated is an expression construct complexed with Lipofectamine (Gibco BRL).

Liposome-mediated nucleic acid delivery and expression of foreign DNA in vitro has been very successful (Nicolau and Sene, 1982; Fraley et al., 1979; Nicolau et al., 1987). Wong
et al., (1980) demonstrated the feasibility of liposome-mediated delivery and expression of foreign DNA in cultured chick embryo, HeLa and hepatoma cells.

In certain embodiments of the invention, the liposome may be complexed with a hemagglutinating virus (HVJ). This has been shown to facilitate fusion with the cell membrane and promote cell entry of liposome-encapsulated DNA (Kaneda et al., 1989). In other embodiments, the liposome may be complexed or employed in conjunction with nuclear non-histone chromosomal proteins (HMG-1) (Kato et al., 1991). In yet further embodiments, the liposome may be complexed or employed in conjunction with both HVJ and HMG-1.


b. Electroporation

In certain other embodiments of the present invention, the expression construct is introduced into the cell via electroporation. Electroporation involves the exposure of a suspension of cells and DNA to a high-voltage electric discharge.

Transfection of eukaryotic cells using electroporation has been quite successful. Mouse pre-B lymphocytes have been transfected with human kappa-immunoglobulin genes (Potter et al., 1984), and rat hepatocytes have been transfected with the chloramphenicol acetyltransferase gene (Tur-Kaspa et al., 1986) in this manner. Examples of electroporation of islets include Soldevila et al., (1991) and PCT application WO 91/09939.

c. Calcium Phosphate Precipitation or DEAE-Dextran Treatment.

In other embodiments of the present invention, the expression construct is introduced to the cells using calcium phosphate precipitation. Human KB cells have been transfected with adenovirus 5 DNA (Graham and Van Der Eb, 1973) using this technique. Also in this manner, mouse L(A9), mouse C127, CHO, CV-1, BHK, NIH3T3 and HeLa cells were transfected with
a neomycin marker gene (Chen and Okayama, 1987), and rat hepatocytes were transfected with a variety of marker genes (Rippe et al., 1990).

In another embodiment, the expression construct is delivered into the cell using DEAE-dextran followed by polyethylene glycol. In this manner, reporter plasmids were introduced into mouse myeloma and erythroleukemia cells (Gopal, 1985).

d. **Particle Bombardment**

Another embodiment of the invention for transferring a naked DNA expression construct into cells may involve particle bombardment. This method depends on the ability to accelerate DNA-coated microprojectiles to a high velocity allowing them to pierce cell membranes and enter cells without killing them (Klein et al., 1987). Several devices for accelerating small particles have been developed. One such device relies on a high voltage discharge to generate an electrical current, which in turn provides the motive force (Yang et al., 1990). The microprojectiles used have consisted of biologically inert substances such as tungsten or gold beads. Gainer et al., (1996) have transfected mouse islets with a luciferase gene/human immediate early promoter reporter construct, using ballistic particles accelerated by helium pressure.

e. **Direct Microinjection or Sonication Loading.**

Further embodiments of the present invention include the introduction of the expression construct by direct microinjection or sonication loading. Direct microinjection has been used to introduce nucleic acid constructs into *Xenopus* oocytes (Harland and Weintraub, 1985), and LTK⁻ fibroblasts have been transfected with the thymidine kinase gene by sonication loading (Fechheimer et al., 1987).

f. **Adenoviral Assisted Transfection.**

In certain embodiments of the present invention, the expression construct is introduced into the cell using adenovirus assisted transfection. Increased transfection efficiencies have been reported in cell systems using adenovirus coupled systems (Kelleher and Vos, 1994;
Cotten et al., 1992; Curie, 1994), and the inventors contemplate using the same technique to increase transfection efficiencies into human islets.

**g. Receptor Mediated Transfection.**

Still further expression constructs that may be employed to deliver the tissue-specific promoter and transforming construct to the target cells are receptor-mediated delivery vehicles. These take advantage of the selective uptake of macromolecules by receptor-mediated endocytosis that will be occurring in the target cells. In view of the cell type-specific distribution of various receptors, this delivery method adds another degree of specificity to the present invention. Specific delivery in the context of another mammalian cell type is described by Wu and Wu (1993; incorporated herein by reference).

Certain receptor-mediated gene targeting vehicles comprise a cell receptor-specific ligand and a DNA-binding agent. Others comprise a cell receptor-specific ligand to which the DNA construct to be delivered has been operatively attached. Several ligands have been used for receptor-mediated gene transfer (Wu and Wu, 1987, 1988; Wagner et al., 1990; Ferkol et al., 1993; Perales et al., 1994; Myers, EPO 0273085), which establishes the operability of the technique. In the context of the present invention, the ligand will be chosen to correspond to a receptor specifically expressed on the neuroendocrine target cell population.

In other embodiments, the DNA delivery vehicle component of a cell-specific gene targeting vehicle may comprise a specific binding ligand in combination with a liposome. The nucleic acids to be delivered are housed within the liposome and the specific binding ligand is functionally incorporated into the liposome membrane. The liposome will thus specifically bind to the receptors of the target cell and deliver the contents to the cell. Such systems have been shown to be functional using systems in which, for example, epidermal growth factor (EGF) is used in the receptor-mediated delivery of a nucleic acid to cells that exhibit upregulation of the EGF receptor.

In still further embodiments, the DNA delivery vehicle component of the targeted delivery vehicles may be a liposome itself, which will preferably comprise one or more lipids
or glycoproteins that direct cell-specific binding. For example, Nicolau et al. (1987) employed lactosyl-ceramide, a galactose-terminal asialganglioside, incorporated into liposomes and observed an increase in the uptake of the insulin gene by hepatocytes. It is contemplated that the tissue-specific transforming constructs of the present invention can be specifically delivered into the target cells in a similar manner.

2. Viral Infection
   a. Adenoviral Infection.

   One of the preferred methods for delivery of the transforming constructs involves the use of an adenovirus expression vector. Although adenovirus vectors are known to have a low capacity for integration into genomic DNA, this feature is counterbalanced by the high efficiency of gene transfer afforded by these vectors. "Adenovirus expression vector" is meant to include those constructs containing adenovirus sequences sufficient to (a) support packaging of the construct and (b) to ultimately express a tissue-specific transforming construct that has been cloned therein.

   The expression vector comprises a genetically engineered form of adenovirus. Knowledge of the genetic organization of adenovirus, a 36 kb, linear, double-stranded DNA virus, allows substitution of large pieces of adenoviral DNA with foreign sequences up to 7 kb (Grunhaus and Horwitz, 1992). In contrast to retrovirus, the adenoviral infection of host cells does not result in chromosomal integration because adenoviral DNA can replicate in an episomal manner without potential genotoxicity. Also, adenoviruses are structurally stable, and no genome rearrangement has been detected after extensive amplification.

   Adenovirus is particularly suitable for use as a gene transfer vector because of its mid-sized genome, ease of manipulation, high titer, wide target-cell range and high infectivity. Both ends of the viral genome contain 100-200 base pair inverted repeats (ITRs), which are cis elements necessary for viral DNA replication and packaging. The early (E) and late (L) regions of the genome contain different transcription units that are divided by the onset of viral DNA replication. The E1 region (E1A and E1B) encodes proteins responsible for the regulation of transcription of the viral genome and a few cellular genes. The expression of the E2 region
(E2A and E2B) results in the synthesis of the proteins for viral DNA replication. These proteins are involved in DNA replication, late gene expression and host cell shut-off (Renan, 1990). The products of the late genes, including the majority of the viral capsid proteins, are expressed only after significant processing of a single primary transcript issued by the major late promoter (MLP). The MLP, located at 16.8 m.u., is particularly efficient during the late phase of infection, and all the mRNA's issued from this promoter possess a 5'-tripartite leader (TPL) sequence which makes them preferred mRNA's for translation.

In a current system, recombinant adenovirus is generated from homologous recombination between shuttle vector and provirus vector. Due to the possible recombination between two proviral vectors, wild-type adenovirus may be generated from this process. Therefore, it is critical to isolate a single clone of virus from an individual plaque and examine its genomic structure.

Generation and propagation of the current adenovirus vectors, which are replication deficient, depend on a helper cell line, designated 293, which was transformed from human embryonic kidney cells by Ad5 DNA fragments and constitutively expresses E1 proteins (Graham et al., 1977). Since the E3 region is dispensable from the adenovirus genome (Jones and Shenk, 1978), the current adenovirus vectors, with the help of 293 cells, carry foreign DNA in either the E1, the D3 or both regions (Graham and Prevec, 1991). In nature, adenovirus can package approximately 105% of the wild-type genome (Ghosh-Choudhury et al., 1987), providing capacity for about 2 extra kb of DNA. Combined with the approximately 5.5 kb of DNA that is replaceable in the E1 and E3 regions, the maximum capacity of the current adenovirus vector is under 7.5 kb, or about 15% of the total length of the vector. More than 80% of the adenovirus viral genome remains in the vector backbone.

Helper cell lines may be derived from human cells such as human embryonic kidney cells, muscle cells, hematopoietic cells or other human embryonic mesenchymal or epithelial cells. Alternatively, the helper cells may be derived from the cells of other mammalian species that are permissive for human adenovirus. Such cells include, e.g., Vero cells or other monkey
embryonic mesenchymal or epithelial cells. As stated above, the preferred helper cell line is 293.

Recently, Racher et al., (1995) disclosed improved methods for culturing 293 cells and propagating adenovirus. In one format, natural cell aggregates are grown by inoculating individual cells into 1 liter siliconized spinner flasks (Techne, Cambridge, UK) containing 100-200 ml of medium. Following stirring at 40 rpm, the cell viability is estimated with trypan blue. In another format, Fibra-Cel microcarriers (Bibby Sterlin, Stone, UK) (5 g/l) is employed as follows. A cell inoculum, resuspended in 5 ml of medium, is added to the carrier (50 ml) in a 250 ml Erlenmeyer flask and left stationary, with occasional agitation, for 1 to 4 h. The medium is then replaced with 50 ml of fresh medium and shaking initiated. For virus production, cells are allowed to grow to about 80% confluence, after which time the medium is replaced (to 25% of the final volume) and adenovirus added at an MOI of 0.05. Cultures are left stationary overnight, following which the volume is increased to 100% and shaking commenced for another 72 h.

Other than the requirement that the adenovirus vector be replication defective, or at least conditionally defective, the nature of the adenovirus vector is not believed to be crucial to the successful practice of the invention. The adenovirus may be of any of the 42 different known serotypes or subgroups A-F. Adenovirus type 5 of subgroup C is the preferred starting material in order to obtain the conditional replication-defective adenovirus vector for use in the present invention. This is because Adenovirus type 5 is a human adenovirus about which a great deal of biochemical and genetic information is known, and it has historically been used for most constructions employing adenovirus as a vector.

As stated above, the typical vector according to the present invention is replication defective and will not have an adenovirus E1 region. Thus, it will be most convenient to introduce the transforming construct at the position from which the E1-coding sequences have been removed. However, the position of insertion of the construct within the adenovirus sequences is not critical to the invention. The polynucleotide encoding the gene of interest may also be inserted in lieu of the deleted E3 region in E3 replacement vectors as described by
Karlsson et al. (1986) or in the E4 region where a helper cell line or helper virus complements the E4 defect.

Adenovirus growth and manipulation is known to those of skill in the art, and exhibits broad host range in vitro and in vivo. This group of viruses can be obtained in high titers, e.g., $10^9$-$10^{11}$ plaque-forming units per ml, and they are highly infective. The life cycle of adenovirus does not require integration into the host cell genome. The foreign genes delivered by adenovirus vectors are episomal and, therefore, have low genotoxicity to host cells. No side effects have been reported in studies of vaccination with wild-type adenovirus (Couch et al., 1963; Top et al., 1971), demonstrating their safety and therapeutic potential as in vivo gene transfer vectors.

Adenovirus vectors have been used in eukaryotic gene expression (Levrero et al., 1991; Gomez-Foix et al., 1992) and vaccine development (Grunhaus and Horwitz, 1992; Graham and Prevec, 1992). Recently, animal studies suggested that recombinant adenovirus could be used for gene therapy (Stratford-Perricaudet and Perricaudet, 1991: Stratford-Perricaudet et al., 1990; Rich et al., 1993). Studies in administering recombinant adenovirus to different tissues include trachea instillation (Rosenfeld et al., 1991; Rosenfeld et al., 1992), muscle injection (Ragot et al., 1993), peripheral intravenous injections (Herz and Gerard, 1993) and stereotactic inoculation into the brain (Le Gal La Salle et al., 1993).

Recombinant adenovirus and adeno-associated virus (see below) can both infect and transduce non-dividing human primary cells. In fact, gene transfer efficiencies of approximately 70% for isolated rat islets have been demonstrated by the inventors (Becker et al., 1994a; Becker et al., 1994b; Becker et al., 1996) as well as by other investigators (Gainer et al., 1996).

b. AAV Infection.

Adeno-associated virus (AAV) is an attractive vector system for use in the human cell transformation of the present invention as it has a high frequency of integration and it can infect nondividing cells, thus making it useful for delivery of genes into mammalian cells in tissue
culture (Muzychka, 1992). AAV has a broad host range for infectivity (Tratschin et al., 1984; Laughlin, et al., 1986; Lebkowski, et al., 1988; McLaughlin, et al., 1988), which means it is applicable for use with human neuroendocrine cells, however, the tissue-specific promoter aspect of the present invention will ensure specific expression of the transforming construct. Details concerning the generation and use of rAAV vectors are described in U.S. Patent No. 5,139,941 and U.S. Patent No. 4,797,368, each incorporated herein by reference.

Studies demonstrating the use of AAV in gene delivery include LaFace et al., (1988); Zhou et al., (1993); Flotte et al., (1993); and Walsh et al., (1994). Recombinant AAV vectors have been used successfully for in vitro and in vivo transduction of marker genes (Kaplitt et al., 1994; Lebkowski et al., 1988; Samulski et al., 1989; Shelling and Smith, 1994; Yoder et al., 1994; Zhou et al., 1994; Hermonat and Muzychka, 1984; Tratschin et al., 1985; McLaughlin et al., 1988) and genes involved in human diseases (Flotte et al., 1992; Luo et al., 1994; Ohi, et al., 1990; Walsh, et al., 1994; Wei, et al., 1994). Recently, an AAV vector has been approved for phase I human trials for the treatment of cystic fibrosis.

AAV is a dependent parvovirus in that it requires coinfection with another virus (either adenovirus or a member of the herpes virus family) to undergo a productive infection in cultured cells (Muzychka, 1992). In the absence of coinfection with helper virus, the wild type AAV genome integrates through its ends into human chromosome 19 where it resides in a latent state as a provirus (Kotin et al., 1990; Samulski et al., 1991). rAAV, however, is not restricted to chromosome 19 for integration unless the AAV Rep protein is also expressed (Shelling and Smith, 1994). When a cell carrying an AAV provirus is superinfected with a helper virus, the AAV genome is "rescued" from the chromosome or from a recombinant plasmid, and a normal productive infection is established (Samulski, et al., 1989; McLaughlin, et al., 1988; Kotin, et al., 1990; Muzychka, 1992).

Typically, recombinant AAV (rAAV) virus is made by cotransfecting a plasmid containing the gene of interest flanked by the two AAV terminal repeats (McLaughlin et al., 1988; Samulski et al., 1989; each incorporated herein by reference) and an expression plasmid containing the wild type AAV coding sequences without the terminal repeats, for example
pIM45 (McCarty et al., 1991; incorporated herein by reference). The cells are also infected or transfected with adenovirus or plasmids carrying the adenovirus genes required for AAV helper function. rAAV virus stocks made in such fashion are contaminated with adenovirus which must be physically separated from the rAAV particles (for example, by cesium chloride density centrifugation). Alternatively, adenovirus vectors containing the AAV coding regions or cell lines containing the AAV coding regions and some or all of the adenovirus helper genes could be used (Yang et al., 1994a; Clark et al., 1995). Cell lines carrying the rAAV DNA as an integrated provirus can also be used (Flotte et al., 1995).

The present invention contemplates infection of the target cells with a recombinant adeno-associated virus (AAV) containing an oncogene driven by a tissue specific promoter. Recombinant AAV plasmids with RIP driving T antigen have been constructed.

c. Retroviral Infection.

The retroviruses are a group of single-stranded RNA viruses characterized by an ability to convert their RNA to double-stranded DNA in infected cells by a process of reverse-transcription (Coffin, 1990). The resulting DNA then stably integrates into cellular chromosomes as a provirus and directs synthesis of viral proteins. The integration results in the retention of the viral gene sequences in the recipient cell and its descendants. The retroviral genome contains three genes, gag, pol, and env that code for capsid proteins, polymerase enzyme, and envelope components, respectively. A sequence found upstream from the gag gene contains a signal for packaging of the genome into virions. Two long terminal repeat (LTR) sequences are present at the 5' and 3' ends of the viral genome. These contain strong promoter and enhancer sequences and are also required for integration in the host cell genome (Coffin, 1990).

In order to construct a retroviral vector, a nucleic acid encoding a gene of interest is inserted into the viral genome in the place of certain viral sequences to produce a virus that is replication-defective. In order to produce virions, a packaging cell line containing the gag, pol, and env genes but without the LTR and packaging components is constructed (Mann et al., 1983). When a recombinant plasmid containing a cDNA, together with the retroviral LTR and
packaging sequences is introduced into this cell line (by calcium phosphate precipitation for example), the packaging sequence allows the RNA transcript of the recombinant plasmid to be packaged into viral particles, which are then secreted into the culture media (Nicolas and Rubenstein, 1988; Temin, 1986; Mann et al., 1983). The media containing the recombinant retroviruses is then collected, optionally concentrated, and used for gene transfer. Retroviral vectors are able to infect a broad variety of cell types. However, integration and stable expression require the division of host cells (Paskind et al., 1975).

Concern with the use of defective retrovirus vectors is the potential appearance of wild-type replication-competent virus in the packaging cells. This can result from recombination events in which the intact sequence from the recombinant virus inserts upstream from the gag, pol, env sequence integrated in the host cell genome. However, new packaging cell lines are now available that should greatly decrease the likelihood of recombination (Markowitz et al., 1988; Hersdorffer et al., 1990).

d. Other Viral Vectors.

Other viral vectors may be employed as expression constructs in the present invention. Vectors derived from viruses such as vaccinia virus (Ridgeway, 1988; Baichwal and Sugden, 1986; Coupar et al., 1988) and herpesviruses may be employed. They offer several attractive features for various mammalian cells (Friedmann, 1989; Ridgeway, 1988; Baichwal and Sugden, 1986; Coupar et al., 1988; Horwich et al., 1990).

With the recent recognition of defective hepatitis B viruses, new insight was gained into the structure-function relationship of different viral sequences. In vitro studies showed that the virus could retain the ability for helper-dependent packaging and reverse transcription despite the deletion of up to 80% of its genome (Horwich et al., 1990). This suggested that large portions of the genome could be replaced with foreign genetic material. Chang et al., recently introduced the chloramphenicol acetyltransferase (CAT) gene into duck hepatitis B virus genome in the place of the polymerase, surface, and pre-surface coding sequences. It was cotransfected with wild-type virus into an avian hepatoma cell line. Culture media containing
high titers of the recombinant virus were used to infect primary duckling hepatocytes. Stable CAT gene expression was detected for at least 24 days after transfection (Chang et al., 1991).

In still further embodiments of the present invention, the nucleic acids to be delivered are housed within an infective virus that has been engineered to express a specific binding ligand. The virus particle will thus bind specifically to the cognate receptors of the target cell and deliver the contents to the cell. A novel approach designed to allow specific targeting of retrovirus vectors was recently developed based on the chemical modification of a retrovirus by the chemical addition of lactose residues to the viral envelope. This modification can permit the specific infection of hepatocytes via sialoglycoprotein receptors.

Another approach to targeting of recombinant retroviruses was designed in which biotinylated antibodies against a retroviral envelope protein and against a specific cell receptor were used. The antibodies were coupled via the biotin components by using streptavidin (Roux et al., 1989). Using antibodies against major histocompatibility complex class I and class II antigens, they demonstrated the infection of a variety of human cells that bore those surface antigens with an ecotropic virus in vitro (Roux et al., 1989).

3. Multiple Viral Infection

A further alternative for practicing the present invention is to use adenovirus or AAV infection of primary cells leading to in vitro expansion of a primary cell population that is then amenable to stable oncogene transfer by methods requiring cell growth such as retroviral transduction, plasmid transfection of expanding cells (Lipofectin or electroporation), or a second round of Adenovirus and/or AAV infection.

Another embodiment of the invention is to use alternating AAV and adenovirus infections. Propagation of AAV is dependent upon adenovirus, and using both viruses may lead to more productive infections. Such a method may increase the number of final cells that have oncogenes integrated and expressed.
Multiple, sequential viral infections may allow one of skill in the art to exploit the benefits of various viral delivery systems and avoid their limitations. For example, a limitation of adenoviral gene delivery is that this system affords a very low rate of integration of viral and recombinant DNAs into the host cell genome. Consequently, adenoviral gene expression is diluted when the cells divide and typically is used only for transient gene expression. An advantage that adenoviral gene delivery has over many other viral vectors is that entry of the virus into the cell and the expression of transgenic proteins is not dependent on cellular replication. This benefit of adenoviral gene delivery is in contrast to retroviruses where the integration and sustained expression of virally introduced DNA is dependent on cellular replication.

The coupling of these two viral systems for the transformation of primary tissues minimizes the limitations of each and maximally exploits their distinct biological properties. For example, primary human pancreatic β-cells typically do not divide in culture and are thereby resistant to transformation by immortalizing gene constructs delivered by retroviruses. However, human β-cells can be infected with adenovirus for the purposes of transgenic protein expression.

In a preferred embodiment, human β-cells or pancreatic islets would first be infected with a recombinant adenovirus that provides for the expression of a growth-promoting protein to stimulate cellular division. Cellular replication could be monitored by measuring thymidine incorporation or other techniques that have been developed to monitor DNA replication. In addition or alternatively, dividing cells could be enriched by FACS. Following the stimulation of cellular replication (about 12-96 hours following adenoviral infection), cells could be successfully infected with a recombinant retrovirus that has been engineered to express immortalizing gene products. The genomic DNA of a dividing cell population will be susceptible to stable integration by retrovirus and expression of recombinant proteins. This system of sequential and varied viral infections could further be optimized by the use of tissue-specific promoters for transgene expression in designated cell types and the expression of antibiotic resistance markers to selectively enrich for virally infected cells.
I. Glucose-Responsive Insulin Secretion

Glucose responsiveness is an important parameter in the neuroendocrine cell lines of the present invention. Immortal RIN cells have been shown to lose glucose responsiveness over time. The glucose-responsiveness can be re-engineered into a stable cell that secretes insulin but in which the glucose-response has been lost, diminished or shifted.

The basis for engineering the stable cells to produce a cell with glucose-regulated insulin secretion is disclosed in U.S. Patent 5,427,940, incorporated herein by reference. U.S. Patent 5,427,940 discloses islet and non-islet cell lines of neuroendocrine origin which are engineered for insulin expression and glucose regulation. First, even the insulin gene can be supplied to such an engineered cell and, although this will not be required in many aspects of the present invention, it is also contemplated.

The basis for such engineering originated in part with studies using AtT-20 cells, which are derived from ACTH secreting cells of the anterior pituitary. It has been demonstrated that stable transfection of AtT-20 cells with a construct in which a viral promotor is used to direct expression of the human proinsulin cDNA results in cell lines that secrete the correctly processed and mature insulin polypeptide (Moore et al., 1983). Insulin secretion from such lines (generally termed AtT-20ins) can be stimulated by agents such as forskolin or dibutyryl cAMP, with the major secreted product in the form of mature insulin. These results suggest that these cells contain a regulated secretory pathway that is similar to that operative in the islet β-cell (Moore et al., 1983. Gross et al., 1989). More recently, it has become clear that the endopeptidases that process proinsulin to insulin in the islet β-cell, termed PC2 and PC3, are also expressed in AtT-20ins cells (Smeekens and Steiner, 1990, Hakes et al., 1991).

1. GLUT-2 and Glucokinase

AtT-20ins cells do not respond to glucose as a secretagogue (Hughes et al., 1991). Interestingly, AtT-20 cells express the glucokinase gene (Hughes et al., 1991. Liang et al., 1991) and at least in some lines, low levels of glucokinase activity (Hughes et al., 1991; 1992; Quaade et al., 1991), but are completely lacking in GLUT-2 expression (Hughes et al., 1991;

The studies with AtT-20ins cells are important because they demonstrate that neuroendocrine cell lines that lack glucose-stimulated peptide release may be engineered for this function. Therefore, once a stable human neuroendocrine cell that has a regulated secretory pathway has been generated by the present invention, certain elements of the responsiveness can be reengineered into the stable cell. In contrast, the "regulated secretory pathway", including the secretory granules, endopeptidases and post-translational modification enzymes, cannot be reengineered into a cell lacking such a pathway.

One of the present inventors has previously shown that GLUT-2 and glucokinase work in tandem as the "glucose sensing apparatus" of the β-cell (U.S. Patent 5,427,940). U.S. Patent 5,427,940, incorporated herein by reference, describes methods for conferring glucose sensing in neuroendocrine cells and cell lines by transfection of such cells with one or more genes selected from the insulin gene, the glucokinase gene and the GLUT-2 glucose transporter gene, so as to provide an engineered cell having all three of these genes. The glucokinase and GLUT-2 genes are thus preferred for use in re-engineering stable human cells.

U.S. Patent 5,427,940 discloses that three functional genes are required to give glucose-responsive insulin secreting capacity to a cell: an insulin gene, a GLUT-2 glucose transporter gene and a glucokinase gene. In the practice of the re-engineering aspects of the present invention, therefore, it may be that only one of these three genes needs to additionally supplied, expressed or overexpressed.

Thus, if the stable human cell produces and expresses a reasonable level of insulin, but in a non-regulated manner, the provision of either or both of a functional glucokinase gene and a GLUT-2 gene will be desired. One of ordinary skill in the art will be readily able to test the levels of glucokinase and GLUT-2 expression, either by molecular biological hybridization or biochemical activity assays, in order to determine which one or both of such enzymes is not sufficiently expressed or active and should therefore be supplied in recombinant form. If the
stable cell does not express either of the aforementioned genes in a functional fashion, or at physiological levels, it will be preferred to introduce both genes. In re-engineering glucose-responsiveness using GLUT-2 and/or glucokinase the constructs of GenBank accession numbers J03145 and M25807, respectively, may be used. In other embodiments, even the insulin gene could be re-engineered and overexpressed in a stable cell of the invention.

2. Hexokinase Reduction

In studies in which the stable transfection of AtT-20ins cells with GLUT-2, but not GLUT-1, conferred glucose-stimulated insulin secretion, this was achieved with maximal responsiveness at subphysiological glucose levels. The inventor reasoned that this was likely due to a non-optimal hexokinase:glucokinase ratio (U.S. Patent 5,427,940).

In re-engineering glucose-responsiveness, the stable cells of the invention may be modified to any degree such that they have a reduced a low $K_m$ hexokinase activity relative to the stable parent cell from which the re-engineered cell was prepared. Depending on the intended use of the cells, cells in which a moderate hexokinase inhibition is achieved will have utility. Such inhibition levels are contemplated to be those in which the low $K_m$ hexokinase activity is reduced by at least about 5%, about 10%, about 15%, about 20%, or about 25% relative to control levels.

Re-engineered cells exhibiting more significant inhibition are also contemplated within the invention. Accordingly, cells in which the low $K_m$ hexokinase activity is reduced by about 30%, about 40%, about 50%, about 60% or even about 70% or higher, with respect to control levels, are contemplated as part of this invention and will be preferred in certain embodiments.

In embodiments of re-engineering a stable cell to secrete insulin in response to glucose, other parameters may be applied in assessing useful levels of low $K_m$ hexokinase inhibition. For example, it may be desired to determine the ratio of glucokinase to hexokinase (GK:HK ratio) and to monitor changes in this ratio as hexokinase is inhibited. It will be understood that a cell in which this ratio is changed to reflect the ratio commonly observed in functional or
natural pancreatic β–cells, or in which the ratio is changed towards this, will be an
advantageous engineered cell in the context of this invention.

In certain preferred embodiments, it is contemplated that cells of this invention will
have a low $K_m$ hexokinase activity that has been reduced to a level appropriate to confer more
physiological insulin secretion capacity to the cell. This includes re-engineered cells that have
a near-homeostatic insulin secretion capacity.

"Engineered cells that exhibit more physiological insulin secretion" are cells that exhibit
glucose-stimulated insulin secretion (GSIS) closer to the normal range than the parent stable
cell from which they were prepared. In this regard, the maximal glucose response of previously
described cell lines generally occurs at subphysiological glucose concentrations of between
about 10 mM and about 100 mM.

The GSIS of normal islet β–cells generally occurs at glucose concentrations of between
about 3 mM to 20 mM, with ranges of 5 to 20 mM and 4 to 9 mM being frequently reported.
Insulin responses in these ranges would therefore be described as "near-homeostatic insulin
secretion." Cells that comprise an inhibitor in an amount effective to reduce the low $K_m$
hexokinase activity of the cell to a level sufficient to confer insulin secretion in response to an
extracellular glucose concentration of between about 1 mM and about 20 mM will thus be most
preferred. Extracellular glucose concentrations of "between about 1 mM and about 20 mM"
will be understood to include each and every numerical value within this range, such as being
about 1, 2, 3, 4, 5, 7.5, 10, 12, 14, 16, 18, and about 20 mM or so.

To re-engineer the ratios of glucokinase to hexokinase by inhibiting hexokinase, and
thus to render the glucose-responsive insulin secretion more physiological, any one of a variety
of methods may be employed, including blocking of expression of the gene in the stable human
cells and/or inhibiting or reducing the activity of any protein produced. In creating molecular
biological tools to effect these methods, the hexokinase gene construct of GenBank accession
number J04526 may be utilized.
In molecular approaches suitable for reducing hexokinase activity via inhibiting gene expression, constructs can be designed to introduce nucleic acids complementary to a target endogenous gene, *i.e.*, an antisense approach. Expression of RNAs corresponding to these complementary nucleic acids will interfere with the transcription and/or translation of the target sequences. Inhibitory constructs can still further be designed to homologously recombine into the hexokinase endogenous gene locus, rendering the endogenous gene nonfunctional, *i.e.*, a knock-out approach. Genetic constructs may also be designed to introduce nucleic acids encoding ribozymes, RNA-cleaving enzymes, that will specifically cleave the target hexokinase mRNA. In other embodiments, the hexokinase activity may be abrogated by constructs designed to randomly integrate throughout the genome, resulting in loss of expression of the endogenous hexokinase gene. Further, the endogenous gene can be rendered dysfunctional by genomic site directed mutagenesis. These methods for blocking hexokinase production are well known to those of skill in the art. By way of example, WO publication numbers WO 97/26334 (published July 24, 1997) and WO 97/26321 (published July 24, 1997) describe these methodologies and are specifically incorporated herein by reference.

**J. Screening For Modulators Of Secretion**

The immortalized secretory cell lines described by the present invention have been shown to have a stable neuroendocrine phenotype. They are capable of providing a measurable secretion of the secretory product. Furthermore, they have been shown to be secretagogue responsive, thereby showing that these cell lines respond to modulators of secretory function. Therefore, within certain embodiments of the invention, methods are provided for screening for modulators of secretory function. Such methods may use the cells of the present invention either as adherent cells on a culture dish, as part of an alginate biomatrix, in suspension culture or in any other form that permits the secretion of the polypeptide to be monitored. These cells are then used as reagents to screen small molecule and peptide libraries to identify modulators of secretory function.

Secretory function embodies all aspects of the cell’s capacity to sense the extracellular milieu, respond to that milieu *via* the activation and inhibition of a variety of intracellular signaling mechanisms, and accordingly regulate the secretion of a peptide or hormone from the
secretory pathway. Such intracellular signals may include, but are not limited to, calcium ions, cAMP, calmodulin, phosphorylation, dephosphorylation, membrane polarization, glucose, pH, ATP, ADP, fatty acid pools such as free fatty acids and triglycerides, nitrous oxide and other free radicals, action potentials, glycolytic flux, DNA fragmentation and other events associated with apoptosis, patterns of gene expression, NADPH, NADP, NADH, NAD and enzyme activities.

Regulation from the secretory pathway can occur at any phase in the synthesis and release of a peptide or hormone including gene transcription; stability of the mRNA; translation; post-translational modifications such as proteolytic processing, formation of disulfide bonds, amidation, and glycosylation; trafficking through the secretory tubules and vesicles; storage within the secretory granule; membrane fusions, and exocytosis. In particular embodiments, the secretory function may be manifest as the secretion of a particular polypeptide from a secretory cell.

The polypeptide is generally secreted into the media of the cells, from where it can be quantified using any of a number of techniques. The polypeptide may be detected directly from the media using for example, ELISA, RIA and the like. Alternatively, the polypeptide may be purified prior to detection according to known methods, such as precipitation (e.g., ammonium sulfate), HPLC, immunoprecipitation, ion exchange chromatography, affinity chromatography (including immunoaffinity chromatography) or various size separations (sedimentation, gel electrophoresis, gel filtration). Such techniques of polypeptide separation are well known to those of skill in the art. The purified polypeptide may then quantified through immunodetection methods, biological activity, or radioisotope labeling. These techniques are described herein below.

1. **Assay Formats**

   a. **Stimulators of Secretory Function**

   The present invention provides methods of screening for stimulators of secretory function, by monitoring secretory function in the absence of the candidate substance and
comparing such results to the assay performed in the presence of candidate secretory function stimulators.

In certain embodiments, the present invention concerns a method for identifying such stimulators. It is contemplated that this screening technique will prove useful in the general identification of a compound that will serve the purpose of promoting, augmenting or increasing the secretion of, for example, a polypeptide from a secretory cell as exemplified by the immortalized secretory cells of the present invention. Such compounds will be useful in the treatment of various secretory disorders resulting from impaired secretory function, such as for example, diabetes, Parkinson’s disease, athyrotic cretinism and Adison’s disease.

In these embodiments, the present invention is directed to a method for determining the ability of a candidate substance to stimulate the secretory function of immortalized cells that either naturally secrete molecules or have been engineered to possess secretory function as described herein. The method including generally the steps of:

(a) providing at least one immortalized cell having stable secretory function;
(b) contacting said cell with said candidate substance;
(c) measuring the secretory function of said cell; and
(d) comparing the secretory function of the cell in step (c) with the secretory function of the cell of step (a).

To identify a candidate substance as being capable of stimulating secretory function in the assay above, one would measure or determine the secretory function in the absence of the added candidate substance by determining the secretion of the desired molecule. One would then add the candidate substance to the cell and determine the secretory function in the presence of the candidate substance. A candidate substance which increases the secretory function or capacity relative to the secretory function in its absence, is indicative of a candidate substance with stimulatory capability.
Secretory function may be determined by measuring the amount of secreted molecule. In particular embodiments, the secreted molecule will be a polypeptide such as an amidated polypeptide, glycosylated polypeptide, a hormone or a growth factor. In such circumstances these molecules may be detected using any of a number of techniques well known to those of skill in the art as described herein below. Secretory function may also be monitored by measuring, for example, calcium ions, cAMP, calmodulin, phosphorylation, dephosphorylation, membrane polarization glucose, ATP, ADP, fatty acids and NADPH or membrane potential. Detection of these molecules can be performed using immunoreactive detection, fluorescence luminescence, changes in action potential and the like.

b. Inhibitors of Secretory Function

These assays may be set up in much the same manner as those described above in assays for secretory function stimulators. In these embodiments, the present invention is directed to a method for determining the ability of a candidate substance to have an inhibitory or even antagonistic effect on secretion from the immortalized cells described herein. The method including generally the steps of:

(a) providing at least one immortalized cell having secretory function;
(b) contacting said cell with said candidate substance;
(c) measuring the secretory function of said cell; and
(d) comparing the secretory function of the cell in step (c) with the secretory function of the cell of step (a).

To identify a candidate substance as being capable of inhibiting secretory function one would measure or determine such a secretory activity in the absence of the added candidate substance and monitoring the secretory function. One would then add the candidate inhibitory substance to the cell and determine the secretory function in the presence of the candidate inhibitory substance. A candidate substance which is inhibitory would decrease the secretion from said cell, relative to the amount of secretion in its absence.
c. **Candidate Substances**

As used herein the term "candidate substance" refers to any molecule that is capable of modulating secretory function. The candidate substance may be a protein or fragment thereof, a small molecule inhibitor, or even a nucleic acid molecule. It may prove to be the case that the most useful pharmacological compounds for identification through application of the screening assay will be compounds that are structurally related to other known modulators of secretion. The active compounds may include fragments or parts of naturally-occurring compounds or may be only found as active combinations of known compounds which are otherwise inactive. However, prior to testing of such compounds in humans or animal models, it will be necessary to test a variety of candidates to determine which have potential.

Accordingly, the active compounds may include fragments or parts of naturally-occurring compounds or may be found as active combinations of known compounds which are otherwise inactive. Accordingly, the present invention provides screening assays to identify agents which stimulate or inhibit cellular secretion, it is proposed that compounds isolated from natural sources, such as animals, bacteria, fungi, plant sources, including leaves and bark, and marine samples may be assayed as candidates for the presence of potentially useful pharmaceutical agents. It will be understood that the pharmaceutical agents to be screened could also be derived or synthesized from chemical compositions or man-made compounds. Thus, it is understood that the candidate substance identified by the present invention may be polypeptide, polynucleotide, small molecule inhibitors or any other compounds that may be designed through rational drug design starting from known secretagogues or inhibitors of secretory function.

The candidate screening assays are simple to set up and perform. Thus, in assaying for a candidate substance, after obtaining an immortalized secretory cell of the present invention, one will admix a candidate substance with the cell, under conditions which would allow measurable secretion to occur. In this fashion, one can measure the ability of the candidate substance to stimulate the secretory function of the cell in the absence of the candidate substance. Likewise, in assays for inhibitors after obtaining an immortalized secretory cell, the candidate substance is admixed with the cell. In this fashion the ability of the candidate
inhibitory substance to reduce, abolish, or otherwise diminish secretion from said cell may be detected.

"Effective amounts" in certain circumstances are those amounts effective to reproducibly stimulate secretion from the cell in comparison to their normal levels. Compounds that achieve significant appropriate changes in activity will be used.

Significant increase in secretory function, e.g., as measured using RIA, HPLC, ELISA, biological activity and the like are represented by an increase/decrease in secretion of at least about 30%-40%, and most preferably, by increases of at least about 50%, with higher values of course being possible. The active compounds of the present invention also may be used for the generation of antibodies which may then be used in analytical and preparatory techniques for detecting and quantifying further such inhibitors.

It will, of course, be understood that all the screening methods of the present invention are useful in themselves notwithstanding the fact that effective candidates may not be found. The invention provides methods for screening for such candidates, not solely methods of finding them.

2. **Purification of Secreted Products**

Purification techniques are well known to those of skill in the art, and will be used to purify the molecules secreted from the immortalized secretory cells of the present invention. These techniques tend to involve the separation the secreted protein or other secretory molecule from other components of the mixture. Having separated the secreted product from the other components, the sample may be purified using chromatographic and electrophoretic techniques to achieve complete purification. Analytical methods particularly suited to the preparation of a pure peptide are ion-exchange chromatography, exclusion chromatography; polyacrylamide gel electrophoresis; isoelectric focusing. A particularly efficient method of purifying peptides is fast protein liquid chromatography or even HPLC.
In certain aspects, the secreted molecule is a polypeptide and may be isolated from the conditioned media and analyzing the extracts by HPLC as described (Halban et al., 1986, Sizonenko and Halban, 1991). Solvent systems, gradients and flow rates used were as described by Halban et al., (1986) however it is well within the skill of the ordinary person ion the art to adapt the chromatography conditions to suit individual need. Standards may be used to obtain optimization of chromatography conditions and methods.

Certain aspects of the present invention concern the purification, and in particular embodiments, the substantial purification, of a secreted product. The term "purified" as used herein, is intended to refer to a composition, isolatable from other components, wherein the product is purified to any degree relative to its naturally-obtainable state, i.e., in this case, relative to its purity within a hepatocyte or β-cell extract. A purified protein or peptide therefore also refers to a protein or peptide, free from the environment in which it may naturally occur.

Generally, "purified" will refer to a protein or peptide composition that has been subjected to fractionation to remove various other components, and which composition substantially retains its expressed biological activity. Where the term "substantially purified" is used, this designation will refer to a composition in which the protein or peptide forms the major component of the composition, such as constituting about 50% or more of the proteins in the composition.

Various methods for quantifying the degree of purification of the protein or peptide will be known to those of skill in the art in light of the present disclosure. These include, for example, determining the specific activity of an active fraction, or assessing the number of polypeptides within a fraction by SDS/PAGE analysis. A preferred method for assessing the purity of a fraction is to calculate the specific activity of the fraction, to compare it to the specific activity of the initial extract, and to thus calculate the degree of purity, herein assessed by a "-fold purification number". The actual units used to represent the amount of activity will, of course, be dependent upon the particular assay technique chosen to follow the purification and whether or not the expressed protein or peptide exhibits a detectable activity.
Various techniques suitable for use in protein purification will be well known to those of skill in the art. These include, for example, precipitation with ammonium sulphate, PEG, antibodies and the like or by heat denaturation, followed by centrifugation; chromatography steps such as ion exchange, gel filtration, reverse phase, hydroxylapatite and affinity chromatography; isoelectric focusing; gel electrophoresis; and combinations of such and other techniques. As is generally known in the art, it is believed that the order of conducting the various purification steps may be changed, or that certain steps may be omitted, and still result in a suitable method for the preparation of a substantially purified protein or peptide.

There is no general requirement that the protein or peptide always be provided in their most purified state. Indeed, it is contemplated that less substantially purified products will have utility in certain embodiments. Partial purification may be accomplished by using fewer purification steps in combination, or by utilizing different forms of the same general purification scheme. For example, it is appreciated that a cation-exchange column chromatography performed utilizing an HPLC apparatus will generally result in a greater -fold purification than the same technique utilizing a low pressure chromatography system. Methods exhibiting a lower degree of relative purification may have advantages in total recovery of protein product, or in maintaining the activity of an expressed protein.

It is known that the migration of a polypeptide can vary, sometimes significantly, with different conditions of SDS/PAGE (Capaldi et al., 1977). It will therefore be appreciated that under differing electrophoresis conditions, the apparent molecular weights of purified or partially purified expression products may vary.

High Performance Liquid Chromatography (HPLC) is characterized by a very rapid separation with extraordinary resolution of peaks. This is achieved by the use of very fine particles and high pressure to maintain and adequate flow rate. Separation can be accomplished in a matter of minutes, or a most an hour. Moreover, only a very small volume of the sample is needed because the particles are so small and close-packed that the void volume is a very small
fraction of the bed volume. Also, the concentration of the sample need not be very great because the bands are so narrow that there is very little dilution of the sample.

Gel chromatography, or molecular sieve chromatography, is a special type of partition chromatography that is based on molecular size. The theory behind gel chromatography is that the column, which is prepared with tiny particles of an inert substance that contain small pores, separates larger molecules from smaller molecules as they pass through or around the pores, depending on their size. As long as the material of which the particles are made does not adsorb the molecules, the sole factor determining rate of flow is the size. Hence, molecules are eluted from the column in decreasing size, so long as the shape is relatively constant. Gel chromatography is unsurpassed for separating molecules of different size because separation is independent of all other factors such as pH, ionic strength, temperature, etc. There also is virtually no adsorption, less zone spreading and the elution volume is related in a simple matter to molecular weight.

Affinity Chromatography is a chromatographic procedure that relies on the specific affinity between a substance to be isolated and a molecule that it can specifically bind to. This is a receptor-ligand type interaction. The column material is synthesized by covalently coupling one of the binding partners to an insoluble matrix. The column material is then able to specifically adsorb the substance from the solution. Elution occurs by changing the conditions to those in which binding will not occur (alter pH, ionic strength, temperature, etc.).

A particular type of affinity chromatography useful in the purification of carbohydrate containing compounds is lectin affinity chromatography. Lectins are a class of substances that bind to a variety of polysaccharides and glycoproteins. Lectins are usually coupled to agarose by cyanogen bromide. Concanavalin A coupled to Sepharose was the first material of this sort to be used and has been widely used in the isolation of polysaccharides and glycoproteins other lectins that have been include lentil lectin, wheat germ agglutinin which has been useful in the purification of N-acetyl glucosaminyl residues and Helix pomatia lectin. Lectins themselves are purified using affinity chromatography with carbohydrate ligands. Lactose has been used to purify lectins from castor bean and peanuts; maltose has been useful in extracting lectins from
lentils and jack bean; N-acetyl-D galactosamine is used for purifying lectins from soybean; N-acetyl glucosaminyl binds to lectins from wheat germ; D-galactosamine has been used in obtaining lectins from clams and L-fucose will bind to lectins from lotus.

The matrix should be a substance that itself does not adsorb molecules to any significant extent and that has a broad range of chemical, physical and thermal stability. The ligand should be coupled in such a way as to not affect its binding properties. The ligand should also provide relatively tight binding. And it should be possible to elute the substance without destroying the sample or the ligand. One of the most common forms of affinity chromatography is immunoaffinity chromatography. The generation of antibodies that would be suitable for use in accord with the present invention is discussed below.

3. **Methods of Detection**

The present invention encompasses methods for determining the effects of active compounds on the secretory function of the immortalized secretory cells of the present invention. Generally, this will be achieved by determining the secretion of, for example a secretory polypeptide, of the immortalized secretory cell in the presence of the active compounds and comparing the level of secretion with those levels observed in normal cells of the same type. In this manner the secretory function of the immortalized secretory cells may be quantitated.

The immunodetection methods of the present invention have evident utility in the detecting polypeptide secretion. Here, a sample containing the secreted moiety is contacted with a corresponding antibody. Thus, in an exemplary assay, a modulator screening assay is performed in which cells secreting a polypeptide are exposed to a test or candidate substance under suitable conditions and for a time sufficient to permit the agent to effect secretion of the polypeptide. The secretion of the polypeptide is then detected by incubating the reaction mixture with for example a specific antibody, which antibody may be labeled directly or may be detected secondarily. (*e.g.*, using a labeled idiotypic or species specific antibody) under conditions that permit the formation of immune complexes between the polypeptide and its specific antibody. The test reaction is compared to a control reaction which lacks the test
sample. To complete the modulator screening assay, the presence and/or amount of complexes formed between the polypeptide and the antibody is detected in the test sample (e.g. by determining the presence or amount of label bound directly to the antibody or to a secondary antibody directed against the primary antibody). Within this exemplary assay, agents that inhibit polypeptide secretion will demonstrate a reduced binding with polypeptide-specific antibodies relative to the control sample and agents that induce or stimulate polypeptide secretion will demonstrate an increased binding with specific antibodies relative to the control sample.

Those of skill in the art are very familiar with differentiating between significant secretion of a protein, which represents a positive identification, and low level or background secretion of such a protein.

a. Immunodetection Methods

In still further embodiments, the present invention concerns immunodetection methods for binding, purifying, removing, quantifying or otherwise generally detecting biological the secreted components. The steps of various useful immunodetection methods have been described in the scientific literature and are well known to those of skill in the art.

In general, the immunobinding methods include obtaining a sample suspected of containing a compound of interest (i.e. the secreted molecule), and contacting the sample with an antibody under conditions effective to allow the formation of immunocomplexes.

The immunobinding methods include methods for detecting or quantifying the amount of a reactive component in a sample, which methods require the detection or quantitation of any immune complexes formed during the binding process. Here, one would obtain a sample to be measured as containing a secreted molecule, and contact the sample with an antibody or encoded protein or peptide, as the case may be, and then detect or quantify the amount of immune complexes formed under the specific conditions.

In terms of detection, the biological sample analyzed may be any sample that is suspected of containing a secreted molecule from the immortalized, secretory cells of the
present invention. Such a cell may be an immortalized neuroendocrine cell, an immortalized pancreatic β-cell, or even any biological fluid that comes into contact with the secretory cells in vivo.

Contacting the chosen sample with the antibody under conditions effective and for a period of time sufficient to allow the formation of immune complexes (primary immune complexes) is generally a matter of simply adding the composition to the sample and incubating the mixture for a period of time long enough for the antibodies to form immune complexes with, i.e., to bind to, any antigens present. After this time, the sample-antibody composition, such as a tissue section, ELISA plate, dot blot or western blot, will generally be washed to remove any non-specifically bound antibody species, allowing only those antibodies specifically bound within the primary immune complexes to be detected.

In general, the detection of immunocomplex formation is well known in the art and may be achieved through the application of numerous approaches. These methods are generally based upon the detection of a label or marker, such as any radioactive, fluorescent, biological or enzymatic tags or labels of standard use in the art. U.S. Patents concerning the use of such labels include 3,817,837; 3,850,752; 3,939,350; 3,996,345; 4,277,437; 4,275,149 and 4,366,241, each incorporated herein by reference. Of course, one may find additional advantages through the use of a secondary binding ligand such as a second antibody or a biotin/avidin ligand binding arrangement, as is known in the art.

The secreted protein, peptide or corresponding antibody employed in the detection may itself be linked to a detectable label, wherein one would then simply detect this label, thereby allowing the amount of the primary immune complexes in the composition to be determined.

Alternatively, the first added component that becomes bound within the primary immune complexes may be detected by means of a second binding ligand that has binding affinity for the encoded protein, peptide or corresponding antibody. In these cases, the second binding ligand may be linked to a detectable label. The second binding ligand is itself often an antibody, which may thus be termed a "secondary" antibody. The primary immune complexes
are contacted with the labeled, secondary binding ligand, or antibody, under conditions effective and for a period of time sufficient to allow the formation of secondary immune complexes. The secondary immune complexes are then generally washed to remove any non-specifically bound labeled secondary antibodies or ligands, and the remaining label in the secondary immune complexes is then detected.

Further methods include the detection of primary immune complexes by a two step approach. A second binding ligand, such as an antibody, that has binding affinity for the encoded protein, peptide or corresponding antibody is used to form secondary immune complexes, as described above. After washing, the secondary immune complexes are contacted with a third binding ligand or antibody that has binding affinity for the second antibody, again under conditions effective and for a period of time sufficient to allow the formation of immune complexes (tertiary immune complexes). The third ligand or antibody is linked to a detectable label, allowing detection of the tertiary immune complexes thus formed. This system may provide for signal amplification if desired.

b. ELISA

It is contemplated that the secreted proteins or peptides of the invention will be detected in a preferred embodiment in ELISA assays. Antibodies against such secreted proteins are readily available to those of skill in the art.

Immunoassays, in their most simple and direct sense, are binding assays. Certain preferred immunoassays are the various types of enzyme linked immunosorbent assays (ELISA) and radioimmunoassays (RIA) known in the art.

In one exemplary ELISA, antibodies binding to the secreted proteins of the invention are immobilized onto a selected surface exhibiting protein affinity, such as a well in a polystyrene microtiter plate. Then, a test composition containing the secreted polypeptide is added to the wells. After binding and washing to remove non-specifically bound immune complexes, the bound antibody may be detected. Detection is generally achieved by the addition of a second antibody specific for the target protein, that is linked to a detectable label.
This type of ELISA is a simple "sandwich ELISA". Detection may also be achieved by the addition of a second antibody, followed by the addition of a third antibody that has binding affinity for the second antibody, with the third antibody being linked to a detectable label.

In another exemplary ELISA, the samples containing the secreted polypeptide are immobilized onto the well surface and then contacted with the antibodies of the invention. After binding and washing to remove non-specifically bound immune complexes, the bound antigen is detected. Where the initial antibodies are linked to a detectable label, the immune complexes may be detected directly. Again, the immune complexes may be detected using a second antibody that has binding affinity for the first antibody, with the second antibody being linked to a detectable label.

Another ELISA in which the proteins or peptides are immobilized, involves the use of antibody competition in the detection. In this ELISA, labeled antibodies are added to the wells, allowed to bind to the secreted protein, and detected by means of their label. The amount of marker antigen in an unknown sample is then determined by mixing the sample with the labeled antibodies before or during incubation with coated wells. The presence of marker antigen in the sample acts to reduce the amount of antibody available for binding to the well and thus reduces the ultimate signal. This is appropriate for detecting antibodies in an unknown sample, where the unlabeled antibodies bind to the antigen-coated wells and also reduces the amount of antigen available to bind the labeled antibodies.

Irrespective of the format employed, ELISAs have certain features in common, such as coating, incubating or binding, washing to remove non-specifically bound species, and detecting the bound immune complexes. These are described as follows:

In coating a plate with either antigen or antibody, one will generally incubate the wells of the plate with a solution of the antigen or antibody, either overnight or for a specified period of hours. The wells of the plate will then be washed to remove incompletely adsorbed material. Any remaining available surfaces of the wells are then "coated" with a nonspecific protein that is antigenically neutral with regard to the test antisera. These include bovine serum albumin
(BSA), casein and solutions of milk powder. The coating of nonspecific adsorption sites on the immobilizing surface reduces the background caused by nonspecific binding of antisera to the surface.

In ELISAs, it is more customary to use a secondary or tertiary detection means rather than a direct procedure. Thus, after binding of a protein or antibody to the well, coating with a non-reactive material to reduce background, and washing to remove unbound material, the immobilizing surface is contacted with the control and/or clinical or biological sample to be tested under conditions effective to allow immune complex (antigen/antibody) formation. Detection of the immune complex then requires a labeled secondary binding ligand or antibody, or a secondary binding ligand or antibody in conjunction with a labeled tertiary antibody or third binding ligand.

"Under conditions effective to allow immune complex (antigen/antibody) formation" means that the conditions preferably include diluting the antigens and antibodies with solutions such as BSA, bovine gamma globulin (BGG) and phosphate buffered saline (PBS)/Tween™. These added agents also tend to assist in the reduction of nonspecific background.

The "suitable" conditions also mean that the incubation is at a temperature and for a period of time sufficient to allow effective binding. Incubation steps are typically from about 1 to 2 to 4 hours, at temperatures preferably on the order of 25° to 27°C, or may be overnight at about 4°C or so.

Following all incubation steps in an ELISA, the contacted surface is washed so as to remove non-complexed material. A preferred washing procedure includes washing with a solution such as PBS/Tween™ or borate buffer. Following the formation of specific immune complexes between the test sample and the originally bound material, and subsequent washing, the occurrence of even minute amounts of immune complexes may be determined.

To provide a detecting means, the second or third antibody will have an associated label to allow detection. Preferably, this label will be an enzyme that will generate color
development upon incubating with an appropriate chromogenic substrate. Thus, for example, one will desire to contact and incubate the first or second immune complex with a urease, glucose oxidase, alkaline phosphatase or hydrogen peroxidase-conjugated antibody for a period of time and under conditions that favor the development of further immune complex formation (e.g., incubation for 2 hours at room temperature in a PBS-containing solution such as PBS-Tween™).

After incubation with the labeled antibody, and subsequent to washing to remove unbound material, the amount of label is quantified, e.g., by incubation with a chromogenic substrate such as urea and bromocresol purple or 2,2'-azido-di-(3-ethyl-benzthiazoline-6-sulfonic acid [ABTS] and H₂O₂, in the case of peroxidase as the enzyme label. Quantitation is then achieved by measuring the degree of color generation, e.g., using a visible spectra spectrophotometer.

In a one embodiment, the screening assay uses live cells in the 96-well format has long been the standard used as the format lends itself to automation and robotics handling. The 96-well plate format allows for a variety of different candidate substances to be tested in one plate. However, use of live cells for the purpose of drug screening has inherent problems associated with the handling of the cells. Handling attachment dependent cell culture in the 96-well format becomes difficult when there is a need for several exchanges of solution. The forces of surface tension associated with the meniscus on the well wall stress and even damages cells on the bottom of the well as aqueous solutions are removed or added. The shear forces created by a suction device (e.g., a pipet tip) as it is close to the cell layer removing the last microliter of solution may also damage and remove cells. In order to overcome these problems, the cells are encapsulated in highly porous, biocompatible hydrogels in a bead form. In a preferred assay format, the encapsulated cells are placed in a 96-well plates that incorporates a filter-bottom. The cells are then incubated with the candidate substance for a suitable period of time to allow the cell to secrete the polypeptide of interest. This incubation step is followed by the harvesting of the media from the cells by the application of a vacuum below the plate to empty all wells in one step. The collected samples are then assayed for the presence of the secreted peptide using standard ELISA techniques well known to those of skill in the art.
In other preferred embodiments, the detection assays may be radioimmunoassays as described by various groups (Halban et al., 1986; Pieber et al., 1994). Standard commercially available radioimmunoassays are available from Coat-a-count, Diagnostic Products Corp., Los Angeles for insulin, and rat amylin immunoassay (Peninsula Laboratories, EIAH-7323. Immunoreactive species of glucagon, glucagon-like peptide 1 (7-37, non amidated) and glucagon-like peptide (7-36, amide) were determined as described by the suppliers of the respective commercial kits (all purchased from Peninsula Laboratories Inc. Cat #'s RIK-7165, RIK-7123 and RIK-7168, respectively)

c. Non-Immunologic Methods

Alternatively, one may employ non-immunologic procedures in the measuring the secretory function according to the of the present invention. For example, when examining molecules that are involved in receptor interactions, it is possible to set up assays that look at the occupancy of relevant receptor molecules. This can be performed, for example, by using labeled ligand molecules that will be compete with the ligand (stimulators and inhibitor) in the sample. The more ligand in the sample, the less labeled receptor that will be bound to the receptor. Such studies can be performed on whole cells as well as on purified receptors. Labels include radiolabels, fluorescent labels and chemiluminescent labels.

Other non-immunologic forms of diagnostic assays include those that look for the presence of biological activity of the secreted polypeptide.

4. In vivo Assays

The present invention also provides for the testing of candidate substances for their ability to modulate secretory function of cell in in vivo contexts. This approach has the added advantage of assessing (i) the function of cells under normal. physiologic conditions including the presence of various intercellular signaling mechanisms and (ii) the ability of candidate substances to target cells local, regional or distal to their site of administration and (iii) localization and/or tissue distribution of a secreted metabolite or downstream effector metabolite. Different formulations including time release compositions also may be assessed.
Finally, this format permits testing on the basis of physiologic states rather than the mere increase or decrease of secretory function. This provides additional information on the actual potential therapeutic benefit of the substance for the host, testing of therapeutic vs. toxic concentrations to establish therapeutic ranges and drug safety parameters, as well as allowing for *in vivo* interactions to be monitored.

The preferred embodiment for *in vivo* screening of candidate substances involves the use of a nude rodent model. The nude mouse lacks immune functions that might compromise or interfere with testing of implanted cells of the present invention. This system is well characterized and is used for a variety of other purposes including a model of transplanted human cancer. Yet another preferred model is one in which the animal has diabetes (IDDM or NIDDM).

Briefly, immortalized secretory cells of the present invention will be transferred, as part of an implantable device (described elsewhere in this document) into a suitable site of the host animal. Typically, subcutaneous implant on the flank, back or hindlimb of the animal, or intraperitoneal insertion, is preferred. Intramuscular implant, usually on the hindlimb, also is contemplated. Particular issues that will affect the choice of implantation site include (i) similarity to the normal site the cells might be found or implanted clinically and (ii) the importance of establishing a supporting vasculature structure for the implant.

After a suitable period of time for stabilization of the implant, usually 1 to 10 days, the animal is ready for testing. The initial step involves the determination of steady state levels of any metabolite that will be used as a read-out for effects on modulation of the implanted cells. This often will involve taking peripheral blood measurements of the metabolite of interest. Such metabolites include, but are not limited to, glucose, insulin, glucagon, GLP-1, amylin, leptin, somatostatin, growth hormone. Alternatively, one may assess functional attributes of the animal such as alteration in blood glucose in the case of insulin and loss of body fat in the case of leptin. Other parameters that could be monitored include, in body weight, food intake, blood pressure, metabolic rate, body temperature, serum minerals, *etc.*
Once steady state levels and conditions have been determined, the candidate substance is administered. Depending on the location of the implant and the particular purpose for the assay, the candidate substance, formulated in a pharmacologically acceptable fashion, will be administered to the animal. Suitable routes include oral, rectal, vaginal, topical or intravenous or intraarterial injection. Also contemplated are intramuscular, intraperitoneal, intraocular, subcutaneous or submucosal administration.

As stated above, the metabolite will be tested from the appropriate tissue or fluid from the host animal. Fluids include blood, lymph, saliva, sputum, feces, urine, semen or tears. Tissues that may be sampled include liver, brain, muscle, pancreas, spleen, testis, ovarian, stomach, intestine, endocrine glands, adrenal glands and kidney. Depending on the metabolite, different methodology, also described above, will be used to separate and identify the presence, quantity and/distribution of the metabolite. In addition, histologic examination, involving microscopy, may be performed. Modulation of the metabolite or function, in the presence of the candidate substance, as compared with the levels determined prior to provision of the candidate substances, will indicate that the candidate substance is a modulator of that metabolite or function.

Following completion of the experimental phase, rescue of the implant may be performed for the purpose of determining the secretory status of the implant cells. Any change in the behavior or characteristics of the cells could impact the results. Proper controls will include animals implanted with empty devices and animals implanted with devices populated with "placebo" (non-responsive, non-secretory) cells.

K. Cell Propagation

The cells of the present invention may be present propagated as non-anchorage dependent cells growing freely in suspension throughout the bulk of the culture; or as anchorage-dependent cells requiring attachment to a solid substrate for their propagation (i.e., a monolayer type of cell growth). WO publication numbers WO 97/26334 (published July 24, 1997) and WO 97/26321 (published July 24, 1997) are specifically incorporated herein by
reference and describe the different modes of cell culture that can be employed to maintain the cells of the present invention.

In particular embodiments, the cells that will be used for the screening of modulators of secretory function may be in a microcarrier culture van Wezel (1967). This mode of the culture propagation on the microcarriers makes it possible to use this system for cellular manipulations, such as cell transfer without the use of proteolytic enzymes, co-cultivation of cells, transplantation into animals, and perfusion of the culture using decanters, columns, fluidized beds, or hollow fibers for microcarrier retainment.

As described herein, particular embodiments, employ microencapsulation of cells because this system readily lends itself to batch screening methods such as 96-well plate screening and also provides a useful mode of providing the cells to an animal model for in vivo testing. The cells are retained inside a semipermeable hydrogel membrane. A porous membrane is formed around the cells permitting the exchange of nutrients, gases, and metabolic products with the bulk medium surrounding the capsule. Several methods have been developed that are gentle, rapid and non-toxic and where the resulting membrane is sufficiently porous and strong to sustain the growing cell mass throughout the term of the culture. These methods are all based on soluble alginate gelled by droplet contact with a calcium-containing solution. Lim (1982) describes cells concentrated in an approximately 1% solution of sodium alginate which are forced through a small orifice, forming droplets, and breaking free into an approximately 1% calcium chloride solution. The droplets are then cast in a layer of polyamino acid that ioniically bonds to the surface alginate. Finally the alginate is reliquified by treating the droplet in a chelating agent to remove the calcium ions. Other methods use cells in a calcium solution to be dropped into an alginate solution, thus creating a hollow alginate sphere. A similar approach involves cells in a chitosan solution dropped into alginate, also creating hollow spheres.

Microencapsulated cells are easily propagated in stirred tank reactors and, with beads sizes in the range of 150-1500 μm in diameter, are easily retained in a perfused reactor using a fine-meshed screen. The ratio of capsule volume to total media volume can kept from as dense
as 1:2 to 1:10. With intracapsular cell densities of up to $10^8$, the effective cell density in the culture is $1-5 \times 10^7$.

The advantages of microencapsulation over other processes include the protection from the deleterious effects of shear stresses which occur from sparging and agitation, the ability to easily retain beads for the purpose of using perfused systems, scale up is relatively straightforward and the ability to use the beads for use in 96-well screening assays and in implantation.

The cells of the present invention may, irrespective of the culture method chosen, be used in protein production and as cells for *in vitro* cellular assays and screens as part of drug development protocols.

L. *In vivo* Uses

1. **Pharmacetically Acceptable Formulations**

Where clinical applications are contemplated, it will be necessary to prepare pharmaceutical compositions of the stable cells in a form appropriate for the intended application, which will most usually be within a selectively permeable membrane. Nonetheless, the cells will generally be prepared as a composition that is essentially free of pyrogens, as well as other impurities that could be harmful to humans or animals.

One will generally desire to employ appropriate salts and buffers to render stable cells suitable for introduction into a patient within their selectively permeable membrane, implantable device or other delivery vehicle. Aqueous compositions of the present invention comprise an effective amount of stable neuroendocrine cells dispersed in a pharmacetically acceptable carrier or aqueous medium, and preferably encapsulated.

The phrase "pharmacetically or pharmacologically acceptable" refer to molecular entities and compositions that do not produce adverse, allergic, or other untoward reactions when administered to an animal or a human. As used herein, "pharmacetically acceptable carrier" includes any and all solvents, dispersion media, coatings, antibacterial and antifungal
agents, isotonic and absorption delaying agents and the like. As used herein, this term is particularly intended to include biocompatible implantable devices and encapsulated cell populations. The use of such media and agents for pharmaceutically active substances is well know in the art. Except insofar as any conventional media or agent is incompatible with the vectors or cells of the present invention, its use in therapeutic compositions is contemplated. Supplementary active ingredients also can be incorporated into the compositions.

Under ordinary conditions of storage and use, the cell preparations may further contain a preservative to prevent growth of microorganisms. Intravenous vehicles include fluid and nutrient replenishers. Preservatives include antimicrobial agents, anti-oxidants, chelating agents and inert gases. The pH and exact concentration of the various components in the pharmaceutical are adjusted according to well-known parameters.

2. Cell-Based Delivery and Devices

The engineered cells of the present invention may be introduced into animals, including human subjects, so that modulators of secretion identified by the present invention can provide a controlled secretion of a desired polypeptide. In order for the cells to simulate normal human β-cells, ideally cells are engineered to achieve glucose dose responsiveness resembling that of islets. However, other cells will also achieve advantages in accordance with the invention. It should be pointed out that the studies of Madsen and coworkers have shown that implantation of poorly differentiated rat insulinoma cells into animals results in a return to a more differentiated state, marked by enhanced insulin secretion in response to metabolic fuels (Madsen et al., 1988). These studies suggest that exposure of engineered cell lines to the in vivo milieu may have some effects on their response(s) to secretagogues.

A preferred method of providing the cells to an animal involves the encapsulation of the engineered cells in a biocompatible coating. In this approach, the cells are entrapped in a capsular coating that protects the contents from immunological responses. One preferred encapsulation technique involves encapsulation with alginate-polylysine-alginate. Capsules made employing this technique generally have a diameter of approximately 0.5 to 1 mm and should contain several hundred cells.
Cells may thus be implanted using the alginate-polylysine encapsulation technique of O'Shea and Sun (1986), with modifications, as later described by Fritschy et al. (1991; both references incorporated herein by reference). The engineered cells are suspended in 1.3% sodium alginate and encapsulated by extrusion of drops of the cell/alginate suspension through a syringe into CaCl₂. After several washing steps, the droplets are suspended in polylysine and rewashed. The alginate within the capsules is then reliquified by suspension in 1 mM EGTA and then rewarshed with Krebs balanced salt buffer.

An alternative approach is to seed Amicon fibers with stable cells of the present invention. The cells become enmeshed in the fibers, which are semipermeable, and are thus protected in a manner similar to the micro encapsulates (Altman et al., 1986; incorporated herein by reference). After successful encapsulation or fiber seeding, the cells may be implanted intraperitoneally, usually by injection into the peritoneal cavity through a large gauge needle (23 gauge).

A variety of other encapsulation technologies have been developed that are applicable to the practice of the present invention (see, e.g., Lacy et al., 1991; Sullivan et al., 1991; WO 91/10470; WO 91/10425; WO 90/15637; WO 90/02580; U.S. Patent 5,011,472; U.S. Patent 4,892,538; U.S. Patent 5,002,661; U.S. Patent 5,569,462; U.S. Patent 5,593,440; U.S. Patent 5,549,675; U.S. Patent 5,545,223; U.S. Patent 5,314,471; U.S. Patent 5,626,561 and WO 89/01967; each of the foregoing being incorporated by reference). To the extent that these references describe encapsulation techniques that will be useful in combination with the present invention, some of these references are described in further detail herein below.

Lacy et. al. (1991) encapsulated rat islets in hollow acrylic fibers and immobilized these in alginate hydrogel. Following intraperitoneal transplantation of the encapsulated islets into diabetic mice, normoglycemia was reportedly restored. Similar results were also obtained using subcutaneous implants that had an appropriately constructed outer surface on the fibers. It is therefore contemplated that engineered cells of the present invention may also be straightforwardly "transplanted" into a mammal by similar subcutaneous injection.
Sullivan et al. (1991) reported the development of a biohybrid perfused "artificial pancreas", which encapsulates islet tissue in a selectively permeable membrane. In these studies, a tubular semi-permeable membrane was coiled inside a protective housing to provide a compartment for the islet cells. Each end of the membrane was then connected to an arterial polytetrafluoroethylene (PTFE) graft that extended beyond the housing and joined the device to the vascular system as an arteriovenous shunt. The implantation of such a device containing islet allografts into pancreatectomized dogs was reported to result in the control of fasting glucose levels in 6/10 animals. Grafts of this type encapsulating engineered cells could also be used in accordance with the present invention.

U.S. Patent 5,626,561, specifically incorporated herein by reference, describes an implantable containment apparatus for a therapeutic device and method for loading and reloading the device. The implantable containment apparatus is made of selectively permeable material and can be used to contain a therapeutic device, such as a drug delivery device, a cell encapsulation device, or a gene therapy device. A therapeutic device can be easily placed and replaced in the apparatus without damaging tissues associated with the selectively permeable material of the apparatus.

U.S. Patent 4,402,694, also is incorporated herein by reference and describes a body cavity access device containing a hormone source. In this patent, the device supplies a hormone to a patient. The device is made of an implantable housing which is placed in the body and has an impermeable extracorporeal segment and a semipermeable subcutaneous segment. A hormone source such as live, hormone-producing cells, e.g., pancreatic islet cells or the engineered human cells of the present invention are then removably positioned in the housing to provide a hormone/ and or other peptide supply to the patient. Such a device also can contain a sensor located within the subcutaneous segment and operably associated with a dispenser to release medication into the housing and to the patient.

Hydrophilic polymeric chambers for encapsulating biologically active tissue and methods for their preparation are described in U.S. Patent 4,298,002. In the technology
described therein the tissue refers to those essential cellular components of a particular organ that is capable of receiving, modifying or secreting hormones. A device comprising such chamber and such tissue is fabricated and implanted in a living body so that said tissue is permitted normal function without being rejected by the host's immunological system. The viability of the tissue in the device is maintained by a correlation of factors including pore size and membrane thickness of the hydrophilic chamber. To maintain the viability of the tissue, the implanted device allows the inflow of essential nutrients and gases, and outflow of metabolites and products while simultaneously excluding the ingress of cellular components of the host's immunological system. To the extent that the device described therein can be used to implant the engineered cells of the present invention. U.S. Patent 4,298,002 is incorporated by reference herein.

U.S. Patent 5,011,472 describes devices and methods to provide hybrid, modular systems for the constitutive delivery of appropriate dosages of active factor to a subject and, in some instances, to specific anatomical regions of the subject. This patent is incorporated herein by reference in that it contains devices and methods that may be useful in conjunction with the present invention. This system includes a cell reservoir containing living cells capable of secreting an active agent, which is preferably adapted for implantation within the body of the subject and further includes at least one semipermeable membrane, whereby the transplanted cells can be nourished by nutrients transported across the membrane while at the same time protected from immunological, bacterial, and viral assault. The systems further include a pumping means, which can be implantable or extracorporeal, for drawing a body fluid from the subject into the cell reservoir and for actively transporting the secreted biological factors from the cell reservoir to a selected region of the subject.

Similarly, U.S. Patent 4,892,538 describes methods and compositions for the in vivo delivery of neurotransmitters by implanted, encapsulated cells and the technology described therein may be useful in combination with the present invention.

U.S. Patent 5,002,661 describes an artificial pancreatic perfusion device in which a hollow fiber having an inner diameter of about 5 mm is surrounded by islets of Langerhans
enclosed in a housing. The islets are suspended in a semi-solid matrix which ensures desired distribution of the cells about the hollow fiber. The hollow fiber and suspended islets are enclosed in a housing which further aids the desired distribution of islets about the hollow fiber. The hollow fiber has a porosity which selectively allows passage of substances having a molecular weight of less than about 100,000 Daltons. The semi-solid matrix in which the islets are embedded and suspended is formed of an appropriate supporting material such as alginate or agar. This device may be used with the present invention in that the engineered cells of the present invention may substitute for the islet cells.

U.S. Patent 5,549,675, incorporated herein by reference, describes additional methods for implanting tissue in a host. The method comprises creating an implant assembly for holding cells including a wall for forming a porous boundary between the host tissue and the implanted cells in the device and implanting the device and then adding the cells. The pore size of the boundary is such that it is sufficient to isolate the implanted cells from the immune response. U.S. Patent 5,545,223, describes methods of making and using ported tissue implant systems and is therefore incorporated herein by reference.

In certain instances it may be necessary to enhance vascularization of implant devices, methods for achieving such an aim are disclosed in U.S. Patent 5,569,462. The methods involve placing a population of therapeutic substance-producing cells into the cell receiving chamber of an immunoisolation apparatus, implanting the apparatus into a patient, and administering an immunomodulatory agent to the patient. The immunomodulatory agent increases the number of close vascular structures in the vicinity of the implanted device, which increases the long term survival of the cell population housed therein.

In other instances, it may be necessary to supply the cells of the present invention in a relatively high density. Brauker, et. al. (U.S. Patent 5,593,440. and U.S. Patent 5,314,471 each incorporated herein by reference) describe tissue implant systems and methods for sustaining viable high cell densities within a host.
Implantation employing such encapsulation techniques are preferred for a variety of reasons. For example, transplantation of islets into animal models of diabetes by this method has been shown to significantly increase the period of normal glycemic control, by prolonging xenograft survival compared to unencapsulated islets (O'Shea and Sun, 1986; Fritschy et al., 1991). Also, encapsulation will prevent uncontrolled proliferation of clonal cells. Capsules containing cells are implanted (approximately 1,000-10,000/animal) intraperitoneally and blood samples taken daily for monitoring of blood glucose and insulin.

An alternate approach to encapsulation is to simply inject glucose-sensing cells into the scapular region or peritoneal cavity of diabetic mice or rats, where these cells are reported to form tumors (Sato et al., 1962). Implantation by this approach may circumvent problems with viability or function, at least for the short term, that may be encountered with the encapsulation strategy. This approach will allow testing of the function of the cells in experimental animals, which is a viable use of the present invention, but certainly is not applicable as an ultimate strategy for treating human diabetes. Nonetheless, as a pre-clinical test, this will be understood to have significant utility.

In summary, biohybrid artificial organs encompass all devices which substitute for an organ or tissue function and incorporate both synthetic materials and living cells. Implantable immunoisolation devices will preferably be used in forms in which the tissue is protected from immune rejection by enclosure within a semipermeable membrane. Those of skill in the art will understand device design and performance, as it relates to maintenance of cell viability and function. Attention is to be focused on oxygen supply, tissue density and the development of materials that induce neovascularization at the host tissue-membrane interface; and also on protection from immune rejection. Membrane properties may even be further adapted to prevent immune rejection, thus creating clinically useful implantable immunoisolation devices.

An effective amount of the stable cells is determined based on the intended goal. The term "unit dose" refers to a physically discrete unit suitable for use in a subject, each unit containing a predetermined quantity of the therapeutic composition calculated to produce the desired response in association with its administration, i.e., the appropriate route and treatment
regimen. The quantity to be administered, both according to number of treatments and unit dose, depends on the subject to be treated, the state of the subject, and the protection desired. Precise amounts of the therapeutic composition also depend on the judgment of the practitioner and are peculiar to each individual.

M. Examples

The following examples are included to demonstrate preferred embodiments of the invention. It should be appreciated by those of skill in the art that the techniques disclosed in the examples which follow represent techniques discovered by the inventor to function well in the practice of the invention, and thus can be considered to constitute preferred modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments which are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention.

EXAMPLE 1

Stable response to a variety of secretagogues.

βG 49/206 was chosen as representative of an engineered β–cell line that reproducibly responds to a variety of secretagogues. This line has been engineered to stably express functional glucose transporter (GLUT-2) and glucokinase proteins and biologically active human insulin (Clark et al., 1997).

Prior to testing, cells were plated on polystyrene plastic 48-well or 96-well tissue culture plates at a density of 0.1 x 10⁶ cells/cm² (approx. 50% confluency) — approx. 90 x 10³ cells/well or 30 x 10³ cells/well, respectively. Cells were allowed to recover and propagated for 24-72 hours in regular growth media (βGene media with 3.5% FBS). After propagation and before stimulation, the cells are rinsed once and washed twice for 20 min. each in HEPES Buffered Biological Salt Solution (HBBS; in mmol/l: 114 NaCl, 4.7 KCl, 1.21 KH₂PO₄, 1.16 MgSO₄, 25.5 NaHCO₃, 2.5 CaCl₂, 10 mM HEPES, 0.1% BSA) at 37 °C. 0.5 ml of HBBS supplemented with secretagogue(s) was added to each well and allowed to incubate for 2 hours at 37°C. At the end of the incubation period, HBBS was harvested from each well and
assayed for insulin. Results are expressed in terms of fold stimulation over a basal sample containing HBBSS only.

The cell lines of the present invention show stable insulin secretion with time in continuous culture. The cell lines chosen represent established lines that have undergone more than 100 population doublings (2-3 years) during which two or three genes were iteratively introduced, including time for clonal selection for each gene introduced. The cells therefore already have shown long term stability during the engineering process. Cells thawed from cryogenic storage for experimentation are kept in maintenance culture in parallel. From these cultures, cells are harvested and plated for two more repeat experiments two to three weeks apart. This will prove stability over the course of a couple of months, demonstrating a window of time in which it is possible to validate reproducible results.

Secretagogues have been selected to represent agents that signal via discrete pathways, i.e., glucose and amino acids via metabolic signals, IBMX and GLP-1 via cAMP, carbachol via muscarinic receptors, sulfonylureas via the K⁺-ATP channels, and phorbol esters via protein kinase C. Cells are stimulated with the following:

- 100 μM IBMX; with and without 10 mM glucose
- a mixture of 10 mM each of glutamine, leucine, and arginine; with and without 10 mM glucose
- 100 μM carbachol; with and without 10 mM glucose
- 10 nM glyburide; with and without 10 mM glucose
- 10 nM GLP-1; with and without 10 mM glucose
- 10 nM PMA; with and without 10 mM glucose
- Stimulatory Cocktail (10 mM each of glutamine, leucine, and arginine, 100 μM carbachol, 100 μM IBMX, and 10 mM glucose in BetaGene Medium with 0.1% BSA).

The following controls are incorporated: No stimulant (basal); with and without 10 mM glucose; 100 μM Diazoxide.
The selected engineered cell lines have stable and reproducible responses to the various secretagogues over time in culture (FIG. 3 and FIG. 4). The data shown are from the last of three experiments. In the first experiment the cells had undergone 8 population doublings (PDs) after the engineering and clone selection was completed. The parental cell line had been stable for over two years, undergone more than 100 PDs, and subjected to two more engineering steps. Through all these manipulations, the cells have maintained a remarkably stable phenotype. In the last experiments the cells had undergone further 8 PDs (approx. 16 days) in continuous culture.

On average, in each of the experiments the cells had a 10 fold response (relative to basal, a 1 fold response means no change) to glucose alone. A 1-1.5 fold response to IBMX alone was observed, but there was a 20-30 fold response to IBMX in the presence of glucose, consistent with IBMX’s ability to potentiate the effect of glucose on insulin secretion. The amino acids, in the absence or presence of glucose, elicit a 20-30 fold response, as they serve as fuel molecules in the metabolic pathways. Carbachol as well as GLP-1, when tested alone, have no appreciable effect on secretion, but have a 15-25 fold response in the presence of glucose. Glyburide, in the absence or presence of glucose, elicit a 7-15 fold response, as the sulfonylurea inhibits the K⁺ channel and causes depolarization of the cell membrane. PMA, acting directly on protein kinase C has an 8-10 fold effect on basal secretion in the absence of glucose and a strong 30-40 fold response in the presence of glucose. A stimulatory cocktail that includes glucose, IBMX, amino acids, and carbachol, yields a 30-40 fold response.

EXAMPLE 2

Maintenance of Secretagogue Responsiveness Through Bulk Cell Production

For consistency and reproducibility between experiments, engineered β-cell lines were bulk produced in a bioreactor, harvested and frozen to establish a homogeneous repository of cells. Cells undergoing this process should continue to secrete complex, fully biologically active polypeptides into the growth media with no significant differences in the response to glucose and other secretagogues pre-bulk, post-bulk and post-thaw. βG 49/206 (described in example 1) was selected as a representative engineered β-cell line to undergo the complete process; bulk production, harvest, freeze and thaw. Representative samples from each step were analyzed for
response to various secretagogues. Each of the procedures and the secretion profile are described in detail below.

βG cell lines were bulk produced in the CellCube™ system (Corning Costar) and frozen as described in example 30.

Frozen vials of βG 49/206 representing each stage of the bulk production process were thawed and allowed to recover prior to testing their insulin response to various secretagogues.

The cells were ready to plate for testing of cell response to various secretagogues 48-72 hours after thawing. This assay was done to demonstrate that each of the processes described; bulk production, harvest, freeze and thaw, has no appreciable effect on the secretory response of βG 49/206 cells. The secretory response of pre-bulk, post bulk and harvest, and freeze/thaw samples was studied using the secretagogues listed in the table below. Each of the listed secretagogues and their signaling pathway has been previously described. The data are as follows:

**TABLE 10**

PERFORMANCE OF BG 49/206 CELLS THROUGH BULK PRODUCTION AND CRYOPRESERVATION

<table>
<thead>
<tr>
<th>Stimulus</th>
<th>Fold Stimulation</th>
<th>Pre-Cube PD18</th>
<th>Post-Cube Pre-Freeze</th>
<th>Post-Cube Frz/Thaw PD29</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal</td>
<td></td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>Basal +10mM Glucose</td>
<td></td>
<td>12.01</td>
<td>11.21</td>
<td>11.45</td>
</tr>
<tr>
<td>100μM IBMX</td>
<td></td>
<td>2.64</td>
<td>2.64</td>
<td>4.29</td>
</tr>
<tr>
<td>100μM IBMX + Glucose</td>
<td></td>
<td>31.74</td>
<td>18.82</td>
<td>22.35</td>
</tr>
<tr>
<td>10mM aa</td>
<td></td>
<td>22.17</td>
<td>19.45</td>
<td>21.50</td>
</tr>
<tr>
<td>10mM aa + Glucose</td>
<td></td>
<td>29.19</td>
<td>18.75</td>
<td>22.92</td>
</tr>
<tr>
<td>100μM Carbachol</td>
<td></td>
<td>1.43</td>
<td>1.73</td>
<td>1.60</td>
</tr>
</tbody>
</table>
Table 10 - Continued

<table>
<thead>
<tr>
<th>Stimulus</th>
<th>Pre-Cube PD18</th>
<th>Post-Cube PD29</th>
<th>Post-Cube Frz/Thaw PD29</th>
</tr>
</thead>
<tbody>
<tr>
<td>100μM Carbachol + Glucose</td>
<td>23.22</td>
<td>17.59</td>
<td>18.57</td>
</tr>
<tr>
<td>10nM GLP-1</td>
<td>2.12</td>
<td>2.24</td>
<td>1.82</td>
</tr>
<tr>
<td>10nM GLP-1 + Glucose</td>
<td>22.55</td>
<td>21.37</td>
<td>16.78</td>
</tr>
<tr>
<td>10nM Glyburide</td>
<td>8.31</td>
<td>10.19</td>
<td>7.80</td>
</tr>
<tr>
<td>10nM Glyburide + Glucose</td>
<td>16.55</td>
<td>13.77</td>
<td>11.77</td>
</tr>
<tr>
<td>10nM PMA</td>
<td>10.46</td>
<td>9.80</td>
<td>2.81</td>
</tr>
<tr>
<td>10nM PMA + Glucose</td>
<td>39.48</td>
<td>30.32</td>
<td>23.67</td>
</tr>
<tr>
<td>Stimulatory cocktail</td>
<td>38.61</td>
<td>39.71</td>
<td>38.55</td>
</tr>
</tbody>
</table>

As shown in Table 10, there is no appreciable difference in the overall secretion response of βG 49/206 cells which have undergone bulk production, bulk freezing and thawing.

**EXAMPLE 3**

Use of alginate encapsulated cells to enhance stability of the cells and their secretory response.

The use of living cells for the purpose of drug screening has inherent problems associated with the handling of the cells. For high-throughput screening purposes, the 96-well format has long been the standard used it lends itself to automation and robotics handling. However, handling attachment dependent cell culture in the 96-well format becomes difficult when there is a need for several exchanges of solution. The forces of surface tension associated with the meniscus on the well wall stress and even damages cells on the bottom of the well as aqueous solutions are removed or added. The shear forces created by a suction device (e.g., a pipet tip) as it is close to the cell layer removing the last microliter of solution also is adequate to damage and remove cells. When it is necessary to remove all liquid from a well before addition of liquid in the next step, cells are left exposed directly to air. Any direct exposure to air is undesirable and causes stress to the cells. It may result in impaired and unpredictable function and response. When working with large numbers of wells there will obviously be cells
that will be exposed to air for prolonged periods of time. This only aggravates the problem of drying and of unpredictable results.

The technique of encapsulating the cells was applied for testing to solve all of the above problems and to further advantages. Cells encapsulated in highly porous, biocompatible gels in a bead form enjoy the advantage of being protected from mechanical and physical forces that are at play in a small well. In addition to adding this protection, small beads can be handled in suspension and, thus, are very amenable to be dispensed by standard robotic equipment. This reduces the amount of manual labor involved and increases reproducibility of cell count per well, another factor that is often hard to control when using multiwell plates. An important feature is that beads do not restrict the user to cell culture plasticware. By using 96-well plates that incorporate a filter-bottom one can now apply vacuum below the plate to empty all wells in just one simple step. This makes exchanges of solution more efficient and rapid. For collection of solutions for assaying, it also is possible to collect the contents of each well in the vacuum process. All of the above steps can take advantage of an automated process using robotics.

Cells in suspension in alginate (1.5 x 10⁶ cells/ml) were encapsulated in approx. 800 µm beads (approx. 4,000 cells/ bead) by dripping and congealing the slurry into a Ca⁺⁺ containing solution. A suspension of alginate beads was aliquoted into polystyrene plastic 48-well or 96-well tissue culture plates yielding approx. 50 beads per well.

49/206 cells were encapsulated in alginate using the following procedure. Trypsinized and PBS-washed cells are evenly suspended in a 1.5 - 2% final concentration of sodium alginate (50:50 mixture of LV low viscosity and HV high viscosity, Kelco, CA) in growth medium without serum. The suspension is loaded in a syringe and then dispensed through a 27 gauge needle at approx. 0.3 ml/min. The droplets leaving the tip of the needle are blown off by a continuous air stream. By adjusting the velocity of the air stream, beads averaging approx. 800 µm can be achieved reproducibly. The droplets are blown into a container holding a 1.35% (w/v) CaCl₂/20 mM HEPES solution. The beads are allowed to fully congeal for approx. 10 min in the CaCl₂ solution. Beads are washed twice in growth medium without serum and
placed a T-flask with regular growth medium and incubated for about 72 hours with one feeding at 48 hours.

After incubation the beads were transferred into a 50-ml conical and the total volume adjusted so the settled bead slurry makes up approx. 50% of the volume. Using a repeat pipetker, 50 μl bead slurry (about 30,000 cells) is dispensed into each well. Washing, stimulation, and assaying is performed as described above.

The data presented FIG. 5 demonstrate that it is possible to encapsulate engineered RIN cells and maintain comparable responses to secretagogues relative to non-encapsulated cells. The fold responses are essentially equivalent to the data in FIG. 3 and FIG. 4 with regard to fold stimulation. It should be noted that the data falls within a narrower range as indicated by the smaller standard deviation values derived. This observation is indicative of better control of total remaining cell number at time of stimulation and of more stable conditions for all cells in the individual wells.

EXAMPLE 4

Maintenance of secretion performance in a 96-well format.

Several criteria must be met in order for cell-based screens to meet the current industry standards for high through put screening (HTS). First, the screens must be adaptable to a microtiter plate screening format. Second, the read-out or signal from an assay must be compatible with data management software so information can be tracked and integrated. Third, total screen time should be minimized. Fourth, assays should be sensitive and precise.

When cultured and assayed in 12-well plates, measurements of insulin secretion from BetaGene cell lines are sensitive and precise (Hohmeier et al., 1997). It is important to determine if these properties can be maintained when the cells are cultured and assayed in microtiter plates. As shown in FIG. 6, the performance of βG 49/206 cells was compared in 48-well and 96-well formats. Cells were plated, cultured, and assayed in 48-well dishes (100,000/well) as described in the legend to FIG. 3. For 96-well assays, βG 49/206 cells (30,000 per well) were plated and cultured for 48 hrs. in 150 μl of BetaGene Medium/ 2.5%
fetal bovine serum: washed twice (20 min each, in 200 μl in HBBS), and stimulated with glucose or glucose plus IBMX. The pattern of secretory responsiveness is maintained when βG 49/206 cells were plated, cultured, and assayed in a 96-well format: the inclusion of diazoxide in the medium provides a slight clamp to basal secretion, glucose alone is potently stimulatory, and the glucose response can be augmented by the inclusion of IBMX as a secretagogue.

EXAMPLE 5
Construction of expression plasmids and production of stably transfected RIN cell lines.

To enhance the responsiveness of insulin secretion to various modulators, a number of receptor cDNAs or genes were engineered into RIN cell lines for the stable expression of the receptor proteins. Receptors of interest include the following: alpha-2 adrenergic receptor (ATTC number 59303, HPalpha2GEN Genbank accession numbers M18415, M23533, incorporated herein by reference), glucagon-like peptide I receptor (Genbank accession numbers: L23503, U10037, U01156, U01104: each incorporated herein by reference), somatostatin receptor V (mouse Genbank accession number AF004740; human Genbank accession numbers: L14856, L14856, M81830, M96738, M81829, L07833 each incorporated herein by reference). Other receptors to be used include the SUR channel (Genbank accession numbers L78207, U63455, L78243, incorporated herein by reference), KIR channel (Genbank accession number D50582, incorporated herein by reference), pancreatic polypeptide receptor (Genbank accession numbers: Z66526, U42387, U42389 each incorporated herein by reference), muscarinic receptors (Genbank accession numbers: X52068, X15264, X15265, X15266, AF026263 each incorporated herein by reference); glucocorticoid receptor (Genbank accession numbers: M10901, M11050 each incorporated herein by reference). human (glucose-dependent insulinotropic peptide) GIP receptor (Genbank accession number X81832, incorporated herein by reference) human PACAP/VIP receptor (Genbank accession numbers L36566, D17516, U18810, each incorporated herein by reference) human β-cell type Ca2+ channel (Genbank accession number M83566 incorporated herein by reference) and leptin receptor (Genbank accession numbers: U43168, U52912, U52913, U52914 each incorporated herein by reference), human gastrin/cholecystokinin (CCK) B receptor (Genbank accession numbers: L34339, L07746 each incorporated herein by reference), human CCK A receptor
Genbank accession number L13605 incorporated herein by reference) and human galanin receptor (Genbank accession number L34339 incorporated herein by reference).

Following the appropriate manipulations, DNAs encoding the receptors were ligated into plasmids suitable for the stable transfection of mammalian cells. Such plasmids contain genes that confer resistance to antibiotics and cloning sites for transgene insertion and expression. Resistance to hygromycin (hygromycin phosphotransferase) is encoded in the plasmid designated pCB7 and resistance to zeomycin is encoded in CW102 (pZeoCMV). CW102 was created by replacing the SV40 promoter in pZeoSV with the CMV promoter. pZeoSV was digested with Bam HI and the ends were blunted-ended by a fill-in reaction with Klenow. The CMV promoter was excised from pAC/CMV by digestion with Not I and prepared for blunt-end ligations by treatment with Klenow. There are two copies of the CMV promoter in CW102: one driving the expression of the zeomycin resistance gene and the other for transcribing transgenes of interest.

RIN 1046-38 cells and derived cell lines were grown BetaGene Medium containing 7.8 mM glucose and supplemented with 3.5% fetal bovine serum (JRH Biosciences, Lenexa, KS), 100 milliunits/ml penicillin and 100 µg/ml streptomycin. Cells were passaged weekly using 0.05% trypsin-EDTA solution and cultured in an atmosphere of 95% air and 5% CO₂ at 37°C.

For stable transfections, RIN cell lines were grown to 50 to 75% confluence, harvested by trypsinization, washed once with phosphate-buffered saline (PBS), and resuspended in PBS for counting. For each electroporation, 1 x 10⁷ cells were pelleted by centrifugation at 1000 rpm for 2 minutes and resuspended in 0.4 ml electroporation buffer (137 mM NaCl, 6 mM glucose, 5 mM KCl, 0.7 mM Na₂HPO₄, 20 mM Hepes, pH 7.0; or in BetaGene medium without serum). DNA was added to the cell suspension to achieve a final concentration of 30-50 µg/ml. DNA was electroporated into the cells in a 2 mm cuvette at 170 volts. 510 µF and 129 ohms using an Electro Cell Manipulator 600 (BTX, Inc.). Stably transfected cells were selected by culturing in the appropriate drug for about 2 weeks. The drug concentrations used were: 500 µg/ml active fraction G418 (Geneticin, Gibco Life Sciences); 300 µg/ml for hygromycin (Boehringer Mannheim); 400 µg/ml for zeomicin (InVitroGen).
The gene encoding the human alpha-2A receptor (α2AR) inserted into a plasmid backbone (ATCC number 59303, HPalpha2GEN) was purchased from the American Type Culture Collection. Following replication and preparation of this plasmid at BetaGene, the DNA was designated BX700. BX700 plasmid DNA was digested with restriction endonucleases Kpn I, Nhe I, and Hind III to release the α2AR genomic fragment. This fragment was ligated into pBluescript II SK plasmid that had been digested with Spe I, treated with the large fragment (Klenow) of DNA polymerase I to fill-in the overhangs created by Spe I digestion, and dephosphorylated with calf intestinal alkaline phosphatase (CIAP). The plasmid resulting from this ligation, CE406, was digested with Kpn I and Xba I, and the α2AR DNA was ligated in to pCB7 to create CE616 plasmid DNA.

The full-length human glucagon-like peptide I (GLP-1) receptor mRNA (Genbank accession number: L23503) and a portion of the rat GLP-1 mRNA (Genbank accession number: M97797) were reverse transcribed and amplified by the polymerase chain reaction (RT-PCR). Total RNA was isolated from tissues using RNAzol B RNA isolation reagent (Cinna/Biotex Laboratories International). RT-PCR was performed using the Titan™ One Tube RT-PCR System (Beohringer Mannheim). For the amplification of a portion of the rat GLP-1 receptor mRNA, 100 ng of B17/I total RNA was transcribed at 55° C using AMV reverse transcriptase and amplified with a blend of Taq DNA polymerase and Pwo DNA polymerase. 35 rounds of amplification were performed with denaturation at 94° C (30 secs), annealing at 59° C (45 secs) and extension at 68° C (2 min.) using oligonucleotides IDK4 (5’CAGCCTGCCCCTGGAGGGAGC3’ SEQ ID NO:1) and IDK5 (5’CCGAGAAGGCCAGCAGTGTTAC3’ SEQ ID NO:2). Utilizing the same RT-PCR conditions, the full-length human GLP-1 mRNA was amplified from RNA isolated from a human small cell lung line (ATCC: HTB-184, NCI: H510A) using oligonucleotides IDK3 (5’TGGTGGAATTCTGAACTCCACCC3’ SEQ ID NO:3) and IDK6 (5’GATTGCGACCCCGCTGC3’ SEQ ID NO:4). The rat GLP-1 cDNA was subcloned into pNOTA/T7 (5’ to 3’, Inc) to create plasmid CU201. The human PCR product was subcloned into pBluescript KS that had been digested with EcoR V and the resulting plasmid was designated CX800. The GLP-1 receptor fragment was isolated from CX800 following
digestion with *EcoR* I and *Hind* III, and ligated with CW102 that had been digested with *EcoR* I and *Hind* III.

The human pancreatic polypeptide receptor (PPR) mRNA was amplified from RNAs isolated from human lung cell lines (ATCC number: CRL-5816; NCI-H810) using the Titan® One Tube RT-PCR System. 100 ng of total RNA was transcribed at 55°C; 35 rounds of amplification were performed with 94°C denaturation (30 secs), 57°C annealing (30 secs), and 68°C extension (2 min). PCR products were subcloned into pBluescript SK that had been digested with Hind III and filled in with Klenow to create plasmid DG105. The PPR fragment form DG105 was ligated into CW102 as a *EcoR* I/ *Kpn* I fragment.

The mouse somatostatin receptor, type V gene ligated into pBluescript was received from the Dr. F. Charles Brunicardi, Baylor Medical Center, Houston, Texas. Following replication of the plasmid at BGene the DNA was designated CW000. CW000 was digested with *PpuM* I and treated with Klenow. The SSTRV DNA was ligated in CW102 that had been digested with *Bam H* I and treated with Klenow and CIAP, and the resulting plasmid was designated CX503.

**EXAMPLE 6**

**Transgenic overexpression of the α2AR improves the response of RIN cells to Clonidine, an analogue of epinephrine.**

Epinephrine participates in regulating circulating glucose levels by stimulating glucose production from the liver and inhibiting insulin secretion from the pancreatic β-cell. In comparison to human pancreatic islets, βG18/3E1 cells are relatively refractory to epinephrine and Clonidine, an analogue of epinephrine. As determined by the capacity of Clonidine to inhibit insulin-secretion, human pancreatic islets are about 10-fold more sensitive to this compound than βG18/3E1 cells. It was reasoned that the sensitivity of βG18/3E1 cells to Clonidine could be increased by the transgenic overexpression of the α2AR. βG18/3E1 cells were electroporated (EP265) with plasmid CE616. Following selection with hygromycin and
growth, single colonies were assayed by immunocytochemistry for the expression of the transgenic α2AR.

βG18/3E1 cells and single clones derived from EP265 were plated on Falcon 8-chamber culture slides and maintained for 2 days in BetaGene Medium. Following fixation, cells were incubated with α2AR antibody (diluted 1:200; Dr. John Regan, University of Arizona, Tucson). Following incubation with a secondary antibody (antichicken IgG .alkaline phosphatase) immune complexes were detected colormetrically. The specificity of the α2AR antibody was confirmed by competition assays with a α2AR-glutathione-S transferase fusion protein. Eight individual clones were analyzed for sensitivity to Clonidine in insulin-secretion assays. The capacity of the cell lines to secrete mature insulin during stimulation of the regulated secretory pathway was determined by incubating for 1 hour in a mixture of secretagogues. The mixture consisted of RPMI medium (JRH BioSciences) with 5 mM glucose, supplemented with 0.1% BSA, 100 μM carbachol, and 100 μM of isobutylmethylxanthine (IBMX). As evidenced by the capacity of Clonidine to inhibit insulin secretion, one clonal cell line overexpressing α2AR (βG265/2), was about 10-fold more sensitive to Clonidine than human pancreatic islets (FIG. 8) and about 100-fold more sensitive than βG18/3E1 cells (FIG. 7).

βG265/2 cell lines were encapsulated in alginate and injected into the intraperitoneal cavity of Zucker diabetic rats to test if an enhanced sensitivity to Clonidine would extend to in vivo conditions. Beads were maintained in vivo for 3 - 5 days, or until blood glucose normalized. Animals were injected with Clonidine, an agonist of the α2AR (50μg/kg) or Yohimbine, an antagonist of the α2AR (75μg/kg). Blood glucose, rat C-peptide II, and human insulin levels were monitored at 20 minute intervals post-injection. As shown in FIG. 9, Clonidine injection resulted in a 50% reduction of human insulin in plasma; whereas, Yohimbine had no effect on human insulin in plasma. Measurement of rat C-peptide II demonstrated that the vascularized endogenous β-cells were similarly inhibited by Clonidine, but unaffected by Yohimbine. These results indicate that the overexpression of α2AR in RIN cell lines (βG 265/2) confers an in vivo sensitivity to Clonidine.
EXAMPLE 7

Loss of insulin secretion in the absence of FBS.

The progression of NIDDM is characterized by metabolic failure of the pancreatic β-cell and the subsequent extinction of glucose-stimulated insulin secretion. There is currently no optimal \textit{in vitro} system that models the progressive β-cell dysfunction that occurs \textit{in vivo} in NIDDM. FIG. 10A graphically represents insulin secretion from engineered cell lines that have been maintained in culture for one week with βGene medium, supplemented or non-supplemented with 3% fetal bovine serum (FBS). As shown, withdrawal of serum for one week results in the complete ablation of stimulated insulin secretion; however, basal secretion is relatively unchanged relative to controls cells that have been maintained in FBS-supplemented growth medium. In contrast to the dramatic effect on stimulated insulin secretion, the lack of FBS in BetaGene medium has very mild effects on growth, resulting in only a 10 - 20% reduction in total cell number relative to controls following 9 days of culture (FIG. 10B).

The loss of stimulated insulin secretion from engineered β-cell lines that occur in the absence of FBS in the culture medium (FIG. 10A and FIG. 10B) provides an \textit{in vitro} system for modeling the loss of insulin secretion that occurs in NIDDM. All the aspects of engineered β-cells that make them suitable for the identification of insulin-modulating compounds also create an ideal reagent for modeling β-cell dysfunction. The effects of FBS-deprivation shown in FIG. 10 are relatively rapid, reproducible, and amenable to high-throughput screening. Experiments could be designed to identify serum factors that are involved in the maintenance of stimulated secretion, identify candidate genes and proteins whose expression patterns are modulated by FBS-deprivation, or screen for compounds that maintain stimulated insulin secretion despite the absence of FBS in the culture medium. Information from any of these screens could be informative as to biology of β-cell dysfunction in NIDDM and provide new insights into the design of therapeutic compounds.

EXAMPLE 8

Overexpression of Somatostatin Receptor to Enhance Sensitivity to Somatostatin

Somatostatin (SS-28) is a peptide hormone (SerAlaAsnSerAsnProAlaMetalaProArgGluArgLysAlaGlyCysLysAsnPhePheTrpLysThrPheTh
rSerCys **SEQ ID NO:11** that has been shown to inhibit the release of growth hormone, thyroid stimulating hormone, insulin, and glucagon. In addition, SS-28 and its analogue Octreotide may inhibit growth of some tumors. Preliminary studies indicated that RIN 1046-38 clonal cell lines were insensitive to SS-28. Described here is the overexpression of mouse somatostatin receptor, type V gene (**SSTRV**) in a clonal derivative of RIN 1046-38 cell lines. An SSTRV-expressing cell line is analyzed with regard to the effects of SS-28 on insulin secretion.

The cell-based delivery of insulin for the treatment of diabetes is a therapy that requires precise regulation of insulin release in order to achieve tight glycemic control. Historically, BetaGene, Inc. has introduced transgenes to achieve physiologically relevant glucose-sensing in beta-cell lines. More recently as described in the present invention, the introduction of transgenic receptors also has been contemplated so that implanted cell lines can sense and respond to a variety of post-prandial and/or hypoglycemic signals. Included in this effort are the overexpression cDNAs encoding the following cell-surface proteins: subunits of the K\textsubscript{ATP} channel, SUR and Kir, alpha-2 adrenergic receptor, pancreatic polypeptide receptor, glucagon like peptide receptor, glucocorticoid receptor, and somatostatin receptor.

The mouse somatostatin receptor, type V gene (**SSTRV**, Genbank accession number AF004740) ligated into pBluescript and a rabbit polyclonal antibody that recognizes the receptor (**Ab9462**) were received from the Dr. F. Charles Brunicardi, Baylor Medical Center, Houston, Texas. Following replication of the plasmid at BetaGene the DNA was designated CW000. CW000 was digested with PpmM I and treated with Klenow. The SSTRV DNA was ligated in CW102 that had been digested with Bam HI, filled in with Klenow, and treated with CIAP, and the resulting plasmid was designated CX503. BG 40/110 cells (clonal derivatives of RIN 1046-38 overexpressing human insulin and glucokinase) were transfected (EP 603) with plasmid CX503. Following selection in Zeomycin, 13 colonies were selected for further analysis and growth. Portions of the clones were plated onto cover slides and assayed by immunocytochemistry for the expression of SSTRV. The primary antibody **Ab9462** was diluted 1/1000 and immune complexes were colorimetrically detected following incubation with a secondary antibody, goat anti-rabbit linked alkaline phosphatase. Of the 13 clones, one was a high expressor of SSTRV (BG 603/11), and two expressed low levels of the receptor (**BG**
603/8 and 10). In the other clones (including βG 603/7) levels of SSTRV were below detection limits of the assay and indistinguishable from the unengineered clones.

Two clones (a high expressor, βG603/11 and a non-expressor, βG 603/7) were tested at basal and stimulatory conditions with various concentrations of SS-28 added to media under 2 hr. stimulation conditions. As shown in FIG. 11A, glucose-stimulated (10 mM glucose) insulin secretion from βG 603/11 cells were potently inhibited by 50 pM SS-28; whereas, βG 603/7 cells were resistant to all concentrations of SS-28. Furthermore, the effects of SS-28 were such that stimulated secretion from βG 630/11 could be reduced to levels below those observed for basal. In a second set of experiments, SS-28 was tested as an inhibitor of various secretagogues of insulin secretion. As shown in FIG. 11B, at 5 nM SS-28 effectively inhibits stimulated insulin secretion in the presence of BetaGene Medium with no glucose and under conditions of maximum stimulation, Stimulatory Cocktail (BetaGene Media supplemented with 10 mM glucose, 10 mM each of glutamine, leucine, and arginine, 100 µM carbachol, and 100 µM IBMX.).

EXAMPLE 9

Insulin expression and processing in βG H03 cells

Stable transfection of βG H03 with BetaGene plasmid AA603 (simian virus promoter 40 (SV40) driving expression of neomycin phosphotransferase and cytomegalovirus (CMV) promoter driving expression of human insulin) resulted in new clonal cell lines that were resistant to G418 and expressed variable levels of human insulin (~100 - fold differences among various clones). Three clones expressing relatively high levels of insulin were selected for further study: βG 498/20, βG 498/44, and βG 498/45: secreting about 100, 20, and 50 ng/million cells/ 24 hrs, respectively. The cellular contents and culture medium of βG 498/20 were extracted with acetic acid and fractionated by high-performance liquid chromatography. Immunoreactive insulin species were quantified by radioimmunoassay using human insulin standards. Proinsulin was effectively processed to mature insulin, with mature insulin representing the majority of the total insulin both in whole cell and media extracts (FIG. 12A and FIG. 12B). The chromatography in FIG. 12A was derived the cellular contents of βG 498/20, and FIG. 12B is derived from insulin secreted into the media. These data verify that
the proconvertases are active and function efficiently to process insulin in the clonal cell lines. The stability of insulin output for βG 498/45 has been maintained for > 50 population doublings.

EXAMPLE 10

Regulated secretory pathway in βG H03 cells

βG 498/20, βG 498/44, and βG 498/45 were also studied for the capacity to secrete insulin from the regulated pathway and respond to modulators of secretion. Cells were plated in 12-well tissue culture dishes (250,000 cells/well), maintained for 72 hrs in BetaGene Medium, and washed twice, 20 min each, in HEPES/bicarbonate-buffered salt solution (HBBSS; in mmol/l: 114 NaCl, 4.7 KCl, 1.21 KH₂PO₄, 1.16 MgSO₄, 25.5 NaHCO₃, 2.5 CaCl₂, 10 mM HEPES) supplemented with 0.1% BSA but lacking glucose. Insulin secretion was stimulated by incubating the cells for 2 hrs in HBBSS containing 0.1% BSA and supplemented with 10 mM IBMX. 100 μM carbachol, or 10 nM of the phorbol ester, PMA; all in the presence of absence of 10 mM glucose. As shown in FIG. 13A, βG 498/20 respond robustly to carbachol and PMA (about 10 - 15 fold over basal), however, the cells were unresponsive to glucose and IBMX. βG 498/44 and βG 498/45 were nearly identical in their secretion profiles as compared to βG 498/20. These data are consistent with the presence of a regulated secretory pathway; and it appears that protein kinase C-mediated events dominate in the regulation of secretion. However, as expected, these lung neuroendocrine cell lines do not mimic the response of pancreatic β-cells or β-cell lines to glucose alone or the glucose-potentiator, IBMX.

βG 498/45 was further engineered for increased levels of insulin expression by the introduction of number of plasmids, all of which encoded human insulin but varied in the genes encoding antibiotic resistance. The 793, 794, and 796 cell lines are resistant to mycophenolic acid, puromycin, and hygromycin, respectively. The data in FIG. 13B show the presence of a regulated secretory pathway in the progenitor cell line (498/45) and the maintenance of this capacity through a second round of engineering (793, 794, and 796 cell lines). Insulin content and secretion were increased by about 3- to 4-fold in second generation clonal cell lines. The insulin secreted from two of these high-producing clones (793/28 and 793/15) was fractionated by high-performance liquid chromatography, and immunoreactive insulin species were
quantified by radioimmunoassay using human insulin standards. Proinsulin was effectively processed to mature insulin, with mature insulin representing the majority of the total insulin in media extracts.

EXAMPLE 11

In vivo analysis of βG H03 cells

Due to high levels of insulin secretion, efficient prohormone processing, and stable transgene expression, βG 498/20. was analyzed for performance in vivo in rodent models of diabetes. Cells were encapsulated in alginate beads prior to implantation. Following treatment of cultures with trypsin/EDTA, cells were washed in PBS and resuspended in unsupplemented BetaGene media. Cells were mixed and evenly suspended in a 2% final concentration of sodium alginate (50:50 mixture of LV low viscosity and HV high viscosity, Kelco. CA) in HEPES buffered BetaGene Medium. Cells were transferred to a syringe and the suspension was dispensed through a 25 gauge needle at approx. 0.3 ml/min. Droplets were blown into a container holding a 1.35% (w/v) CaCl₂/20 mM HEPES solution. The beads were allowed to fully congeal for approx. 10 min in the CaCl₂ solution. Beads were washed twice in growth medium without serum and placed a T-flask with regular growth medium and incubated for about 72 hours with one feeding at 48 hours.

EXAMPLE 12

Correction of hyperglycemia in diabetic rats by βG H03 cells.

NIH nude rats (Strain F344/Ncr-1nu strain the National Cancer Institute, Frederick, MC) were housed in a sterile isolation facility with free access to sterile standard laboratory diets and water. Immune-competent Wistar and Zucker rats were housed in standard facilities and had free access to standard laboratory diets and water. To create models of insulin-dependent diabetes mellitus (IDDM), pancreatic beta cells were selectively destroyed in nude and Wistar rats by intracardiac administration of streptozotocin (STZ (70 mg/kg body weight). Blood glucose was monitored to confirm induction of diabetes; all animals that received cellular transplants had blood glucose levels of greater than 375 mg/dl within 2 days of STZ treatment. Alginate-encapsulated cells were surgically implanted into the intraperitoneal cavity of anesthetized animals.
For each in vivo study, animals were divided into two groups: a control group that received the parental cells, βG H03 or low doses of βG 498/20, and an experimental group that received high doses of βG 498/20. Data in FIG. 14A, FIG. 14B, and FIG. 14C demonstrate that βG 498/20 cells can reverse hyperglycemia in nude and immunocompetent hosts, and insulin delivery in vivo by this cell line is an effective treatment for both IDDM and NIDDM. In FIG. 14A cells were implanted into STZ-treated, diabetic nude NIH rats (25 million/100 grams body weight). The blood glucose values of the control group (n=3) show that the unengineered parental line (βG H03) does not impact blood glucose. In contrast, animals receiving βG 498/20 (n=5) had a rapid reduction of blood glucose within 2 days implant, and this effect was sustained for greater than 20 days.

To study the effectiveness of βG 498/20 cells in an immune-competent model of IDDM, encapsulated cells were implanted into STZ-treated, diabetic male Wistar rats (FIG. 14B). βG 498/20 cells were implanted at two doses, 12.5 million (n = 2) and 25 million cells (n = 1) per 100 grams of body weight. Control cells (βG H03) were also implanted at 25 million cells per 100 grams of body weight (n = 2). As shown, both doses of βG 498/20 affected a correction in hyperglycemia with the following differences: (1) The higher dose of cells reduced blood glucose more rapidly; 2 days versus 4-6 days for the lower dose. (2) The higher dose of βG 498/20 stabilized blood glucose in the normoglycemic range for a longer period of time; 27 days post implant, versus 17 days for the lower dose.

Serum analysis of human insulin and C-peptide and rat C-peptide are consistent with the effects on hyperglycemia resulting from secretions from βG 498/20. In STZ-treated Wistar rats, rat C-peptide was reduced to about 4% of normal pre-implant, and on day 7, post-implant, was less than 10% of normal C-peptide levels. In contrast, secretion by βG 498/20 of human insulin and detection of human C-peptide in the serum, correlated well with cell number (FIG. 15) and the effects observed on blood glucose (FIG. 14B).

The study of in vivo performance of βG 498/20 cells was extended to a model of NIDDM, Zucker diabetic fatty (ZDF) rats. Encapsulated βG 498/20 cells were implanted at
three doses into obese male (fa/fa) rats: 5 (n=3), 15 (n=3), and 25 (n=2) million cells per 100 grams body weight, and blood glucose was monitored as shown in FIG. 14C. Whereas the lowest dose of βG 498/20 cells failed to affect hyperglycemia, the two higher doses of βG 498/20 were both effective in nearly normalizing blood glucose by day 5 post-transplantation. Human C-peptide levels in the serum correlate with both cell dosing and correction of hyperglycemia. The lowest dose of βG 498/20/cells, that failed to affect hyperglycemia, did not result in detectable human C-peptide in the serum In contrast, two groups receiving 15 and 25 million cells per 100 grams body weight, created a dose-dependent increase in serum levels of human C-peptide.

EXAMPLE 13

Improved Glucose Tolerance by Treatment with Cells Derived from βG H03

As shown in FIG. 16A and FIG. 16B, doses of βG 498/20 that reverse hyperglycemia in STZ-treated Wistar and ZDF rats also result in improved glucose tolerance. For tolerance tests, animals were fasted overnight, tested for levels of blood glucose, and given a bolus of glucose (1 ml of 20% glucose/ 100 gm body weight, injected IP). Blood glucose levels at "time zero" in FIG. 16 represent pre-bolus values, and the 15 min. time point was the first measurement of glucose post-bolus.

Glucose tolerance testing was performed on the STZ-treated Wistar rats on day 19 post-transplantation. As shown in FIG. 16A, only the higher dose of cells is functioning to correct hyperglycemia at this time point. Likewise, with 25 million βG 498/20 cells per 100 gm body weight, there is a clamping of hyperglycemic excursion following a glucose bolus. The rats that received a low dose of βG 498/20 and βG H03 were glucose intolerant. As shown in FIG. 16B, treatment of ZDF rats with βG 498/20 cells results in a similar pattern: low cell doses that fail to correct hyperglycemia do not correct glucose intolerance; however cell doses sufficient to reduce blood glucose also improve glucose tolerance. The glucose tolerance test in ZDF rats was performed on day 13 post-transplantation.
EXAMPLE 14

Reduction of Glycated Hemoglobin using βG H03 cells.

A marker that predicts susceptibility to the long-term complications associated with diabetes is glycated hemoglobin (GHb). Sustained, poor glycemic control correlates with an excessive glycation of hemoglobin and the subsequent development of retinopathy, neuropathy, and nephropathy. Consequently, an important criterion for the effectiveness of any given therapy for diabetes is a reduction in percent of hemoglobin that is glycated. Blood samples were taken from rats and glycated hemoglobin in the blood was determined with Helena GlycoTek affinity columns (cat number 5351, Helena Laboratories) as recommended by the manufacturer. As shown, in FIG. 17A, in STZ-induced nude rats receiving βG H03 cells, GHb is greater than 13% of total hemoglobin; whereas, rats receiving insulin from βG 498/20 cells have normal levels of GHb (5.4 +/- 0.76 % of total hemoglobin is glycated). Likewise, measurements at day 20 of post-transplantation in STZ-treated Wistars treated with βG 498/20 show about a 33% reduction in percent of total hemoglobin that is glycated as compared to untreated controls (FIG. 17B).

EXAMPLE 15

Indications of Immuno-Resistance of Engineered βG H03 cells.

Surprisingly, βG 498/20 cells were as effective and durable in immune-competent rats as they were in nude rats (FIG. 14A versus FIG. 14B and FIG. 14C). Though alginate provides time-limited and partial protection from the immunotoxicity of the host, it was expected that graft survival in the Wistar and Zucker rat strains would be much reduced from that observed in the nude rat. The prolonged survival of encapsulated βG 498/20 in two immune-competent hosts suggests that this cell line may be intrinsically resistant to the effects of immune-mediated killing and cytotoxicity, and/or somewhat invisible to immune surveillance. In an initial set of experiments to test this possibility, βG 498/20 and βG H03 cells were exposed to a number of human cytokines that are known to participate in immune-mediated cytotoxicity (FIG. 18A). Cells were plated into 96- well plates (50,000 /well), grown in BetaGene Medium for 24 hours and switched into medium supplemented with cytokines for 48 hours. Viability of the cultures was determined by an MTT staining assay. As shown, both βG H03 and its clonal derivative βG 498/20 were resistant to various concentrations of Interleukin -1beta (IL-1β), interferon
gamma (IFNγ), tumor necrosis factor-alpha (TNFα), and the combined actions of all three of these cytokines.

The effect of cytokines on insulin secretion from βG 498/20 was also tested, and as shown in FIG. 18B, the cells' secretory function was unaffected by the effects of cytokines. Cells were plated in 48-well plates (~90,000 cells/well) and cultured for 2 days. For secretion studies, cells were washed twice, 20 min each, in HEPES/bicarbonate-buffered salt solution (HBBSS; in mmol/l: 114 NaCl, 4.7 KCl, 1.21 KH2PO4, 1.16 MgSO4, 25.5 NaHCO3, 2.5 CaCl2, 10 HEPES) supplemented with 0.1% BSA but lacking glucose. Insulin secretion was stimulated by incubating the cells in HBBSS containing 0.1% BSA and supplemented with 10 mM glucose, or 10 mM glucose plus either 100 µM carbachol or 10 nM PMA. After a 2 hr incubation, medium was collected and assayed for insulin by radioimmunoassay. Two sets of cultures were exposed to cytokines for 24 hours, prior to secretion studies (24h cytokines, and 24h cytokines + HBBSS + cytokines); and two sets of cultures were supplemented with cytokines for the 2 hr secretion period (HBBSS + cytokines, and 24h cytokines + HBBSS + cytokines). The control culture (HBBSS) was not exposed to cytokines. As indicated, cultures were exposed to the following mixture of human cytokines that have been shown to impair cellular function and cause cell-killing in multiple cell types: IL-1β (5 ng/ml) IFNγ (200 units/ml), TNFα and TNFB (10 ng/ml). Surprisingly, neither short-term exposure during the secretion period, nor a long-term 24 hr. pre-exposure to cytokines had any effect on insulin secretion from βG 498/20 cells.

An explanation that is consistent with the data shown in the data shown in FIG. 18 and that shown in FIG. 14 (comparable function in immune-deficient and immune-competent hosts), is that the alginate provides a time-limited barrier against the cellular-mediated aspects of immunotoxicity (20 - 25 days in nude and immune-competent hosts). Since βG 498/20 cells are resistant to cytokines (small molecules that diffuse freely across the alginate barrier) the function of the cells is equivalent in nude and immune-competent hosts as long as cellular-mediated immune killing is prevented by the encapsulation device. The results shown in FIG. 18 are contrary to the studies of rodent beta-cell lines that have been shown by multiple groups to have impaired secretory function and diminished viability when exposed to cytokines.
Previously, the inventors have shown that both INS-1 and RIN beta-cell lines are susceptible to cell-killing by IFNγ and that IL-1β is cytotoxic to INS-1 cells (Hohmeier et al., 1998). Although protection from IL-1β in these studies was induced by the overexpression of manganese superoxide dismutase in the INS-1 cells, a cell line that is inherently cytokine-resistant may be a preferred starting material for cell-based delivery of therapeutic products in immune-competent hosts.

EXAMPLE 16

High levels of transgene expression in βG H03 and βG H04 cells

In addition to human insulin, βG H03 cells have been engineered to express glucagon like peptide 1 (GLP-1) and human growth hormone (hGH). The former peptide was efficiently processed from the precursor pre-proglucagon and amidated. Clonal cell lines capable of secreting 1 ng GLP-1/million cells/24 hr were isolated.

The human neuroendocrine cell line βG H04 was stably transfected with BetaGene plasmid AA603 (SV40 driving the expression of neomycin phosphotransferase and CMV driving expression of human insulin) resulting in monoclonal cell lines βG 707/55, 707/63, 707/76, 707/94 and 707/96. The clonal cell lines were studied for their ability to secrete insulin in response to various modulators of secretion, as previously described. In each of the 5 clonal cell lines insulin secretion did not change with respect to basal in response to stimulation by 10 mM IBMX, 100 mM carbachol, or 10 nM PMA; and 10 mM glucose. In a subsequent secretion study, the cells were compared at basal (0 mM) and stimulated (25 mM KCl + 2.5 mM Forskolin + 50 mM IBMX) conditions in HBBSS. A high concentration of KCl causes cell membrane depolarization and a subsequent release of all peptides destined for secretion. Forskolin and IBMX enhance the cascade by increasing the production of cAMP, thereby stimulating secretion. Thus, this combination of secretagogues should cause the cells to void any peptides stored in secretory granules. FIG. 19 illustrates the secretion response of βG 707/55, 63, 76, 94, 96 clones and the a clonal derivative of βG H03 (βG 498/45) to the secretagogue cocktail described above. As expected, βG 498/45 cellos, secrete in excess of 500 ng/flask/hour of insulin. In contrast, βG 707 clonal lines secrete a negligible amount of insulin under these conditions. Cell content of βG 707/55 was analyzed by HPLC for insulin.
A small proinsulin peak was detected, however no mature insulin was detected within these cells.

In a separate stable transfection of βG HO4 cells. BetaGene plasmid CD303 (CMV driving expression of human growth hormone, SV40 driving neomycin resistance) was used to establish cell lines resistant to G418. The monoclonal cell line βG 785/5 was analyzed for cell content versus secreted human growth hormone on a Western blot. The results indicated a small fraction of human growth hormone stored within the cells and a large fraction of this peptide in the medium.

These data suggest that the βG HO4 cell line, despite the presence of multiple proteins associated with a neuroendocrine phenotype is not a preferred candidate for secretion of transgenic peptides from the regulated secretory pathway. These cells use a constitutive mode of secretion, rather than a regulated secretory pathway, perhaps due to an inability to depolarize the cell membrane or an absence of dense core granules for peptide storage. Several factors controlling peptide trafficking also may be missing in these cells, further complicating regulated peptide release. In addition to falling short of the regulated peptide secretion requirements, the βG HO4 cells do not process insulin to its mature form. Unprocessed proconvertase 1/2 (PC1/PC2) is present in this cell line, and only proinsulin is detected by HPLC. These data highlight the inefficiencies of neuroendocrine cells with respect to creating a beta-cell phenotype. Although this cell type displays many desirable factors needed to mimic the beta-cell, it also may display many undesirable characteristics, emphasizing the need to thoroughly study and analyze each candidate neuroendocrine cell line.

In order to express high levels of a processed peptide hormone from clonal derivatives of the βG H04 cell line, it may be necessary to create fusion proteins containing a furin site between a given prohormone sequence and the sequence encoding the mature peptide hormone. Such a site may provide the capacity for processing of the transgenic fusion protein through the constitutive pathway that apparently dominates secretion from these cells. Alternatively, the overexpression of PC1 and/or PC2, proteins involved in granule formation such as chromagranin A and chromagranin B, or proteins required for trafficking through the regulated
secretory pathway such as carboxypeptidase E may be required for restoration of a functional regulated secretory pathway in the βG H04 cell line. Carboxypeptidase E is a particularly attractive candidate, as carboxypeptidase E is not expressed in βG H04 cells.

EXAMPLE 17

Overexpression of GLUT-2 transporter in 498/20 cells results in increased sensitivity to STZ.

For reasons of safety and/or enhanced mechanisms for regulating secretory function, a preferred embodiment in the in vivo delivery of peptides via transplantation of engineered cell lines, is the installation of a mechanism that allows for the transplanted cells to be "turned-off" in both secretory function and growth potential. Scenarios where this "off switch" may need to be employed include a malfunction in the graft, an alteration in the physiology of the host creating an incapability with the graft, or a breach in the encapsulation device rendering it permeable to cells. Ideally, an "off switch" for the transplanted cells will be non-invasive to the host; easy to administer; have short-term, immediate effects; and be selective for the grafted cells and non-toxic to the host. One "off switch" that can fulfill these criteria is the installation of a negative selection system into the transplanted cells. By definition, the cells would be engineered to express a protein that converts a non-toxic substance to a cytotoxic one, through catalysis, transport, or binding. Examples of negative selection systems include herpes simples virus thymidine kinase in combination with ganciclovir; cytosine deaminase in combination with 5-fluorocytosine; glucose transporter, type 2 (GLUT-2) in combination with streptozotocin (STZ) and the use of nitroreductase. U.S. Patent application serial number 08/546,934 and PCT publication WO 97/15668 are specifically incorporated herein by reference in that the referenced documents provide methods and compositions comprising GLUT-2 and GLUT-2 chimeras, as such the techniques described therein emphasize the utility of negative selection aspects with the present invention.

βG 498/20 cells, a human neuroendocrine cell line engineered to express insulin, was tested for sensitivity to STZ and found to be resistant to cell killing at concentrations up to 10 mM. βG 498/20 cells were electroporated (EP642) with plasmid AD402 (CMVp-GLUT2/SV40p-Hygro), selected for resistance to hygromycin, and tested by Western blotting
for the expression of the GLUT-2 transporter. βG 642 clones expressed variable levels of the transgenic GLUT-2, and those cells transfected with a plasmid conferring hygromycin resistance alone (βG 640-v) did not express detectable levels of the transporter. The levels of GLUT-2 in the βG 642 clones as detected by Western blot analysis, correlate with functional transport capacity. High GLUT-2 expressors were most sensitive to STZ, with some cell lines effectively killed at less than 3 mM. These data prove the feasibility of converting a human cell line such as βG 498/20 from one that is STZ-resistant to a STZ-sensitive phenotype by the overexpression of the GLUT-2 transporter.

EXAMPLE 18

Methods for Detection of Secretory Function.

Although measuring insulin in the media of cultured cells is a convenient method for assessing secretory function, it may be desirable to create additional read-outs of cellular performance that require less time, are compatible with assays currently in use in the drug discovery industry, and relate to various aspect of beta-cell metabolism. The present example discusses such alternative detection methods.

Two important molecules in β-cell signaling are ATP and Ca^{2+}. The metabolism of glucose is converted to a secretory signal in large part by altering ATP/ADP ratios. Increases in this ratio, resulting from increased glycolytic flux, cause closing of the $K_{\text{ATP}}$ channel, depolarization of the plasma membrane, and increases in cytosolic Ca^{2+}. Increasing cytosolic Ca^{2+} is a common mechanism by which secretagogues stimulate insulin exocytosis.

Intracellular Ca^{2+} and ATP can both be detected with assays that are compatible with HTS. Numerous methods are currently in use for the detection of Ca^{2+} by fluorescence including those that use dyes, or more recent techniques that depend on transgenic expression of proteins that fluoresce in a Ca^{2+}-dependent fashion (Scheenen and Pozzan, 1998). Calcium-binding dyes that increase in intensity of fluorescence in a dose-dependent fashion, such as Fluo-3 and Calcium green, are widely used in cell-based assays in the pharmaceutical industry. In examples of such assays, βG 49/206 and βG 40/110 cells are washed to achieve a basal state.
in secretion, loaded with Calcium green, and stimulated with various secretagogues. Insulin secretion should correlate with increases in calcium-dependent fluorescence.

As an alternative to dyes, it may be desirable to stably express the Ca\textsuperscript{2+}-sensitive photoprotein aequorin in either βG 49/206 or βG 490/110 cells. It has recently been shown that this protein could be targeted to either the cytoplasm and/or mitochondria of the rodent β-cell line INS-1, and stably transfected clonal derivatives provided a model for studying the effects of nutrient-stimulated insulin secretion on subcellular Ca\textsuperscript{2+} (Maechler et al., 1997; Kennedy et al., 1996). Studies from this same group have shown that INS-1 cells transfected with cytosolic luciferase served as a model to monitor ATP changes in living cells. Luciferase-expressing clones were monitored by photon detection, and shown to be a model for tracking ATP changes simultaneously with stimulated insulin secretion (Maechler et al., 1998).

Based on these results, it is reasonable to assume that the secretory function of engineered RIN cell lines can be monitored by Ca\textsuperscript{2+}-dependent fluorescence or luciferase/ATP-dependent luminescence. 96-well and 384-well detection systems for fluorescence and luminescence are currently in use; therefore, these assays may provide an attractive alternative or adjunct to the detection of insulin. BetaGene has begun studies with fluorescent Ca\textsuperscript{2+} dyes in βG 49/206 cells, and is poised to express aequorin and/or luciferase as needed to enable or enhance HTS of secretory function.

**EXAMPLE 19**

**Methods for Creating a Human β-Cell Line**

As described in the previous examples, there may be potential drawbacks for the use of existing human neuroendocrine cell lines for glucose-regulated delivery of human insulin. For this reason, a two-step transformation procedure has been devised to create a human β-cell line (FIG. 22) Preferred starting materials will consist of either a surgically removed human neuroendocrine tumor such as an insulinoma, or isolated primary tissue such as human islets. The β-cells in these tissues proliferate at a very slow rate, therefore, the first step is to get them to grow. This will be accomplished by infecting insulinoma and/or islets with a recombinant adenovirus expressing an oncogene under the control of the rat insulin 1 gene promoter (RIP).
Adenovirus is the preferred viral vector because it will infect and express its transgene in nondividing cells. RIP will selectively express the oncogene, in this case, temperature sensitive SV40 T-antigen (tsTAG), in only β cells. In response to tsTAG expression the β cells should proliferate while other cell types remain quiescent. The drawback and advantage is that adenovirus does not integrate into the genome and thus will not give long-term expression of tsTAG, therefore, a second transformation step is required.

The second step is to immortalize the proliferating β-cells by infection with a recombinant retrovirus also expressing an oncogene like tsTAG under the control of RIP. Retroviruses require cellular division in order to integrate into the genome. Once integrated the transgene should be stably expressed resulting in an immortalized cell.

**EXAMPLE 20**

**Culturing of Human Insulinomas**

The present invention contemplates the use of cell lines derived from human insulinomas as starting cells for the instant methods to produce immortalized human neuroendocrine cells. This example describes the culturing of human insulinomas.

Patients with insulinomas are treated by surgical excision of the tumor. At the time of excision, the tumor tissue is immediately placed in sterile, tissue culture media (BetaGene medium supplemented with 3.5% fetal bovine serum (FBS), 200 U and μg/ml penicillin/streptomycin, and 50 μg/ml gentamycin). The tissue is kept on ice and sterile, keeping the transit time to less than 30 minutes. Using sterile techniques, the tissue is minced with iris scissors until it is reduced to pieces 1 mm³ or smaller. The tumor is then transferred to 40 mesh tissue sieve through which the large pieces are forced using rubber pestle. The cells are then washed twice for a period of 15 minutes each with fresh culture media containing antibiotics.

The tissue is then split onto standard Falcon tissue culture dishes and dishes coated with matrigel extracellular matrix. The tissue is maintained under standard tissue culture atmospheric conditions of 37°C; 5% CO₂/95% air; and humidified. The tissue is then cultured
with media composed of 30% conditioned tissue culture media (BetaGene medium containing 3.5% fetal bovine serum (FBS) conditioned by culture with βG 261/13, a rat β-cell line stably transfected with pCB6 expressing the full length human growth hormone coding region), 70% BetaGene Medium product # 62469-79P, 1% FBS, 50 μg/ml gentamycin. To prevent loss of unattached cells, only 75% of media is replaced by removing old media from the top of the dish. Using this approach a human insulinoma (HT6#2) was cultured, and has been found to secrete insulin for over 150 days (FIG. 23).

EXAMPLE 21

Rat Islet Isolation

Rat islets from adult animals weighing 150-200 g were isolated using the following protocol. Rats were anesthetized with i.p. injection of Nembutal, placed on their back ventral side up, and the abdominal area was wetted with 70% alcohol. Using large forceps and large scissors a midsagittal cut through the skin and musculature from hip level to xiphoid process was made to expose the abdominal cavity. Lateral cuts through skin and musculature were made at the level of the ribs to fold abdominal walls down. The duodenum was located under and adjacent to the liver on the animals right side. The bile duct was clamped where it enters the duodenum with a hemostat, which was positioned so the bile duct was straightened out but not stretched.

The bile duct was blunt dissected from liver adhesions and connective tissue at the level of the liver hilus, while being careful not to rupture the descending aorta directly beneath bile duct. The bile duct was held with fine forceps as close to the hilus bifurcation as possible. While the bile duct was lifted slightly, microscissors were used to nick the bile duct just downstream of the forceps hold. The beveled end of cannula was inserted into the bile duct lumen through the nick, and the end of the cannula was worked down the bile duct to a level past the bile duct branches to the liver lobes. With slow steady pressure, 6 ml of ice cold collagenase solution (2 mg collagenase P/ml, Boehringer 1 249 002) was injected into pancreas through the bile duct. The dorsal aspect of the diaphragm was cut to allow access to the heart, and the left ventricle was bisected with the scissors.
Using forceps, the pancreatic attachments to the large intestine, the mesenteric attachment of the duodenum, and the spleen attachment to greater curvature of stomach were dissected. Then the pancreatic fat from the spleen to the stomach was cut, and while holding the duodenum at the pylorus, the gut was bisected on the duodenal side of the pylorus. The duodenum and attached pancreas was removed from abdominal cavity by cutting the connections to the dorsal cavity wall, the spleen and the gut. The pancreas was then placed in weight dish and any remaining fat and lymph nodes were trimmed off. The pancreas was transferred to a 50 cc tube on ice, and digested in a 37°C water bath for 17 minutes. The digestion was stopped by adding ice cold M199/5% NBS to the 40 ml mark. The tube was then shaken sharply for 5 strokes, and then centrifuged at 1000 rpm for 2 minutes. The supernatant was decanted, and the 40 ml wash was repeated with ice cold M199/5% NBS a total of 3 times. Any remaining undigested connective tissue was removed.

The pellet was resuspended in 20 ml of media, and the digest was poured through a tissue sieve and collected in a fresh 50 ml tube. The original tube was rinsed with 20 ml of media, and the rinse was poured through the tissue sieve. The sample was centrifuged at 1000 rpm for 2 min, the media was poured off, and the tube was drained upside down on a paper towel to remove as much media as possible. Then 10 ml of Histopaque-1077 (Sigma 1077-1) was added, and the pellet was resuspend by vortexing maximally for an instant (2 sec). At this point, 10 ml of media was slowly added to form the top layer of the gradient. The sample was centrifuged in a swinging bucket rotor centrifuge at 2400 rpm for 20 min. The islet tissue settled at the interface between the histopaque and the media. The islets were removed with a pipette, placed in a fresh 50 cc tube, and washed twice with media. The islets can be stored for several hours at 4°C. The islets were transferred to a petri dish and visualized with a stereoscopic dissecting microscope and a lateral fiber optic light source. The islets were separated from non-islet tissue debris prior to use with an eppendorf microtip.

1. **Islet Cell Dispersal and Culturing of Islets**

6000 islet equivalents were placed in a 50 ml tube, brought up in PBS (calcium and magnesium free), and then centrifuged to pellet the islets. The islets were resuspended in 5 ml of trypsin/DNase solution (1 mg/ml trypsin, 30 μg/ml DNase final in PBS), and incubated for
15 min at 37°C, shaking vigorously every 5 minutes. The sample was refluxed through a 10 ml pipet if large pieces were visible. To stop the digestion 5 ml of ice cold media was added, and the sample was placed on ice. The cells were pelleted at 600 rpm for 5 min, and resuspend in 6 ml fresh media. The islets were cultured on two types of matrices. In some studies, Matrigel (collaborative Biomedical, #40234) was used as described by supplier with the following changes. Matrigel was thawed overnight at 4 °C and then diluted 1:4 with Medium 199 without FBS. 2 ml was added to each well of a 6 well plate, and the excess was removed. The matrix was polymerized for 1 hour at room temperature, followed by a rinse with PBS. Coated plates were then placed at 50°C for 2 hours to further dry the matrix. Coated plates are stored at -20°C, and then thawed and rinsed once with PBS prior to plating cells.

Alternatively, an extracellular matrix produced by the human bladder carcinoma line HTB-9 (American Type Culture Collection, ATCC HTB-9 (5637)) was used. HTB-9 matrix was prepared by culturing the cells to confluency in RPMI 1640 with 10% FBS as indicated by the supplier. Media was aspirated and cells washed and lysed in water. This was repeated two times to ensure complete cell lysis. The remaining matrix was incubated for 10 to 15 minutes in PBS, rinsed two more times with PBS, and then stored indefinitely under PBS at 4°C. Prior to plating of dispersed cells, the PBS is aspirated. Cells are plated onto both Matrigel or the HTB-9 matrix in Medium 199 containing 4% FBS.

Whole or dispersed rat islets isolated from the pancreas of Sprague-Dawley rats by collagenase digestion and density gradient centrifugation were plated on culture dishes coated with either matrigel matrix or extracellular matrix derived from the bladder epithelial cell line HTB-9. In the case of dispersed and whole islets on HTB-9 matrix, the cells attached and spread to form a discontinuous monolayer rapidly. Greater than 90% of the islet cells in dispersed islet cultures had formed monolayer plaques after two days culture. Whole islets, probably due to their greater cell mass, formed monolayers slower than dispersed islets.

Typically in the whole preparations the islet cells spread from the periphery to form a monolayer ring comprising approximately 50% of the islet cells with the remaining islet cells in the central multilayer islet remnant after 2 days culture on HTB-9 matrix. Attachment and
spreading of both dispersed and whole islets on matrigel extracellular matrix was slower and less complete than that observed for HTB-9 matrix. After 6 days culture, about 70% of dispersed islet cells were in monolayer plaques, and peripheral monolayer zones were just forming on whole islet plaques. In general islet cells on matrigel matrix tended to be taller and rounded in contrast to HTB-9 cultures in which the cells were flattened and spread over a larger area. Fibroblasts from the islets were observed in both the matrigel and HTB-9 matrix cultures but were a minor population (1 to 5%) compared to the epithelial like presumed endocrine cells.

EXAMPLE 22

Human Islet Function in BetaGene Medium

Human islet preparations were obtained from the distribution center of The Diabetes Research Institute, Miami FL. The volume of islets received are expressed in islet equivalents (IEQ). An islet equivalent is the number of cells/volume that is found in an islet with a diameter of a 150 μm. Insulin content and secretory response of the islets was assayed first upon receipt and second after culture in BetaGene medium. Proper insulin processing was also analyzed before and after culture in BetaGene medium.

Methods

1. Receipt and preparation of islets

Islet preparation suspensions were spun down in a bench top centrifuge at 1000 rpm for 2 minutes at room temperature to pellet the cells. The shipping medium was aspirated leaving approximately 5 ml behind to avoid disrupting the pellet. The pellets were resuspended in the remaining 5 ml medium and transferred to a new 50 ml conical tube. About 40 ml of BetaGene medium supplemented with 2% fetal bovine serum, 500 mg/ml gentamycin, 200 units/ml penicillin, and 200 mg/ml streptomycin was added to each suspension and allowed to incubate at room temperature for 15 minutes. The samples were spun down a second time, all but 5 ml of the medium was aspirated, and a fresh aliquot of BetaGene medium with supplements was added and allowed to incubate for another 15 minutes. After the second and final incubation, the islets were spun down and all of the medium was removed. The pellet was resuspended in complete BetaGene medium at a density of 1000 IEQ per milliliter.
2. Alginate Encapsulation of Human Islets

The study of islets in long term culture is facilitated by encapsulating the cells in alginate. Islet cells do not divide in culture and may be overrun by various replicating cells which are present in islet preps as shipped. Encapsulating the cells immediately upon receipt minimizes the growth of fibroblasts and other cell types.

The islets were resuspended in a 2% sodium alginate solution (50% high viscosity and 50% low viscosity sodium alginate made up in complete BetaGene medium) at a concentration of 1000 IEQ per 1 milliliter of alginate. The suspension is transferred to a syringe and allowed to sit at room temperature for 5 minutes to allow all air bubbles to rise to the surface. A 25 gauge needle is attached to the syringe and the islet/alginate slurry is dispensed through the syringe into a 50 ml conical tube containing approximately 35 mls of 1.35% CaCl₂ /20 mM HEPES. Beads are formed as the slurry hits the surface of the CaCl₂ solution, and are completely polymerized after about 10 minutes. The CaCl₂ solution is removed carefully and the beads are washed with two volumes of BetaGene medium / 20 mM HEPES. The encapsulated islets were then cultured with the medium under conditions described for each study.

The glucose concentration of BetaGene Medium was based on the glucose concentration that was provided 50% maximal stimulation during acute secretion studies. Initial studies (n=5 independent islet preparations) indicated that 50% maximal stimulation of human islets was provide by a medium glucose concentration of 7.4±0.2 mM. The glucose concentration of unmodified BetaGene Medium was manufactured at 7.8 mM glucose (or 140 mg/dl).

3. Insulin Content and Processing of Human Islets

A portion of each islet preparation was used to assess insulin content of the islets upon receipt. Prior to culture in BetaGene medium, 2000 IEQ were removed from the stock and spun down to pellet the islets. The medium was removed completely without disturbing the cell pellet. The islets were washed one time with phosphate-buffered saline (PBS) and spun
down. The pellet was dispersed in 0.5 ml content buffer (1M acetic acid, 0.1% BSA) and frozen at -80°C. The cells were thawed, sonicated (3 bursts at setting 5-6) and the insoluble debris were pelleted at 14,000 rpm for 10 minutes at 4°C. The supernatant was then transferred to a clean tube and a portion was analyzed by HPLC.

The HPLC system used for resolving insulin from its precursor, proinsulin:
Beckman System Gold with 166 Detector, 126 Solvent Module, 502 Autosampler
Gilson FC 204 Fraction Collector (set up to collect in deep 96 well plates)
Columns used for separation:
Merck RP-C18 LiChroCART 250-4 (250mmx4.6mm)
LiChrospher 100 (5 μm)
Guard column:
Merck RP-C18 LiChroCART 4-4
LiChrospher 100 (5 μm)
Column and guard in column heater set to 30°C.

Ultraviolet absorption was monitored at 213nm. The 2 buffers used were following:
A= TEAP pH 3.0

20 mM triethylamine (2.8 ml/l)
50 mM ortho-phosphoric acid (3.4 ml/l)
50 mM sodium perchlorate (7.03g/l)

pH to 3 with NaOH

B= 90% acetonitrile (ACN)/ 10% water (degassed RO/DI water)

Gradient used:
33% B –35% B over 10 minutes and after 40 minutes the gradient was increased to 40% B over 90 minutes. Samples were collected from 0-96 minutes.

Flow rate was 1 ml/min and 1-min fractions were collected in deep-well 96 well plates.

To each well 100μl of 0.5M boric acid (31g/l), 1% BSA (10g/l) pH 9.3 (NaOH) was added and samples were frozen at -80°C and lyophilized.
Samples were then reconstituted in 1ml PBS/0.1% BSA (RIA grade) and immunoreactive insulin was assayed using a commercially available radioimmunoassay kit ("Coat-A-Count Diagnostic Products Corp., Los Angeles, CA).

Encapsulated islets were cultured in BetaGene medium and fed 3 times weekly. The islets were removed from the alginate to extract the insulin content. To recover the islets, the beads were incubated in 6 mM EDTA/10 ml BetaGene medium and the alginate was dispersed by pipetting until the mixture became homogeneous. The mixture was centrifuged at 1/2 speed in a benchtop centrifuge for 5 minutes, the supernatant with alginate was removed and the islets washed with 10 ml PBS/2mM EDTA. The solution was spun again and the pellet was resuspended in 5 ml PBS to remove EDTA, spun again and resuspended in content buffer for analysis by HPLC as described above.

4. Glucose Dose-responsive Secretion

Alginate encapsulated islets were cultured in 24 well plates for at least 4 days, with ñ50 IEQ/ well (or 5 beads) in 2 ml of medium. The day before the study the culture medium was replaced with fresh medium. The day of the study the islets are equilibrated for 90 minutes with BetaGene Medium with low glucose. The medium was then removed and replaced with 1 ml of RPMI without glucose, or Modified BetaGene Medium, (manufactured without glucose), that was supplemented with glucose to provide concentrations between 2.2 and 22 mM glucose, and 22 mM + IBMX. The islets were then incubated at 37°C for 90 minutes and samples collected at the end of 90 minutes for assay of insulin. Each experimental value usually represents results from 6 replicate wells. The glucose concentration providing 50% of maximal stimulation (Stim-50) was calculated from the fitted line of the glucose dose-response curve.

Results

Cultured Human Islets and Insulin Secretion

Results in the literature indicate that culture of human islets with high glucose (11-22 mM) is deleterious to secretory function. However, others have indicated that the effects of high glucose were dependent on culture medium used. Culture (> 2 weeks) of human islets was
reported to result in a progressive loss of glucose-responsive insulin secretion over 2 weeks. Different media or glucose concentrations slowed but did not prevent this loss. The glucose concentration of BetaGene Medium was based on the concentration that gave 50% of the maximal glucose-induced response. The effect of different glucose concentrations were tested to ensure that a medium glucose concentration based on 50% stimulation was appropriate. Islets were cultured in BetaGene Medium with 3.9, 7.8 and 22 mM glucose for ≈2 weeks. The secretory responses to glucose concentrations of 3.9 mM, 22 mM and 22 mM +50 μM IBMX were then compared. Although lower glucose was less deleterious than the higher concentration, both resulted in impaired secretory response (FIG. 24). The results demonstrate that neither lower nor higher concentrations of glucose provide improved performance. These results confirm that 7.8 mM glucose in BetaGene Medium is sufficient to sustain secretory function of human islets.

The serum requirements of human islets were tested in long term (≥ 2 month) cultures supplemented with various amounts of serum, 1%, 3.5%, or 10% FBS and 5% horse serum (ES). In four independent isolations the average daily insulin output for 60-90 days was minimally affected by amount of serum supplementation. However, the overall tendency was for higher FBS to yield lower output. Similarly, in an acute secretion experiment, insulin secretion from islets cultured in 10% FBS exhibited lower response to glucose or to a stronger mixed secretagogue stimulus (FIG. 25). The sustained insulin output from human islets with 1% FBS supplementation (in BetaGene Medium) suggested that human islets also may secrete insulin and survive under serum-free conditions.

In order to compare the effect of different media, per se, on human islets, the insulin output over 2-3 months was studied without serum. Islets were cultured with BetaGene Medium, Medium 199, alpha MEM, and CMRL, all with equivalent glucose, and 0.1% BSA. In all four isolations the insulin output was the highest with islets cultured in BetaGene Medium (FIG. 26). In fact, the average insulin output of BetaGene Medium without serum-supplementation was not markedly different from cultures, of the same isolation, cultured with 3.5 or 10% FBS (average serum-free with 4 isolations studied was 112% of cultures supplemented with 3.5 & 10% FBS). Many transplant surgeons consider CMRL the medium
of choice for use with human islets (Warnock et al.). However, CMRL performed the poorest, essentially with no islet survival past 2 months with all 4 isolations studied.

The capacity of BetaGene Medium to sustain the dose-responsive nature of the insulin secretory response was evaluated with continuous cultures. Human islets were stimulated with varied glucose concentrations at intervals to monitor secretory changes that may occur with time. It has been previously noted that the capacity of human islets to respond to glucose is impacted by isolation methods and conditions, in particular, cold ischemia time. Cold ischemia of the preparations studies varied between 10 and 22 h. Variables related to donors and isolations produce considerable variation among islet isolations. As a result, the magnitude of response shown in FIG. 27 is not found with all preparations. However, a common finding was an initially poor response, with increased function with time of culture in BetaGene Medium, and a maintained capability to secrete insulin in response to glucose for times >4 months (FIG. 27).

The sustained secretory function for months in culture was also accompanied by maintained insulin content and insulin processing. This is illustrated with both islets that have initially low or initially high insulin contents, and with islets that initially exhibit minimal insulin processing capacity. The insulin content of islets from HI28 was low upon arrival, 0.3 μg/1000 IEQ with >90% mature, processed insulin. The islets secreted in response to glucose, with 50% stimulation at 7.5 mM. The insulin content of mature, processed insulin with HI28 islets cultured 1.5 months in BetaGene Medium was increased 4 fold to 1.3 μg/1000 IEQ. An islet preparation, HI26, with an initially high insulin content of 5.0 μg/1000 IEQ, and 94% mature insulin, was cultured long term. The insulin content of these islets were maintained for 72 d of culture with BetaGene Medium. The final insulin content was 4.97 μg/1000IEQ, with 89% mature, processed insulin. Finally, culture in BetaGene Medium restored processing with 2 islet isolations that initially had almost no mature insulin. FIG. 28 shows the fractionation of insulin extracted from islets of HI21. Initially (FIG. 28A), 99% of the insulin was unprocessed insulin, with only 29 ng mature insulin/1000 IEQ. The mature insulin content was increased 18-fold to 512 ng/1000IEQ after 4 weeks of culture in BetaGene Medium; this represents >90% of the insulin content (FIG. 28B). Another islet preparation initially making only proinsulin
arrival, HI27. Essentially 100% of the insulin content was proinsulin, prior to culture. HI27 islets were cultured 8 weeks and then insulin content was fractionated by HPLC, with this isolation as well, islets had regained the capacity to process insulin. In both of these preparations, while insulin processing improved the total insulin content (mature + unprocessed insulin) was decreased.

These data demonstrate that human islets cultured in BetaGene medium exhibit improved secretory function, maintained glucose-responsiveness, while maintaining or even increasing proteolytic processing of insulin and insulin content.

EXAMPLE 23

Expression Plasmid Constructs for Enhanced Proliferation and Immortalization

A temperature-sensitive mutant of the SV40 large T antigen, tsA58 (Bourre and Sarasin 1983) was isolated from PBS/tsA58. The tsA58 coding region (tsTAG) was isolated by partial digestion with HpaI, treatment with Klenow fragment, followed by digestion with EcoRI. The resulting 2532 base fragment was ligated into pCMV8/IRES/Neo (Clark et al., 1995) previously digested with BamHI, Klenow treated, then digested with EcoRI. The resulting expression plasmid, pCMV/tsTAG/IRES/Neo, expresses a bicistronic message driven by the human cytomegalovirus promoter with the tsTAG upstream of the G418 resistance gene. Drug resistance to G418 results from translation of the downstream Neo gene due to the internal ribosome entry site (IRES, Macejak and Sarnow, 1991). A second tsTAG expression plasmid was constructed in which the CMV promoter was replaced with the rat Insulin 1 promoter (RIP). pCMV/tsTAG/IRES/Neo was digested with SpeI and EcoRI, removing the CMV promoter, and replaced with RIP on a 440 bp SpeI/EcoRI fragment derived from pRIP7/INS (Clark et al., 1996), generating pRIP/tsTAG/IRES/Neo.

Recombinant adenoviruses expressing tsTAG under the control of either the RIP promoter or the CMV promoter were constructed. The tsTAG encoding fragment was isolated from pCMV/tsTAG/IRES/Neo by digestion with SalI, treatment with Klenow fragment, followed by EcoRI digestion. The fragment was ligated into pAC/RIP that had been digested with BamHI, Klenow treated and digested with EcoRI, generating pAC/RIPtsTAG.
pAC/CMVtsTAG was constructed by removing tsTAG from pBS/tsA58 and ligating into pAC/CMV to produce pAC/RIPtsTAG.

293 cell culture and generation of recombinant adenovirus stocks, conditions for adenovirus stocks, as well as conditions for adenovirus infection of cells are done as described (Becker et al., 1994b; incorporated herein by reference).

Retroviral expression plasmids were constructed in order to produce recombinant retroviruses capable of expressing tsTAG under the control of the tissue-specific rat insulin promoter. A fragment containing RIP/tsTAG was isolated from pRIP/tsTAG/IRES/Neo by digestion with SalI, Klenow treatment followed by SpeI digestion. This fragment was ligated into pBS/hGH PolyA that had been treated with XbaI, Klenow treated and digested with SpeI, generating pBS/RIP/tsTAG/hGH PolyA. The hGH PolyA sequence in pBS/hGH PolyA is a 625 base sequence which directs efficient transcriptional termination and polyadenylation of mRNAs. Finally, pBS/RIP/tsTAG/hGH PolyA was digested with SacI, Klenow treated, followed by digestion with SalI allowing isolation of a RIP/tsTAG/hGH PolyA containing fragment. This fragment was ligated into two retroviral plasmids, pBabeNeo and pBabePuro (Morgenstern and Land 1990), following digestion with SnaBI and SalI, generating pBabeNeo/RIPtsTAG and pBabePuro/RIPtsTAG, respectively. Additionally, the same SacI, Klenow treated, SalI digested fragment from pBS/RIP/tsTAG/hGH PolyA was ligated into pXT1 (Stratagene, Inc.) that had previously been digested partially with SalI, Klenow treated, then digested with XhoI, generating pXT1/RIPtsTAG. Rat insulin promoter driven transcription of tsTAG is in the opposite orientation with respect to the retroviral LTR in all three plasmids. Several packaging cell lines for production of recombinant retroviruses are available (Miller and Buttimore, 1986; Danos and Mulligan, 1988; Miller, 1992).

An alternate approach to ensure that tsTAG is driven by the RIP promoter and not by the viral LTRs is to replace the normal untranslated region (UTR) of the retrovirus with a mutated UTR (von Melchner et al., 1990; GenBank accession numbers M33167 through M33172, inclusive), which results in the loss of promoter/enhancer activity of the retroviral LTR. The mutated UTR strategy has been used previously for promoter trapping (von
Melchner et al., 1990). In the present invention, the inventors contemplate using this technique to confer specificity to the RIP promoter incorporated into the mutated retrovirus.

In addition to tsTAG, two more immortalization genes, the human papilloma virus E6/E7 genes, were obtained from Dr. Jerry Shay and Dr. Woody Wright at the University of Texas Southwestern Medical Center. These genes were cloned into the viral vector backbone LXSN (Miller and Rosman, 1989). E6/E7/LXSN was then introduced into the PA317 packaging cell line to produce replication-defective recombinant retrovirus.

The full length IGF-1 receptor mRNA (Genbank accession number: X04434) was reverse transcribed and amplified by the polymerase chain reaction (RT-PCR). Total RNA was isolated from A549 cells using RNAzol B RNA isolation reagent (Cinna/Biotex Laboratories International). RT-PCR was performed using SuperScript™ Preamplification System (Life Technologies) followed by amplification with High Fidelity Platinum Taq polymerase (Life Technologies). One microgram of total RNA was transcribed at 42°C followed by 35 rounds of amplification with denaturation at 94°C (30 sec), annealing at 55°C (30 sec) and extension at 68°C (4 min and 30 sec) using oligonucleotides AT242; 5'GAGAAAGGGAATTCCATCCCCAAATA SEQ ID NO:12 and AT249; 5'TTCAGGATCCAAGGTACGCAGG SEQ ID NO:13. The IGF1 receptor cDNA was gel purified and cloned as an EcoRI/BamHI fragment into EcoRI/BamHI digested CW102 resulting in plasmid DM202.

EXAMPLE 24

Cell-Specific Expression of tsTAG and beta-galactosidase

Human islet preparations and insulinomas contain many other cell types besides β-cells, therefore, it is important to target oncogene expression to β-cells. The use of RIP or a modified RIP promoter (discussed in Example 8) linked to an oncogene like tsTAG should target expression solely to β-cells as long as there is not promoter interference from viral promoters like retroviral LTRs. LTR interference could result in expression of tsTAG in other cell types besides β-cells, thus creating a more difficult task to isolate an immortalized β-cell line.
1. **Stable Transfection of tS\textsc{TAG}**

   Cell-specific transcription of tS\textsc{TAG} was determined for both pB\textsc{ABE}/Neo/RIPt\textsc{S}\textsc{TAG} and pXT1/RIPt\textsc{S}\textsc{TAG} (construction of these plasmids is detailed in Example 6) in RIN cells and in 293 human fibroblast cells. The retroviral plasmids were stably transfected into both cell types and levels of tS\textsc{TAG} mRNA and protein were determined by Northern and Western blotting, respectively. Significant levels of tS\textsc{TAG} mRNA and protein were detected in RIN cell extracts containing either retroviral plasmid, whereas no expression of tS\textsc{TAG} mRNA or protein was observed in 293 cell extracts containing either retroviral construct. Temperature sensitivity of tS\textsc{TAG} was also observed in RIN cells as significantly more tS\textsc{TAG} protein was produced at the permissive temperature of 33.5°C than at the nonpermissive temperature of 37.0°C. This result corroborates the findings detailed by Frederiksen et al. (1988) in which high-level expression of tS\textsc{TAG} was observed by immunostaining at 33.0°C, but almost no expression was observed at 39.0°C. Because there was no tS\textsc{TAG} present in 293 cells, these results confirm that the viral LTR is not interfering with RIP to express tS\textsc{TAG} in non-\(\beta\)-cells.

2. **Viral Delivery of tS\textsc{TAG}**

   RIN 1046-38 cells were infected with adeno/RIP-tS\textsc{TAG} at varying multiplicities of infection (MOI). The virus was left on the cells for 2 hours then washed off and the cells received fresh medium. The infected cultures were incubated at 37°C for 48 hours then were shifted to 33.5°C for an additional 48 - 72 hours. The cells were washed with PBS and then fixed in Carnoy's fixative for immunocytochemistry. The anti-TAG antibody used to detect TAG expression in RIN cells was from Santa Cruz Biotechnology. Roughly, 10 to 20% of the RIN cells were intensely stained for TAG expression at MOIs of approximately 30 to 300 viral particles per cell.

To provide a higher probability of obtaining \(\beta\)-cell lines, recombinant plasmids utilizing the insulin promoter engineered for enhanced activity are constructed (see Example 8). These constructs provide \(\beta\)-cell specific expression of the oncogene, and in the case of the insulin promoter with enhanced function, also provide a level of gene expression nearly equivalent to that achievable with the CMV promoter.
3. Adenovirus Infection of Rat Islet Primary Cultures

The media was aspirated off 6 well cluster dishes containing primary cultures of islets with the cells well attached to dishes. Then 2 ml of M199 media/10% FBS containing 1000 pfu/cell was added (estimating 500,000 cells from 500 islets and $5 \times 10^8$ pfu/2 ml media). The sample was incubated at 37°C for 1 hour, the media was aspirated, and then 6 ml of M199/4% FBS was added. The sample was cultured for 24 hours, and then expression was checked.

Representative cultures of dispersed and whole islet preparations on both HTB-9 matrix (2 day old cultures) and matrigel matrix (6 day old cultures) were infected with adenovirus expression vectors for β-galactosidase under control of either the CMV promoter (pAC-CMV-β-gal) or the rat insulin 2 gene promoter (pAC-RIP-β-gal). After 24 hours, cultures were cytochemically stained using the β-galactosidase substrate X-gal to characterize expression efficiency. Fresh stain containing 1.75 mM K$_4$Fe(CN)$_6$, 1.75 mM K$_3$Fe(CN)$_6$, 2 mM MgCl$_2$, 1 mg/ml X-gal in water was made up. The cells were washed once with PBS, and then fixed for 20 min at room temperature in 0.5% formaldehyde. The cells were washed again with PBS, 1 ml of stain was added, and the sample was incubated for 30 min at 37°C. The cells were then washed once with PBS. In all culture preparations, dispersed and whole islet on matrigel or HTB-9 matrix staining appeared faster, more intensely, and with higher frequency (greater than 80% of cells) in cultures infected with pAC-CMV-β-gal than in cultures infected with pAC-RIP-β-gal (about 50% of cells).

These results indicate that CMV is a more efficient gene promoter in cultured rat islet cells than the rat insulin promoter although at this time it cannot be ruled out that the difference in β-gal expression under these promoters was due to differences in the titer of viable adenovirus used to infect the islet cultures. It was also observed that fibroblasts stained for the presence of β-galactosidase in cultures infected with pAC-CMV-β-gal but did not stain in cultures infected with pAC-RIP-β-gal indicating a specificity for RIP promoter expression in islet β-cells. These studies demonstrate the feasibility of maintaining primary cultures of islet tissue and using adenovirus expression systems to modify protein production of these cultures.
1. **Additional promoters for expression of transgenes in neuroendocrine cells; the α-glycoprotein promoter.**

The pituitary gland secretes a number of different hormones including leutenizing hormone (LH), thyroid stimulating hormone (TSH) and follicle stimulating hormone (FSH) using a regulated secretory pathway. Each of these hormones contain an alpha and beta subunit. The beta subunits are expressed only in the appropriate pituitary cell types, giving specificity to each hormone. The alpha subunit, called α-glycoprotein, is common to all pituitary hormones and is expressed in all pituitary cell types. Although expression of this protein is fairly ubiquitous in the pituitary, it is postulated to be specific to neuroendocrine cell types only. In transforming pituitary tissue and/or pituitary tumors, the α-glycoprotein promoter may aid in expression of transforming proteins within neuroendocrine cells only and not within non-neuroendocrine cell types which may be also be present in the culture or tumor.

The α-glycoprotein promoter (Genbank accession number LO5632) was amplified by PCR from human liver DNA (Clontech) using Taq Plus Long (StrataGene). Oligonucleotides AT255 (GGGGACTAGTAAACTCTTTGGTGAAG **SEQ ID NO:14**) and AT256 (CTCAGTAACTCGAGTTAATGAAGTCCT **SEQ ID NO:15**) were used in 40 rounds of PCR with denaturation at 94°C (30 sec), annealing at 55°C (30 sec) and extension at 72°C (2 min) to amplify the promoter. The promoter was cloned as a Spe I/Xho I fragment into the SpeI/ SalI site of BetaGene plasmid BL436 (CMV-neo), creating BetaGene plasmid DM102 (α-glycoprotein-neo).

AtT20, RIN38, and H03 cells were transfected with BetaGene plasmids BL436 (CMVneo), BY428 (RIPneo) and DM102 (α-glycoprotein-neo) by electroporation as previously described. Clones resistant to G418 were counted after 13 days of selection. Pituitary cells (AtT20) transfected with BY428 did not survive selection with G418. DM102 created about 75% fewer clones than BL436 in the same cell line. In RIN38 (rat insulinoma) and H03 (human neuroendocrine) cells, DM102 colony formation was equivalent to BY428 with BL436 creating 75% more clones. These data indicate that the α-glycoprotein promoter may provide neuroendocrine-specific gene expression.
EXAMPLE 25

Modified Insulin Promoters

The rat insulin 1 gene promoter fragment (RIP) was modified in an attempt to strengthen its transcriptional activity. The principal modification involved the attachment of varying numbers of Far-FLAT minienhancers (FF minienhancer) (German et al. 1992) to different positions within an intact RIP or to a RIP truncated at -205 (-205RIP). FF minienhancers were constructed by generating oligonucleotides corresponding to the region of RIP between -247 and -196:

5'-GATCCCTTCATCAGGCCATCTGGCCCCCTTGTATAATAATCGACTGACCCTAG

GTCTAA-3' (top strand; SEQ ID NO:5); and 5'-GATCTTAGACCTAGGGTCACTGATTATTACCAAGGGCCAGATGGCCTGGGAAGG-3' (bottom strand; SEQ ID NO:6). The underlined sequences at the ends of the oligonucleotides are BamHI and BgII recognition sites. The oligonucleotides were annealed and ligated in the presence of restriction enzymes BamHI and BgII. Since BamHI and BgII produce compatible DNA ends but can no longer be digested by BamHI or BgII, the only multimers that escaped BamHI and BgII digestion were ligated head-to-tail.

Minienhancers in which the three italicized bases in SEQ ID NO:5 and SEQ ID NO:6 above were mutated are called FFE minienhancers. FFE minienhancers are constructed essentially as described above by generating oligonucleotides corresponding to the region of RIP between -247 and -196: 5'-GATCCCTTCATCAGGCCATCTGGCCCCCTTGTATAATAATAATCGACTGACCCTAG

TAATCTAAATTACCTAGGTCTTAA-3' (top strand; SEQ ID NO:7); and 5'-GATCTTAGACCTAGGGTCACTGATTATTACCAAGGGCCAGATGGCCTGGGAAGG-3' (bottom strand; SEQ ID NO:8). The italicized bases represent the mutated bases. FFE minienhancers were shown to be more active than FF minienhancers when both are attached to a minimal promoter (German et al. 1992). FF and FFE minienhancer dimers, trimers, etc. were separated by polyacrylamide gel electrophoresis and blunt-end cloned into the transient transfection vector, pBS/RIP/hGH, at either an XhoI site immediately upstream of -415 of the intact RIP, into an AvrII site at -206 of an intact RIP, or into an Apal site immediately upstream of -205RIP. The number and orientation of minienhancer repeats were verified by DNA sequencing.
FF and FFE minienhancer/RIP-hGH constructs were transiently cotransfected along with Rous sarcoma virus-chloramphenicol acetyltransferase (RSV-CAT), an internal control plasmid used to monitor differences in transfection efficiencies, into 1 x 10^7 RIN cells by electroporation (Chu and Berg 1987) as modified by Bassel-Duby et al. (1992). The cells were incubated overnight in 199 medium containing 5 mM butyrate. The next day 199 medium containing butyrate was removed and new medium without butyrate was placed on the cells. After 48 to 96 hours, expression of the transfected genes was measured by hGH protein accumulation in the culture medium (Selden et al. 1986) using a radioimmunoassay (Nichols Institute, San Juan Capistrano, CA). The cells were harvested after these time points and extracts were prepared by three successive rounds of freezing and thawing. CAT activity in the cell extracts was determined by the method of Nielsen et al. (1989). Promoter activity as measured by hGH production was then normalized for transfection efficiency differences between samples by the quantitated CAT activity in each sample.

The activities of several FF and FFE/RIP promoters were compared to RIP activity in transiently transfected RIN cells. The best results were obtained with one of the types of FFE minienhancer-RIP constructs. This type of RIP derivative had either three or six copies of the 55 bp FFE minienhancer fused immediately upstream of -410 of intact RIP (pFFE3/-415RIP/hGH and pFFE6/-415RIP/hGH). These modRIP promoters were consistently five- to 6-fold more active than unmodified RIP in RIN cells (FIG. 29). A number of other RIP derivatives were also more active than RIP in transient transfection assays although not to the same extent as FFE3/-415RIP and FFE6/-415RIP.

The strength of the modified RIP promoters was also determined in stably transfected RIN cells. The stable transfection vector, pFFE3/RIP8/INS/IRES/NEO containing three copies of FFE minienhancers (FFE3), was generated by inserting a blunt-ended KpnI/HindIII FFE3/RIP into pCMV8/INS/IRES/NEO in which the CMV promoter was removed with SpeI and SacI. pFFE6/RIP8/INS/IRES/NEO was constructed by inserting an Apal/blunt-ended HindIII FFE6/RIP fragment into pRIP8/hGH polyA in which RIP was removed by
ApaI/EcoRV. A BglII/StuI INS/IREs/NEO fragment was then inserted into pFFE6/RIP8/hGH polyA to complete pFFE6/RIP8/INS/IREs/NEO.

In some of the stable transfection vectors, the normally used adenovirus-immunoglobulin hybrid intron was replaced with the rat insulin 1 gene intron (RIPI). RIPI was obtained by polymerase chain reaction from rat genomic DNA using oligonucleotides 5′-CTCCCAAGCTTAAGTGACCAGCTACAA-3′ (SEQ ID NO:9) and 5′-GGGCAA CCTAGGTACTGGACCTTCTATC-3′ (SEQ ID NO:10). These oligos produced a 185 bp product containing the 119 bp RIPI (Cordell et al. 1979) and a HindIII site on the 5′ end and a BamHI site on the 3′ end. The PCR product was digested with HindIII and BamHI and ligated into pNoTA/T7, whereupon it was removed with XbaI blunt-ended with Klenow/HindIII and inserted into EcoRV/HindIII digested pRIP8/INS/IREs/NEO to generate pRIP8/RIPI/INS/IREs/NEO. pFFE6/RIP8/RIPI/INS/IREs/NEO was constructed by replacing the 5′ adenovirus-immunoglobulin hybrid intron/INS/IREs of pFFE6/RIP8/INS/IREs/NEO with RIPI/INS/IREs from pRIP8/RIPI/INS/IREs/NEO.

As with the transient transfection data, several modRIP promoters also appear to have increased activity compared to that obtained for RIP in stably transfected RIN cells. Both insulin mRNA and secreted insulin protein levels in stably transfected RIN cells were three to four times higher in FFE6 derivatives than levels obtained for RIP alone (FIG. 30A, FIG. 30B, FIG. 30C). In fact, the activity of FFE6 derivatives approached the level of activity exhibited by CMVP in stably transfected RIN cells.

FFE6 promoters also proved to be cell-specific. FFE6 promoters were fused with the neomycin gene to generate FFE6/RIP8/NEO. This plasmid was stably transfected into RIN cells, 293 cells, and pituitary AtT-20 cells. When challenged with G418, drug-resistant colonies were only present in RIN cells. As a control, CMV/NEO was also stably transfected into RIN cells. 293 cells, and pituitary AtT-20 cells. After selection in G418, a large number of drug-resistant colonies were present in all three lines.
Therefore, RIP derivatives like FFE6/RIP8/RIPi possess two important characteristics necessary for optimal expression of linked transforming genes in human β-cells: 1) they will direct expression of the transforming gene to β-cells and remain silent in other cell types associated with the islet preparation; and 2) they will deliver high levels of the transforming gene similar to those obtained from the very strong, non-cell-specific CMVp.

EXAMPLE 26

Induction of β-Cell Growth

1. Background

Although diabetes can be alleviated by insulin injections or sulfonylureas, the fine tuning of blood glucose sensing is lost which, more often than not, leads to unfortunate diabetic complications. Islet transplantation is a frequently mentioned alternative treatment for diabetes that retains glucose sensing, but this remains problematic, mostly because the quantity, quality and consistent supply of human isolated pancreatic islets suitable for transplantation is severely limited. One possible means of circumventing this problem is to somehow induce human β-cells to grow, either to increase the number of human islet β-cells or to produce a human β-cell line suitable for transplantation. However, little progress has been made to find a means that urges islet β-cells into a growth phase to such an extent where large quantities of β-cells can be produced as a potential alleviation of diabetes, and no human β-cell lines that retain essential traits required from in vivo insulin delivery have been created or isolated.

Pancreatic islet β-cell growth can occur from two separate pathways (Swenne, 1992). New islets can differentiate from budding of pancreatic ductule epithelium (neogenesis), or from the replication of existing islet β-cells. Neogenesis of islets is thought to primarily occur during fetal and perinatal stages of development, but has also been observed in the regenerating adult pancreas (Bonner-Weir, 1992). Replication of existing pancreatic β-cells has been seen in the late fetal stages, but is thought to be the principal means of increasing β-cell mass in the adult (Swenne, 1992). In a population of normal islet β-cells the number that are under going cell-division has been measured to be between 0.5-2%.
Several factors have been shown to increase the number of replicating \( \beta \)-cells, however these effects have only been rather slight. Glucose and other nutrients metabolized by the \( \beta \)-cell can increase the number of replicating adult \( \beta \)-cells 2-fold (Hellerström et al., 1988). Several peptide growth factors have shown stimulation of \( \beta \)-cell replication (Bonner-Weir, 1992). Growth hormone (GH) increases the number of \( \beta \)-cells replicating in islets to around 6% (Nielsen et al., 1989). The expression of the GH-receptor has been identified on \( \beta \)-cells (Hellerström et al., 1991). The GH related peptides, prolactin and placental lactogen have similar stimulatory effects on \( \beta \)-cell replication reflecting lactogenic as well as GH-receptors on the \( \beta \)-cell surface (Moldrup and Nielsen, 1990).

It has been suggested that GH mediates its growth effect on \( \beta \)-cells by stimulating the production of IGF-I in islets which in turn mediates a paracrine or autocrine effect to stimulate \( \beta \)-cell replication (Nielsen, 1982). While this may in part be so, (indeed IGF-I alone has been shown to stimulate fetal \( \beta \)-cell replication 2-fold (Brelje and Sorenson, 1991)), it also is clear that GH can exert a stimulation of adult \( \beta \)-cell replication independently of IGF-I (Swenne et al., 1987). Gastrin and cholecystokinin can instigate a small increase in \( \beta \)-cell replication (Bonner-Weir, 1992).

In contrast, EGF does not appear to affect \( \beta \)-cell replication even though significant EGF binding to \( \beta \)-cells has been observed (Nielsen, 1989), suggesting that the EGF signal transduction pathway is not functional in pancreatic \( \beta \)-cells. Similarly, PDGF does not appear to affect \( \beta \)-cell replication, but this is due to there being very few PDGF-receptors on \( \beta \)-cells. However, when the PDGF \( \beta \) receptor is transfected into \( \beta \)-cells only a 50% increase in DNA synthesis was observed upon stimulation with PDGF \( \beta \)-chain (Welsh et al., 1990), suggesting that a post-receptor signal transduction mechanism for \( \beta \)-cell replication is only partly present.

Very little work has been done on establishing key elements in mitogenic signal transduction pathways in pancreatic \( \beta \)-cells. However, insulin promoter driven SV40 T-antigen overexpression in transgenic mice has significantly induced \( \beta \)-cell growth and dedifferentiation resulting in the generation of insulinoma cell lines (Efrat et al., 1988; Miyazaki et al., 1990). In other cell types, the T-antigen mitogenic signaling pathway is thought to be mediated by
inducing Shc tyrosine phosphorylation, recruitment of Grb2 and Ras activation via induction of SOS (the Ras guanine exchange factor; Dikworth et al., 1994). This suggests that in pancreatic β-cells a mitogenic signal transduction pathway mediated via Ras activation can be induced. However, in normal islet β-cells proto-oncogene expression is undetectable or extremely low, but in islets transfected with v-src, or a combination of c-myc and c-Ha-ras, only a modest 50% increased cell replication rate was observed (Welsh et al., 1987). Thus, these studies imply that it is important to appropriately activate a mitogenic signal transduction pathway in β-cells as well as to overexpress certain key elements within that pathway.

Several growth factors have been shown to modestly induce β-cell mitogenesis, but as of yet no potent stimulator(s) of β-cell growth has been identified. Furthermore, the necessary stimulator(s) for signal transduction pathways of growth factor stimulated β-cell mitogenesis is quite poorly defined. Indeed, a given growth factor stimulation of islet β-cells could actually be ineffective because certain elements of the signal transduction pathway are either not appropriately expressed and/or activated.

The present Example concerns the identification of mitogenic signal transduction pathways in pancreatic β-cells, which in turn indicates an appropriate growth factor and signaling pathway to exploit for inducing β-cell growth in vitro and/or establishing novel β-cell lines. The inventors have found that IGF-1 and activation of a signal transduction pathway via IRS-2 and p70S6K (Fig. 28) can induce up to a 30-fold increase in β-cell growth in insulinoma cells. Recombinant adenoviruses were generated to overexpress the IGF-1 receptor and/or IRS-2 in primary isolated islets (preferably human islets) to determine the effects of IGF-1 induced β-cell growth.

2. Methodological Approach

Recombinant Adenovirus Construction- In order to obtain high overexpression and efficient gene transfer of mitogenic signal transduction proteins in primary islets, the recombinant adenovirus system was used. Essentially, replication deficient recombinant adenoviruses were constructed as previously described (Becker et al., 1994a; Becker et al. 1994b). Initially, adenoviral constructs to markedly increase in vitro islet β-cell expression of
IRS-1 (as a putative control for IRS-2), IRS-2, IGF-1 receptor, and insulin receptor (as a putative control for IGF-1 receptor) were produced. Both human and rat forms of these genes (GenBank Accession number S62539 (Rat IRS-1) and X58375 (Human IRS-1)) were obtained for expression in both human and rat isolated islets. IRS-1 and IRS-2 cDNAs were obtained from Morris White (Joslin Diabetes Center/Harvard Medical School, Boston, MA). A series of constitutively on/off IRS-1 and -2 variants are also available.

Recombinant adenoviruses where IRS-1, IRS-2, IGF-1 receptor, or insulin receptor expression is driven by the ubiquitous CMV-promoter (using pAC-CMV) were generated, and confirmed by restriction enzyme and sequence analysis. Alternatively, recombinant adenoviruses for specific β-cell expression driven by the insulin promoter (using pAC-RIP) are generated. Recombinant adenoviruses expressing β-galactosidase and luciferase driven by the CMV-promoter are used as controls. For insulin promoter driven expression a pAC-RIP driven luciferase expressing recombinant adenovirus is used as a control. Recombinant adenovirus infection of isolated islets is followed as previously described (Becker et al., 1994). Confirmation of IRS-1, IRS-2 IGF-1 receptor and insulin receptor overexpression in islets by Northern- and immunoblotting is performed.

Recombination of the pAC and pJM17 vectors to generate E1A deficient recombinant adenovirus can only accommodate an ~3.8 kb insert into pAC shuttle vector. However, the IRS-1, IRS-2, IGF-1 receptor, and insulin receptor cDNA inserts are all >3.8 kb. Therefore, the pBHGI1 (E3 deficient vector) instead of pJM17 is used to generate E1A and E3 deficient recombinant adenovirus. The pBHGI1 vector enables inserts of up to 9 kb into pAC to be used which is suitable for IRS-1, IRS-2, IGF-1 receptor, and insulin receptor cDNAs. The pBHGI1 vector was obtained from Larry Moss (New England Medical Center, Boston, MA).

Cell Preparations– Isolated rat islets are used as a primary model, which are isolated as previously described (Alarcón et al., 1993). The studies are then repeated in human islets. Further characterization of mitogenic signal transduction pathways in the RITz and the INS-1 cell lines is conducted, and these cells are used as positive controls for investigation of IRS-2/IGF-1 receptor overexpression in islets. RITz-cells are isolated from the well granulated line
of NEDH-rat transplantable insulinoma tissue by cellular sieving and Percoll™ centrifugation gradient purification. They are maintained in culture under identical conditions for INS-1 cells (Alarcón et al., 1993). In terms of insulin secretion, RITz-cells are not responsive to glucose in the physiological range, but are when elevating intracellular cAMP, phorbol esters, and/or depolarization.

The occurrence of increased IRS-2 expression in human insulinoma tissue when compared with human islets also is confirmed. Human insulinoma tissue (for example, obtained from the Mayo Clinic) is used for this purpose.

*Measurement of Cell Growth*- Several parameters are used for measurement of cell growth. First, the cell number is counted in a standard volume using a hemocytometer. ³H-thymidine incorporation into cellular DNA is used as a predictor of β-cell growth (Myers and White, 1996). Following addition of ³H-thymidine (1μCi/ml) to cells, incubations were performed under various conditions for 2–4 hrs. at 37° C. Cells were then washed three times in ice-cold HBBS, lysed in 2 ml of 1mg/ml SDS solution on ice, and transferred to 12-ml tubes. Following addition of 2.5 ml ice cold 20% (wt/vol) trichloroacetic acid, the cell extract was poured over a Whatman glass-fiber filter in a millipore filtration apparatus. The filter was washed twice with ice-cold 10% (wt/vol) trichloroacetic acid, air dried, and counted by liquid scintillation counting. The Individual replicating β-cells in islets or β-cell lines are identified and counted using a BrdU-staining kit (Amersham Int.). This technique has the advantage of readily distinguishing between islet β-cells and non β-cells by double staining with a second antibody against insulin. An increase in a population of β-cells could result in part from an inhibition of β-cells entering apoptosis. Thus the number of apoptotic IRS-2/IGF-1 receptor overexpressing cells also is measured by the TUNEL (terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling) method (kit obtained from Upstate Biotechnology Inc.).

*Measurement of β-cell Differentiation*- Ideally, when inducing primary islet β-cells to grow, the maintenance of as much of the β-cell's differentiation state as possible is desired. However, when either the growth rate of β-cells is increased or β-cells are transformed (e.g., by X-ray exposure (RIN-cell lines) or β-cell specific T-antigen expression (BTC3- and MIN6 cells)
there appears to be some degree of loss of differentiation (e.g., glucose-regulated insulin release and biosynthesis). Thus, the differentiation state of primary rat and/or human islets induced to grow by in IRS-2/IGF-1 receptor overexpression is determined.

The (de)differentiation state is assessed using three parameters: 1) **Glucose-regulated proinsulin biosynthesis translation** - To date all the available transformed β-cell lines (except the relatively well differentiated low passage MIN6 cells) do not possess a phenotype of specific regulated proinsulin biosynthesis by a physiologically relevant range of glucose concentrations. Maintenance of correct glucose stimulated proinsulin biosynthesis in IRS-2/IGF-1 receptor overexpressing islet β-cells (Alarcón et al., 1993) is an indication of maintaining a differentiation state. 2) **Regulated (pro)insulin release** - Dedifferentiated transformed β-cell lines have a tendency to constitutively secrete an increased proportion of proinsulin, and also lose their response to relevant secretagogues, especially glucose in the 2-20 mM range. Pulse-chase radiolabeling protocols (Alarcón et al., 1995) are used to assess the proinsulin:insulin ratio released from IRS-2/IGF-1 receptor overexpressing islet β-cells in response to glucose and a stimulatory cocktail containing multiple secretagogues and potentiatators of glucose-stimulated insulin secretion and thus assess the differentiation state. 3) **p70S6K phosphorylation state** - The extent of p70S6K phosphorylation in β-cell lines tends to correlate with dedifferentiation state and growth rate of the cells. A rank order of serum-starved cultured β-cells is seen from differentiation (slow growing) to dedifferentiation state (rapid growth rate) of primary islets > MIN6 > INS-1 > RITz > other RIN lines = βTC3 cells. The phosphorylation of p70S6K occurs on multiple sites on the molecule, with 5 phosphorylated forms observed by immunoblotting (due to electrophoretic retardation of the p70S6K phospho-forms on SDS-PAGE); the upper 3-5 multiple phosphorylated p70S6K forms are activated. In islets only the non-phosphorylated form of p70S6K is observed, but in βTC3 cells only the fully phosphorylated active p70S6K is observed. Other β-cell lines rank in between these extremes. Thus, immunoblot measurement of p70S6K phosphorylation state is a rapid and convenient indication of β-cell differentiation state.

*Characterization of β-cell Mitogenic Signal Transduction Pathways* - Induction of β-cell growth/transformation requires not only overexpression of a particular element in a
mitogenic signal transduction pathway (i.e., IRS-2), but also activation of that pathway by an appropriate growth factor (i.e., IGF-1). Therefore, in IRS-2/IGF-1 receptor overexpressing islet cells it is important to assess activation of the IGF-1 signal transduction pathway(s). This is performed using established methods (Myers and White, 1996). Changes in protein-protein interactions is measured (e.g., IGF-1 instigated IRS-2-PI3 kinase association by immunoprecipitation with p85 PI3 kinase antibody followed by immunoblotting with either anti-phosphotyrosine and/or IRS-2 antibodies); the phosphorylation state of a particular protein is determined (e.g., using specific antibodies that recognize only phosphorylated MAP kinase, or immunoblotting for gel retardation analysis of p70Sk or immunoprecipitation followed by anti-phosphotyrosine immunoblotting); and induction of enzyme activity is measured (e.g., MAP kinase or PI3 kinase assays). Necessary reagents or assay kits are purchased from UBI.

3. Results

To screen for factors that might be important in mitogenic signal transduction pathways in pancreatic β-cells, the expression of certain genes in a model of rapidly growing β-cells (i.e., NEDH-rat transplantable insulinoma cells (Chick et al., 1977)) versus a model of slow growing β-cells (i.e., NEDH-rat normal isolated islet cells) was compared by Northern blot hybridization. Preproinsulin mRNA levels drop by 75% in insulinoma cells compared to islets. Levels of mRNA for c-jun, c-fos, and IRS-1 did not change when comparing islets to insulinoma cells. In contrast to IRS-1, mRNA levels for IRS-2 were increased >50-fold in insulinoma cells compared to isolated islets. These very high levels of IRS-2 mRNA were also found in RIN 1046-38, RIN-m5F, INS-1, βTC3, HIT and MIN6 cell lines, but not in βTC-1, AtT20, PC-12, GH-3, 293, Cos, CHO or 3T3-L1 cell-lines where IRS-2 mRNA levels were comparable to those in isolated rat islets.

The elevated IRS-2 levels appear to be peculiar to insulinoma cell lines. The only other gene product found so far to be overexpressed to such an extent in insulinoma cells is a Ha-Ras containing VL30 transposon element (i.e., an endogenous retroviral like transposon that contains the Ha-Ras sequence within it). However, the overexpressed VL30 element mRNA is not reflected in Ha-Ras expression at the protein level which is unchanged compared to normal rat islets. Thus, this particular VL30 is acting like a typical transposon that is quite common to
tumor cells. The elevated levels of IRS-2 mRNA in insulinoma cells were also reflected at the protein level by immunoblot analysis. Furthermore, the levels of other potential mitogenic signal transduction proteins in the β-cell, namely IRS-1, c-Ha-Ras, PI3-kinase, p70^S6K, Shc, Grb-2, MAP-kinase (erk-1 and -2 isoforms) and CREB were not changed between islets and insulinoma cells.

A polyclonal cell line from the NEDH-rat transplantable insulinoma tissue termed RITz-cells has been obtained. When starved of serum for 48h RITz-cells continue to grow, albeit at a slower rate, so that the rate of $^3$H-thymidine incorporation drops 4-fold compared to fed cells. However, upon refeeding RITz-cells with 10% (v/v) serum the $^3$H-thymidine incorporation rate increases by 20-fold after a further 48h incubation, in line with a parallel increase in RITz-cell proliferation. Interestingly, the expression levels of the aforementioned signaling molecules (including the IRS-2 overexpression) did not significantly change in these ± serum studies. In addition, the differentiation state (as judged by secreted proinsulin:insulin ratio and regulated insulin secretory response to a stimulatory cocktail (20 mM glucose, 10 μM forskolin, 1 mM IBMX, 30 mM KCl, 50 μM PMA) did not alter in the same ± serum studies.

The question as to which growth factor(s) in the serum is responsible for this marked stimulation of β-cell growth was also addressed. IGF-1 at a concentration of $10^{-9}$ M was found to give a maximum stimulation (>30-fold; ED$_{50}$~$10^{-10}$M IGF-1) of RITz-cell growth (as analyzed by $^3$H-thymidine incorporation) after a period of 48h serum deprivation. There is no additive or synergism of serum (10% v/v) + IGF-1 (at $10^{-9}$ M), suggesting that it is IGF-1 in serum that is responsible of stimulating RITz-cell growth. Interestingly, unlike the majority of other cells in tissue culture, the RITz-cells (and also INS-1 and βTC3 cells) do remarkably well in the absence of serum for periods up to 5 days, although they do grow at a slower rate. It is possible that insulin secreted by such insulinoma cells is 'feeding back' via the IGF-1 receptor to maintain the cell line.

Preliminary characterization of the mitogenic signal transduction pathway stimulated by IGF-1/serum in RITz-cells was conducted. Identical observations are obtained whether IGF-1 and/or serum is used as a stimulation, but only IGF-1 stimulation will be referred to below.
Addition of IGF-1 (10^{-9} M) to 48 h serum-starved RITz-cells followed by a 1 h incubation induced autophosphorylation of the IGF-1 receptor, gave a marked stimulation of tyrosine phosphorylation of IRS-2 and an increased association of PI3 kinase (PI3K; 85 kD subunit) which in turn activates PI3-kinase activity. This then increased the phosphorylation state of p70^{S6K}, and hence its activation. Activation of p70^{S6K} has been implicated in mitogenic stimulation in other cell types (Myers and White, 1996). In contrast, no increased tyrosine phosphorylation of Shc or phosphorylation activation of MAPK activity was found.

Next the serum effects on activation of the IGF-1 signal transduction pathway in INS-1 cells was investigated. IRS-2 is activated by tyrosine phosphorylation within the 10-30 min window, resulting in increased association of PI3'K to IRS-2 in INS-1 cells, as shown by immunoprecipitation with a PI3'-kinase 85 kD subunit antibody, and subsequent antiphosphotyrosine and/or IRS2 immunoblotting analyses in 48 h serum-starved INS-1 cells that have been re-fed with serum for 10 min or 30 min. Similar results are observed with IGF-1 stimulation. In INS-1 cells serum starved for 0-72 h, refeeding with 10% (v/v) serum induces an increased activation of p70^{S6K} by increasing its phosphorylation state, as indicated by a retarded mobility on gel electrophoresis.

Similar evidence has been obtained for activation of p70^{S6K} by IGF-1. Several bands of p70^{S6K} can be observed, which correlate to differentially phosphorylated isoforms of the enzyme. In 48h serum starved cells MAPK is endogenously activated, using immunoblotting studies comparing specific antisera that only recognizes the phospho-activated form of MAPK versus antisera which recognizes total MAPK whether phosphorylated or not. However, if INS1 cells are both serum and glucose starved for 48 h, activation of MAPK within 10 min exposure to 15 mM glucose alone can be observed. Conversely, also in INS1 cells both serum and glucose starved for 48 h, adding back 10% serum for 10 min results in activation of MAPK by 10% serum. Thus, both glucose and serum (i.e. IGF-1) can activate MAPK in β-cells. Similar effects of IGF-1 are also being observed to that of adding back 10% serum. Characterization of IGF-1 activation of signal transduction pathways in the β-cell is being studied to identify other elements of this cascade that result in β-cells growth.
The data suggest that the IGF-1 signal transduction pathway in β-cells occurs preferably via a IRS-2/p70^S6K route, rather than a route involving activation of Ras. Because of the massive overexpression of IRS-2 in insulinoma cells, it appears that IGF-1 signaling is mediated via IRS-2 rather than IRS-1. As previously stated, IRS-2 expression levels did not change in response to adding back serum and/or IGF-1. This latter observation suggests that it is not only IRS-2 overexpression, but also activation of IRS-2/p70^S6K signal transduction pathway that is important for IGF-1 mediated stimulation of β-cell growth. This indicates that IRS-2 (and possibly IGF-1 receptor) overexpression in primary (human) islets initiates an IGF-1 mediated potent stimulation of β-cell mitogenesis and/or leads to a novel (human) β-cell line (FIG. 31).

A background level of glucose is required for IGF-1 to stimulate mitogenesis of INS-1 cells (as judged by [³H]-thymidine incorporation). In considering that INS-1 cells respond to glucose in terms of insulin secretion in the appropriate physiological range, for any significant IGF-1 stimulation of INS-1 cell growth glucose must be present > 3 mM glucose (FIG. 32). At 10 nM to 3 mM glucose IGF-1 only has a slight effect in stimulating INS1 cell growth. Glucose alone can instigate INS1 cell growth in a dose dependent manner ~3-fold at 6 mM glucose, ~4-fold at 9 mM glucose and ~10-fold at 18 mM glucose. This effect of glucose on INS1 cell growth is potentiated by IGF1 in a dose dependent manner >10 pM IGF-1 reaching a maximum between 10-100 nM IGF-1. The role that glucose plays in IGF-1 mitogenic signaling pathways in pancreatic β-cells is investigated by studying phosphorylation activation of the 'signal transduction proteins' and protein-protein interactions by IGF-1 ± glucose. It is known that glucose is capable of activating MAPK (via a Ca^{2+}-dependent process), therefore its role in activation of other elements in that pathway is investigated.

Like IGF-1, growth hormone (GH) can also stimulate mitogenesis in β cells. However, it does not signal mitogenesis via the IRS-1/2 pathway, but via the JAK/STAT pathway. In particular, JAK2 and STAT5 A and B are involved in the mitogenic pathway (FIG. 31). The action of rat growth hormone (rGH: from Anne Miller at Eli Lilly) on INS1 cell growth, like that of IGF-1, requires a ‘background’ of glucose (FIG. 30). The rGH has no effect on INS-1 cell growth until a threshold of 6 mM glucose that reaches a maximum (~50-fold increase
compared to "0" glucose) at 15 mM glucose. This is similar to the effect of IGF-1 on INS1 cell growth which has a threshold between 2-4 mM glucose and reaches a maximum at 15 mM glucose. Additionally, there is an additive effect of rGH and IGF-1 on INS-1 cell growth at glucose concentrations up to a maximum of 12 mM (FIG. 34).

In line with the observed effect of IGF-1, glucose and rGH stimulation of INS-1 cell growth, inhibitors of the mitogenic signal transduction pathway were shown to inhibit INS-1 cell growth. Rapamycin (a p70S6K inhibitor), wortmannin and LY294002 (PI3K inhibitors), and PD29083 (a putative MEK inhibitor) all inhibit IGF-1, rGH and glucose induced INS1 cell growth.

Adenoviral-mediated overexpression of IRS-2 in INS1 cells in the presence of IGF-1 and 15 mM glucose resulted in an approximately 200-fold increase in [3H]thymidine incorporation compared to uninfected INS1 cells plus no glucose (FIG. 35). The mitogenic signal was again specific for IRS-2 and not IRS-1 as INS1 cells infected with adenovirus-IRS-1 showed no mitogenic response over and above that for uninfected cells or cells infected with adenovirus-βGal. As before, a background level of glucose was required to stimulate mitogenesis in the adenovirus infected cells. Interestingly, the mitogenic response of INS1 cells to adenoviral overexpression of IRS-2 was greater than that for large T-antigen, a protein known for its ability to induce dedifferentiation and subsequent mitogenesis (FIG. 32).

Overexpression of an element in the signal transduction pathway downstream of IRS-2 (e.g., 'constitutively on' variant forms of p70S6K and/or Ras (RasQ81L)) in islet β-cells also is contemplated. IRS-2 is a multiple tyrosine phosphorylated molecule that appears to be located at a crossroads for many mitogenic signal transduction pathways in a cell (Myers and White, 1996). One particular growth factor induces phosphorylation of only certain IRS-2 tyrosine residues, and thus limits the number of downstream elements that associate with IRS-2 and can then be activated. Furthermore, IRS-2 activation requires exogenous growth factor stimulation (even in IRS-2 overexpressing cells), thus activation of mitogenic signal transduction pathways via IRS-2 can be turned on and off (unlike overexpression of 'constitutively on' downstream
elements). Thus continuing characterization of mitogenic signal transduction pathways in β-cells is being investigated to identify other candidates that induce β-cell growth.

EXAMPLE 27

**BetaGene Medium Maintains Growth and Function of Neuroendocrine Cells**

The biologic activity of peptides considered for biopharmaceutical applications are influenced by a number of complex modifications. These post-translational modifications include correct proteolytic processing of precursor molecules, amidation, glycosylation, disulfide formation, folding, and oligomerization. Production in mammalian cell systems is necessary for many therapeutically relevant peptides to ensure bioactivity and minimize immunogenicity. The latter issue of immunogenicity may even require the use of human cell systems. Neuroendocrine cells are cells that are specialized in the biosynthesis and export (secretion) of biologically relevant peptides. A distinguishing characteristic of neuroendocrine cells is the dominance of a regulated secretory pathway. This pathway involves sorting to and storage of peptides in dense-core or secretory vesicles, in addition to both relatively high level biosynthesis and post-translational modifications of peptides. Neuroendocrine cells are being developed as a cellular therapy for *in vivo* delivery of bioactive peptides. Such an application requires large-scale production of the implantable cells.

A number of enzymes that are essential for the post-translational modifications have been characterized, with many abundantly expressed in neuroendocrine cells. Whether manufacturing processes utilizing neuroendocrine cells involve production of purified peptides or cells for implantation, the process must sustain the activity of these enzymes so that bioactive peptides will be produced. The present invention is directed to optimized culture media for neuroendocrine cells, for the purpose of not only growth, but also function. Specifically, secretory function, and the functional activity of enzymes requisite for post-translational processing. This has involved the use of primary human neuroendocrine cells, and neuroendocrine cell lines, (some specifically engineered to express therapeutically relevant peptides), to empirically determine components critical to secretion and processing.
Assay of Cell Growth- Neutral Red Uptake Assay

A neutral red uptake assay was used for quantification of viable cell mass to allow rapid determinations of cell growth, and for calculation of cell doubling times. Neutral red diffuses across cell membranes, while protonated neutral red does not. Accumulation of neutral red is dependent on an acidic compartment (maintained by H+/ATPase) in metabolically active cells. Accumulation is time and concentration dependent, and with conditions appropriate to cells of interest, uptake is linearly related to viable cell number. The assay is initiated by adding neutral red (from 1 mg/ml stock in acetic acid) to cells to provide a final concentration of 25-50 µg/ml (a minimum of 2 ml medium/cm² culture surface in each well is required). The cells are then incubated with neutral red for 0.5-1 h at 37°C. The medium with neutral red is then aspirated, the cells washed once with medium and the neutral red is extracted from the cells. Neutral red is extracted with a solution containing 50% ethanol and 0.1 M NaH₂PO₄ (pH 5.1-5.5). The soluble neutral red is quantified by determining absorbance at 540 nm in a plate reading spectrophotometer, with a standard curve of neutral red (1-40 µg/ml) dissolved in the same extraction solution.

Assay of Peptides

Insulin in the medium was quantified with a commercial radioimmunoassay (DPC, Los Angeles CA), that is referenced to USP human insulin in the inventors' laboratory (lot G; US Pharmacopeia, Rockville MD). The reference USP human insulin that is included in each assay, is validated by HPLC in the inventors' laboratory (see Example 22 for HPLC methods).

Human growth hormone was determined with a human growth hormone ELISA (Boehringer Mannheim, Indianapolis, IN). The growth hormone (GH) standard was validated by western blotting and HPLC referenced to an independent source of human growth hormone (Bachem, Torrance CA).

Cell Culture

**Cells:** Four different neuroendocrine cell lines were used to evaluate the impact of the current medium on growth. Two cell lines are human. BG785/5 is an engineered version of a neuroendocrine line derived from a lung tumor (BGH04; ATCC CRL-5803); these cells have
been engineered to express human growth hormone. The second human line used, BGH16, is a neuroendocrine gastric carcinoma (ATCC CRL-5974). The other 2 lines are rodent cells. derived from a rat insulinoma. one, BG18/3E1, was engineered to express human insulin (Diabetes 46:958-967. 1997). the other, BG191/26, was engineered to express preproglucagon (transfected with BetaGene plasmid BU503; WO97/26334 and WO97/26321).

5 BGH16 cells were passaged with a 1:3 split ratio into 12 well plates and fed 2-3 times/week with 4 ml per well of BetaGene Medium supplemented with 2% or 5% serum or serum-free.

10 BG785/5 cells were plated (1:30 ) into 24 well plates, ≈1 x 10⁴ /well. and fed 2-3 times/week with 2-3 ml per well of either RPMI or BetaGene Medium supplemented with FBS or serum-free. Media samples were collected for human GH assay, and cell growth determined at 2-3 day intervals for ≈ 2 weeks. RPMI is the medium recommended for this cell type.

15 BG18/3E1 cells were plated (1:8 to 1:16) into 24 well plates, ≈1 x 10⁵ /well. and fed 2-3 times/week with 2-3 ml per well of BetaGene Medium supplemented with 0.5-5% FBS or serum-free. Media samples were collected for human insulin assay, and cell growth determined at 2-3 day intervals for approx. 2 weeks.

20 Medium Supplements- Serum and Serum-free. Serum-supplemented media contained fetal bovine serum (JRH Biosciences, Lenexa KS), supplemented to 2%, unless otherwise indicated. The lot of serum used was selected by screening ≥5 lots of serum by assaying attachment, clonal growth, and maintenance of secretory function (of primary pancreatic beta cells and beta cell lines) at serum supplements of 0.5% to 5%.

25 Serum-free supplement provided 0.1% BSA, 10 μg/ml of transferrin, and 50 μM each of ethanolamine and phosphoethanolamine.

20 Medium. The performance of cells in BetaGene medium (JRH Biosciences) was compared to RPMI, a medium recommended for culture of human cells (Methods in
Enzymology 58, pages 213 and 91: 1979). RPMI is also the medium recommended for the BGH04 cell line (the parental cell line engineered to yield the BG785/5 cell line). BGH16 cells were derived and cultured in DMEM:F12 (50:50) mixture supplemented with a complex mixture of hormones, growth factors. selenium, BSA, transferrin, ethanolamine and phosphoethanolamine (10 µM each). For the present studies the BGH16 cells were switched to BetaGene Medium with either FBS or serum-free supplements and growth was evaluated in this medium.

**Results: Growth & Function**

The BGH16 cell line is a slow-growing suspension culture with a 5-6 day doubling time. The BG785/5 cell line is a rapidly growing monolayer culture that readily reaches confluence with a 2 day doubling time. The BG18/3E1 cell line is a slower-growing monolayer culture that does not readily achieve confluence. Growth in BetaGene Medium for all these cell lines was maintained when serum-free supplements (SF) were used in the place of FBS (Table 11).

**TABLE 11**

**CELL GROWTH IN BETAGENE MEDIUM**

<table>
<thead>
<tr>
<th>Cells</th>
<th>Supplement</th>
<th>Doubling, days</th>
</tr>
</thead>
<tbody>
<tr>
<td>H16</td>
<td>SF</td>
<td>5.91±0.41</td>
</tr>
<tr>
<td></td>
<td>2% FBS</td>
<td>5.03±0.14</td>
</tr>
<tr>
<td></td>
<td>5% FBS</td>
<td>5.31±0.23</td>
</tr>
<tr>
<td>BG785/5</td>
<td>SF</td>
<td>1.97±0.01</td>
</tr>
<tr>
<td></td>
<td>2% FBS</td>
<td>2.03±0.01</td>
</tr>
<tr>
<td>BG18/3E1</td>
<td>SF</td>
<td>2.41±0.01</td>
</tr>
<tr>
<td></td>
<td>0.5% FBS</td>
<td>3.85±0.14</td>
</tr>
<tr>
<td></td>
<td>1% FBS</td>
<td>2.92±0.03</td>
</tr>
<tr>
<td></td>
<td>2% FBS</td>
<td>2.83±0.02</td>
</tr>
<tr>
<td></td>
<td>5% FBS</td>
<td>2.77±0.01</td>
</tr>
</tbody>
</table>
The BG785/5 cell line was derived from BGH04 cells which were derived and routinely cultured in RPMI w/FBS. The growth rate of BG785/5 cells in BetaGene and RPMI media, with FBS or SF, is shown in FIG. 36. Although cells grown in RPMI w/FBS exhibited a longer lag phase, the growth of cells in BetaGene medium and RPMI w/FBS was similar, all with doubling times of 2 days. However, cells in RPMI w/SF essentially failed to grow, with an apparent doubling time of 26±1 days. Three wells of RPMI w/SF were switched to BetaGene Medium w/SF for the last 4 days of the experiment, resulting in a restart of growth and a doubling time of 3.2±0.2 days.

In contrast with growth, the secretory function of cells in RPMI medium did not keep pace with cells grown in BetaGene Medium (FIG. 37). The human growth hormone (hGH) output of cells grown in BetaGene Medium with FBS was approx. 5 times greater than growth hormone output from cells in RPMI w/FBS. Similarly, the hGH output of BetaGene Medium w/SF was more than 5 times that of RPMI w/SF. While BetaGene Medium supplemented with SF sustained hGH output equal to that of RPMI w/FBS, it was not sufficient to support the same secretory function as BetaGene Medium with FBS.

The growth of BG18/3E1 cells was slowed only with low serum — at 0.5%, but not by SF-supplementation (Table 11). The insulin secretory function of these cells was maintained with all supplements until the cells reached the plateau phase of growth. Cells at plateau phase, whether supplemented with 0.5% FBS or SF, do not maintain normal secretory output (FIG. 38). This was confirmed in separate studies with SF and 0.5% FBS cultures. The secretory impairment at plateau phase may be due to decreased biosynthesis or processing of insulin rather than an impairment of secretion. The ability to respond to a secretagogue cocktail is shown in FIG. 39 for SF- and 2% FBS- supplemented cultures in BetaGene Medium (see example 30 for composition of trace mineral and amino acid supplements). This demonstrates that the capability of the regulated secretory pathway has been maintained, only the absolute output has been affected in both unstimulated and stimulated states, while the fold response is maintained. RPMI medium is one of the most commonly used media for culture of rat (and hamster) beta-cell lines. The present results with BetaGene medium stand in contrast with the
literature where insulinoma cells cultured in RPMI medium extinguish insulin production at plateau phase of cell growth (Karlsen, et al. 1991). Insulin output with BetaGene medium supplemented with serum-free supplements is reduced at plateau phase of growth, although it is not extinguished.

The capability of BetaGene medium to sustain processing and secretion of a peptide that yields proteolytically cleaved and amidated products was evaluated by measuring GLP-1 (amidated and non-amidated) production. Cells BG191/26, were plated in T25 flasks with BetaGene Medium and then the medium was switched to RPMI. RPMI with 75 μM ascorbate, or BetaGene Medium, all with 2% FBS. Both the total GLP-1 and the amidated GLP-1 output/day of cells in BetaGene Medium was essentially double that of cells in RPMI. The addition of ascorbate (in the form of the stable ascorbate-2-phosphate) to RPMI increased the amidated GLP-1 output to that of BetaGene Medium, but did not normalize the total GLP-1 output/day (FIG. 40).

EXAMPLE 28

Effects of Ascorbate 2 – Phosphate and Copper on Post-translational Modification in Neuroendocrine Cells

Amidation of a carboxy-terminal glycine is one of the later events in post-translational processing. This modification is essential for the activity of some peptides, including about half of peptide hormones, and appears to be rate-limiting for production of some peptides (Eipper, et al., 1992; Cuttitta 1993). The bifunctional enzyme responsible for amidation is peptidylglycine a-amidating monooxygenase (PAM). The enzyme itself is proteolytically processed and is both N- and O- link glycosylated and is targeted to secretory granules in neuroendocrine cells (Yun et al., 1994). This enzyme requires copper and ascorbate to accomplish amidation; copper is a part of the functional enzyme.

Although several media include ascorbate in the formulation, the value of ascorbate has been most typically considered in the context of extracellular matrix and collagen synthesis. Even for the purpose of collagen formation the addition of ascorbate has been considered impractical in light of the instability of ascorbate (Ham 1979; Mather, 1998). There are several
analogues that are stabilized forms of ascorbate. One of these compounds is ascorbate-2-phosphate (A-2-P; Nomura et al., 1969), this compound is used in some pet foods, and as a supplement in some types of cell cultures. A-2-P has been shown to stimulate collagen synthesis in fibroblast cultures. It has been used for culture of rat hepatocytes, although rat (unlike human) hepatocytes synthesize ascorbate. Recently, A-2-P was shown to acutely improve glucose-induced insulin secretion from pancreatic islets of scorbutic guinea pigs (Wells et al. 1995). The latter authors indicated that normal islets have intracellular levels of 5 μM, with scorbutic levels ≤10% of normal. In contrast, ascorbic acid has been reported to be acutely inhibitory in electrophysiologic and secretory studies with rodent beta-cells (Bergsten et al., 1994); these authors indicate that intracellular ascorbate concentrations in normal mouse islets are 4 mM. Ascorbic acid has been shown to be diabetogenic in vivo, toxic to mouse islets in vitro, and to cultured fibroblasts, hepatocytes and lung carcinoma cells (discussed in Anderson & Granqvist, 1995). It is not clear what concentrations of ascorbate would be required by islets, whether ascorbate would be toxic with chronic culture, or whether there may be species differences in the effect of ascorbate in beta-cells.

Ascorbic acid or a substitute reducing agent is utilized on an essentially equimolar basis for each mole of amidated product. The provision of ascorbate would then be expected to be important for maintaining peptide amidation with neuroendocrine cells cultured, particularly, in the absence of serum, or grown at high-density, production scale. One study of neuroendocrine cells engineered to express an amidated peptide (pancreatic polypeptide) was unable to increase amidation activity by supplementing with 50 mM ascorbic acid (Takeuchi et al., 1991); maximal production achieved was approx. 6 pmol/million cells-day. The present studies have used cultured primary human islets, rat beta-cell lines, and human neuroendocrine cells to determine 1) the chronic cytotoxic effects of ascorbate, and A-2-P; 2) whether A-2-P will support PAM-amidation activity; 3) whether A-2-P has any effect on the secretion of non-amidated peptides, such as insulin.

**Instability of Ascorbate and Stability of A-2-P in media at 37°C.**

The first consideration was to determine whether A-2-P was a more stable form of vitamin C in the cell culture environment. To that end a simple assay was devised that takes
advantage of the dye reducing properties of ascorbic acid. The assay can be coupled with alkaline phosphatase to dephosphorylate A-2-P so that it can be measured with the same assay system used to measure ascorbate. The assay uses alkaline Tris-Mg buffer (pH 7.8-8.0: 2 mM MgCl₂) and nitroblue tetrazolium for ascorbate alone, or for A-2-P the solution contains in addition 10 U/ml of calf intestinal phophatase (C-AlkP). Ascorbate reduces the pale yellow NBT resulting in an intense purple color development. The color is developed whether the source is sodium ascorbate or ascorbic acid produced by the dephosphorylation of A-2-P by C-AlkP.

**Assay Solution**– Nitroblue tetrazolium was dissolved in 70% dimethylformamide to provide a 61 mM dye stock solution. Na ascorbate stock solution, and A-2-P stock solution was made at 100 mM in RO/DI water or culture medium; ascorbic acid stock are stored frozen less than -120°C. These stock solutions are used to construct a standard curve with a range of 1 to 18 mM in culture medium. The assay reaction mixture consists of 0.1 M Tris buffer, 1 mM magnesium, 0.4 μM nitroblue tetrazolium, with or without 10 U/ml of C-AlkP. The standards and samples, 10 μl, are pipetted into individual wells of a 96 well plate. The reaction is started by adding 100 μl of reaction mixture to each well. The reaction is quantified as a rate assay, with kinetic reading of OD at 595 nM at 20s intervals for 15 minutes. The stability of ascorbate was determined by spiking medium samples with ascorbic acid or A-2-P, then incubating the samples in the dark at 4°C, room temperature, and 37°C. The change in concentration with ascorbate and A-2-P, after 1 and 2 days at the various temperatures is presented in Table 12. The results indicate that ascorbate in media is degraded quickly, with marked breakdown occurring at 4°C. In contrast A-2-P was very stable with little loss of activity (98% recovery) after 4 days at 37°C. Refrigerated media exhibited the same A-2-P concentrations as freshly manufactured medium for times of >6 months.
TABLE 13
THE CHANGE IN CONCENTRATION WITH ASCORBATE AND A-2-P, AS A FUNCTION OF TIME (1 AND 2 DAYS) AND TEMPERATURE.

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Ascorbic</th>
<th>Acid, mM</th>
<th>Ascorbate</th>
<th>Phosphate, mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>°C</td>
<td>1 day</td>
<td>2 day</td>
<td>1 day</td>
<td>2 day</td>
</tr>
<tr>
<td>-15</td>
<td>8.9 (99%)</td>
<td>6.8 (75%)</td>
<td>9.2 (102%)</td>
<td>9.6 (107%)</td>
</tr>
<tr>
<td>4</td>
<td>7.0 (78%)</td>
<td>5.4 (59%)</td>
<td>9.2 (102%)</td>
<td>9.4 (105%)</td>
</tr>
<tr>
<td>37</td>
<td>3.4 (38%)</td>
<td>2.4 (27%)</td>
<td>9.6 (107%)</td>
<td>11.4 (126%)</td>
</tr>
</tbody>
</table>

Toxic Concentrations of Ascorbate with Human Neuroendocrine Cells.

BGH03, a lung neuroendocrine cell line was engineered to express human insulin by transfection with BetaGene plasmid AA603. The resultant cell line BG498/45 biosynthesizes, processes, and secretes human insulin. A suspension culture of BG498/45 cells (PD33) were plated in varying concentrations of ascorbate or A-2-P. Samples were collected for insulin assay and medium changed after 2 and 5 days of culture. The insulin RIA is described herein (see example 33).

In the initial 2 days of culture ascorbate altered insulin output by reducing insulin ≈20% only at the highest concentration. In the final 3 days cells were dying and insulin output was reduced to ≈20% of controls by the highest concentrations of ascorbate, while ≈400 μM concentrations of both ascorbate and A-2-P enhanced insulin secretion (FIG. 41). The highest concentration of A-2-P did not inhibit insulin output.

Optimal A-2-P Concentration for PAM-activity of a Rodent β-cell Line.

This present assay used the BG191/26 cells line engineered to overexpress the preproglucagon gene. Production of amidated and nonamidated GLP1 was determined by immunoassay of secreted cell products from cells cultured 1 day in RPMI medium (with 2% FBS) supplemented with varying concentrations of A-2-P. The dose-response shows half-max. and maximal amidation activity with ≈1 and 10-100 μM of A-2-P (FIG. 42). The amount of amidated GLP-1 plateaued from 25-1000 μM. Concentrations of 10 mM consistently (4
separate experiments) resulted in slight decreases in amidated GLP-1, with a similar tendency to reduce non-amidated GLP-1 output. Supplementation with A-2-P results in a decrease in non-amidated GLP-1, such that amidated/ non-amidated exceeds 100%. Maximal output of amidated GLP-1 with this cell line is ≈12 pmol/million cells-day, representing 5 fold increase over 0 μM A-2-P. This result demonstrates that supplementation with A-2-P can effect increased amidation activity with cultured cells, and that maximal amidation activity is reached at lower concentrations (with a related beta-cell line), than the concentrations that increased insulin output (≈400 μM; FIG. 41).

Optimal Copper Concentration for PAM Activity.

BG191/26 cell monolayers in T25 flasks were changed to RPMI medium ± copper, or BG Medium ± additional copper (the latter medium contains 5 nM copper). Medium samples were collected after 24 h and the GLP-1 species were separated and quantified by HPLC. The results in FIG. 43 show that supplementing RPMI (which has no copper in its formulation) increases the output of amidated GLP-1. Further supplementation of BG medium with copper to 250 and 500 nM does not increase amidated GLP-1, whereas 1 μM copper tends to decrease amidated GLP-1. These results indicate that 5 nM copper is adequate for PAM activity in cultured neuroendocrine cells. It should be noted that cells in BetaGene Medium have higher output of non-amidated GLP-1, and thus a lower ratio of amidated product than cells with RPMI. Both forms of GLP-1 are active, so this final processing step is less critical for GLP-1 production.

A human cell line BGH01 was found to naturally express GLP-1. This cell line was used to test the effect of 5 nM copper on amidation. In medium without copper these cells contained 3 ng of GLP-1, with amidated GLP-1 constituting slightly more than half. In the presence of copper the GLP-1 content was increased 4 fold, with amidated GLP-1 constituting more than 80 % of the total. This indicates that with conventional culture conditions the same concentration of copper can be used for both rodent and human cells that make an amidated product.
Lack of Cytotoxic Effect of A-2-P on Primary Human Islets

Human islets encapsulated in alginate beads, (for method details see Example 22), were set up in 24 well plates with \( \approx 50 \) islet equivalents/well and cultured in BetaGene Medium with or without added A-2-P and copper. Secretory function and glucose-sensing was determined by incubating the islets with different concentrations of glucose for 90 minutes (from 2.2 to 22 mM). This glucose dose-response test was performed immediately before adding ascorbate to the cultures and at 2 week intervals. In the first 2 weeks 500 \( \mu M \) A-2-P, and 1 \( \mu M \) copper was supplemented. In the second 2 weeks ascorbate was increased to 2 mM, copper was kept at 1 \( \mu M \).

FIG. 44 shows that A-2-P did not impair function as indicated by sensing of glucose, (EC50 for control and A-2-P islets was the same). Additionally, the maintenance of maximal insulin secretion indicates that there is minimal toxicity of A-2-P for these culture times.

The above findings demonstrate the stability of A-2-P in media, the effectiveness of A-2-P in supporting amidation-activity in cell culture, the beneficial effect on secretory function, and the concomitant lack of cytotoxicity with cultures of neuroendocrine cell lines and primary human islets.

EXAMPLE 29

Defining Levels of Trace Minerals, Redox, Lipid and Lipid-related Compounds

Several trace elements are important for some of the enzymes involved in proteolytic processing. Two neuroendocrine cell lines were used to determine concentrations of trace elements that alter secretion, and concentrations that are cytotoxic. Effects on insulin secretion apart from cytotoxicity are considered evidence of cytostatic action. In addition, because of the importance of redox potential in ER/golgi, cytotoxic and cytostatic concentrations of tocopherol, and lipoic acid were also determined. The utility of ethanolamines are described in Example 2, and the importance of inositol is suggested by the literature. The cytotoxic ranges of these compounds were also defined.
General Methods

Cells were plated as monolayers for short term (3-7 days) studies, for longer term studies, (≥ 2 weeks), cells were cultured encapsulated in alginate spheres (methods describe in example 6). The cell lines used were βG18/3E1 and βG498/45, a rat insulinoma and a human lung carcinoma, respectively, engineered to synthesize and secrete human insulin. Cells were cultured in BetaGene Medium supplemented with 2% FBS or serum-free and the specific test material. Samples were collected at 2-3 day intervals to determine effects on insulin output in continuous culture. Additionally, cells were acutely stimulated to secrete insulin; 1-2 h with carbachol or carbachol and a cocktail of other secretagogues (cocktail consists of 100 μM carbachol, 50 μM IBMX, and 10 mM each of leucine, arginine, and glutamine in BetaGene Medium). Cytotoxicity was quantified by neutral red uptake assay with cells after exposure to the specific test supplements. The concentration yielding 50% inhibition (IC50) was calculated from fitted lines of the data for viable cell mass or from insulin secretion.

Cytotoxic/Cytostatic Concentrations of Trace Minerals

Toxic effects of the following trace minerals were examined in log increments- copper, cobalt, molybdenum, nickel, silicate, tin, vanadate, and zinc in medium with 2% FBS. Cytotoxicity and cytostatic effects were concordant with the tested concentrations of cobalt, molybdenum, nickel, or tin. The IC50s (tested ranges) were: cobalt=64 μM (0.005 to 100 μM); nickel >25, calculated = 169 μM (0.0005 to 25 μM); and vanadate = 17 μM (5-250 μM). Neither molybdenum in concentrations of 0.001 to 30 μM, nor tin concentrations of 0.0005 to 25 μM altered viable cell mass, doubling time, or insulin output. These results indicate that these minerals can be supplemented at concentration less than 10-25 μM with minimal deleterious effects.

Silicate exhibited discordance between cytostatic (insulin output) and cytotoxic effects. Silicate concentrations of 0.5 to 1000 μM had no apparent cytotoxic effects, however, >500 μM silicate significantly decreased insulin output (p<0.005 by ANOVA; 74% of control). Silicate at 5 mM was completely toxic; cytotoxic IC50 was 3 mM. These results indicate that silicate should be used at concentrations ≤ 1 mM.
Zinc and copper inhibition of insulin was fairly proportional to the cytotoxic effects. Zinc, (3 to 300 μM), had a cytotoxic IC50 of 178 μM and a cytostatic IC50 of 159 μM. Copper (0.01 to 100 μM) had a cytotoxic IC50 of 86 μM and a cytostatic IC50 of 62 μM. Deleterious effects of copper and zinc were observed with concentrations greater than 20 and 100 μM, respectively. In serum-free cultures 30 μM zinc enhanced secretion 17% over 3 μM zinc; 100 μM did not enhance secretion. Copper had its maximal effect on amidation between 0.01 and 1 μM (see example 28). In consideration of the potential toxicity of these compounds, copper supplementation should be ≤ 1μM, and zinc between 3 and 30 μM. These concentrations should be adequate for enzymes requiring these minerals for activity.

Cytostatic and Cytotoxic Levels of Lipoic Acid and Tocopherol


In FBS-supplemented medium the cytotoxic IC50 for lipoic acid (0.5 to 1000 μM) was 420 μM, however, it was inhibitory to secretion at lower concentrations; the cytostatic IC50 was 160 μM. The cytostatic effect was seen at 50 μM in serum-free cultures. The 0.5 μM concentration of BetaGene Medium is in the physiologic range, apparently in serum-free conditions a log increase in concentration above physiologic has deleterious actions on cell function. The inhibitory effect of lipoic acid in serum-free conditions may be related to inhibition of biotin or pantothenate uptake, because fatty acid-like toxicity of lipoic acid is in the millimolar range (Sen et al. Free Radic Biol Med 22:1241-57). This indicates that biotin
and/or pantothenate levels may need to be increased for serum-free cultures, but are not rate-limited with FBS-supplemented medium.

Tocopherol (phosphate) supplemented to 1, 5, and 50 μM was compared to BG Medium with 6.8 nM tocopherol. High concentrations of tocopherol were more inhibitory to secretion than cytotoxic. The cytotoxic IC50 was 62 μM, and the cytostatic IC50 was 38 μM. This in spite of the fact that 1 and 5 μM enhanced secretory responsiveness 30%. These results indicate that tocopherol concentrations of 1 μM should be nontoxic and useful, particularly with serum-free cultures.

Cytostatic and Cytotoxic Levels of Ethanolamine, Phosphoethanolamine, and Inositol.

Ethanolamine (EA) and phosphoethanolamine (PEA) were found to be utilized rapidly in bulk-scale cultures, and these compounds promote growth and exert some protective effects in serum-free cultures (see Example 32). The current set of experiments examined the toxic limits of these two compounds. PEA (50, 500, and 1000 μM) exhibited no cytotoxicity, while ethanolamine (50, 500 and 1000 μM) reduced viable cell mass with an IC50 of 1.7 mM. Both compounds inhibited cell function with cytostatic IC50s of 1.2 and 1.7 mM for EA and PEA, respectively. Even though 500 μM PEA alone was neither cytotoxic nor cytostatic, the combination of 500 μM EA and 500 μM PEA reduced insulin secretion to the same extent as 1000 μM of EA, but without the cytotoxicity of 1000 μM EA. These results indicate that these compounds should be effectively supplemented at concentrations of 50 to less than 500 μM each of EA and PEA.

Inositol was tested at 0.6, 0.9, and 1.8 mM with no cytotoxic or cytostatic effects. Early studies indicated that inositol was essential for glucose-induced insulin secretion from rat islets under serum-free conditions (Pace and Clements, 1981. Myo-inositol and the maintenance of beta-cell function in cultured rat pancreatic islets. Diabetes 30(8):621-5). The present studies do not address whether inositol is essential for glucose-induced secretion (all of the present studies have included inositol in the base media). Nonetheless, the present results indicate that inositol can be supplemented to very high concentrations without deleterious effects.
Cytotoxicity of Manganese

In the literature, acute depletion studies of manganese (Mn) indicated that manganese was important for O- and N-linked glycosylations of proteins. This effect of manganese may be related to the manganese requirement of the transferases involved in oligosaccharide addition (Kaufman et al., 1994. Depletion of manganese within the secretory pathway inhibits O-linked glycosylaton in mammalian cells. Biochemistry 33:9813-9819). Initial studies with manganese indicated that 1 mM manganese was completely, and rapidly cytotoxic (within 24 h) when supplemented into BG medium. Cytotoxicity was almost complete (>90%) with 100 μM. With BG498/45 cells 10 μM Mn addition to serum-free medium maintained acute secretory responsiveness equal to that of FBS-supplemented cultures for one week. However, after 2 weeks of culture in serum-free conditions, Mn no longer provided equivalency with the secretory response of FBS-supplemented cultures. It is unknown whether 1-10 μm Mn is sufficient to maintain glycosylation in serum-free cultures, (other facets of this processing may be rate-limiting). The present results do indicate that 1-10 μM of Mn, the more physiologic concentrations of Mn, are not toxic and effect some benefit for serum-free cultures.

EXAMPLE 30

Maintenance of Cell Function with Bulk Cell Production

Engineered and unengineered neuroendocrine cell lines (rodent and human) have been produced in large scale (bulk produced), harvested and frozen to establish a homogeneous repository of cells. Cells undergoing this process continue to secrete complex, biologically active polypeptides into the growth media with no significant differences in the response to secretagogues as compared to before and after bulk production and after freeze-thaw. BG18/3E1 (see examples 32, 33, 29) and BG498/45 (see examples 32, 29) were studied as representative neuroendocrine cell lines for bulk production, harvest, freeze and thaw. Thawed aliquots were tested for secretory response to secretagogues. The procedures and the secretion studies are described below.

Cell lines are bulk produced in the CellCube™ system (Corning Costar). The Cellcube module provides a large surface area for the growth of substrate dependent cells. It is a sterile single-use device that has a series of parallel culture plates with thin laminar flow spaces.
between adjacent plates. The inlet and outlet ports are diagonally placed to distribute the flow of media to the parallel plates. The medium is constantly recirculated from the module through an oxygenator and back to the cube. The external oxygenator provides a bubble free replacement of oxygen to the medium and exchange of CO₂ to provide control of the medium pH. Fresh medium is added continuously at an adjustable rate and medium with secreted product and waste is harvested at the same rate, while retaining the cell population.

The culture was sustained by the initial amount of BetaGene Medium contained within the system for the first 36-60 hours after seeding. Medium circulation was started twenty-four hours after the initial seeding in order to supply the cube with nutrients. Fresh medium addition and ‘spent’ medium harvest began 48 hours post-seeding. (The amount of time between seeding and the start of the media perfusion is dependent on the density of cells in the seeding inoculum and the cell growth rate.) A typical CellCube™ run with a 21,000 cm² surface, contains approximately 1.2 liters of media within the module. The final cell density exceeds 2.5 x 10⁶ cell/cm² or 5 x 10⁷ cells/ml in the culture vessel. In accordance with the cells’ growth rate, the medium addition is adjusted such that the culture is fed approx. 1–2 ml of fresh medium per million cells per day. Media feed required to support BG18/3E1 cells at confluence is in the range of 4-16 CellCube module volumes per day. The nutrient concentration in the circulating media is measured to indicate the status of the culture.

Control of each bulk culture was maintained by monitoring pH and dissolved oxygen content, as well as glucose, lactate, and ammonia concentrations. Bursts of CO₂ were injected at 30-second intervals and the amount controlled by a pH electrode. Oxygen was controlled by adjustment of the partial pressure of oxygen in the oxygenator headspace and by adjustment of the medium recirculation rate. Glucose, lactate, and ammonia levels were measured (IBI Biolyzer) daily in order to monitor nutrient availability and waste removal. Samples were taken prior to the daily increase of medium addition.

Cells were harvested from the CellCube with a sterile, closed system. The CellCube was removed from the circulating medium flow and attached to two bottles at the inlet; one of which contains trypsin (37°C) the other contains phosphate buffered saline (PBS, 37°C). The
outlet of the CellCube module was attached to a harness for cell collection consisting of a vent bottle, two sterile cell culture bags (Baxter), and four bags containing cold horse serum-supplemented (controlled herd, irradiated, JRH Biosciences) or trypsin inhibitor-supplemented BetaGene medium. The growth medium in the CellCube module was emptied into the first sterile culture bag. The CellCube was subsequently rinsed with one volume of PBS and the rinse collected in the second sterile cell culture bag. The CellCube was then filled with warmed trypsin, incubated for an additional two minutes, and the trypsin/cell suspension emptied into the two bags containing BetaGene medium. Trypsin treatment was repeated and the suspension collected into the two remaining bags.

The bags of medium with cells were removed from the collection harness with the use of a tubing heat sealer (Sebra). A sterile tube welder (Terumo) was used to assemble the Cell Harvester tubing pathway and to attach the bags to the Cell Harvester (Baxter-Fenwall). The Cell Harvester system consists of a harvester manifold attached to the cell bags, a reservoir set (Baxter), a centrifuge belt (Baxter), a blood collection bag (Baxter), and a waste collection bag (Stedim, France). PBS was used to prime the harvester tubing prior to the addition of the cells. The cells were pumped into the reservoir set and then into the centrifuge belt where they were pelleted. The cell pellet was washed with 1 liter of cold PBS. The PBS was exchanged for cold BetaGene ChillSolution (5 mM KH$_2$PO$_4$; 25 mM KOH; 30 mM NaCl; 0.5 mM MgCl$_2$; 20 mM L(+) lactic acid; 30 mM trehalose; 5 mM glucose; 167 µM myo-inositol; 200 mM sorbitol; 1 mM pyruvate). The cells were resuspended in 250 to 300 ml BetaGene ChillSolution, transferred to a sterile Blood Collection Bag, and placed on ice. Cell density was determined (by hemocytometer) and the cells were diluted to 40–60 million per milliliter in BetaGene ChillSolution. An equal volume of BetaGene CryoSolution (5 mM KH$_2$PO$_4$; 25 mM KOH; 30 mM NaCl; 0.5 mM MgCl$_2$; 20 mM L(+) lactic acid; 30 mM trehalose; 5 mM glucose; 167 µM myo-inositol; 200 mM sorbitol; 1 mM pyruvate; 15% DMSO; 0.4M propylene glycol) is added to the cells to yield a final concentration of 20–30 million cells per milliliter. The serum-free cell suspension was then dispensed by peristaltic pump from a reservoir in 1 ml aliquots into 2 ml cryovials (Nalgene). The cell solution reservoir was kept on ice and agitated constantly. The cells were aliquoted into vials. The filled vials were assembled in a freezing rack for transfer to a controlled rate freezer (Cryomed System, Forma Scientific).
Each rack was placed in the freezing chamber which was pre-cooled to 0°C. A sample probe was used to record sample temperatures from one vial for each run. The sample temperature lagged behind the chamber temperature. The samples were cooled and frozen with the following step changes in temperature:

1) Hold at 0°C until all samples have been placed in the chamber
2) Cool at 1°C decrements until the sample probe reads -14°C
3) Hold at -14°C for 15 minutes after sample temperature stabilizes
4) Cool at 1°C increments until the sample reaches -40°C
5) Transfer to liquid phase nitrogen to provide rapid cooling to storage temperature.

The frozen vials were stored in liquid phase nitrogen (-196°C).

The cryopreservation solutions were developed to minimize deleterious metabolic effects of DMSO. This involved two approaches, first, salt solutions with low sodium and high lactate were used to suppress cell metabolism (Borrelli. et al. 1987. A method for freezing synchronous mitotic and G1 cells. Exp Cell Res 170:363-368). Second, the solutions included propylene glycol and sugars with cryoprotectant properties (sorbitol and trehalose; Honadel and Killian. 1988. Cryopreservation of murine embryos with trehalose and glycerol. Cryobiology 25:331-337) to allow the lowest DMSO concentrations to be used. In addition, it was found that pyruvate protects against the cytotoxic effects of such free radical generators as hydrogen peroxide; similar to the protective effects with renal cells (Salahudeen et al., 1991. Hydrogen peroxide- induced renal injury. J Clin Invest 88:1886-1893). The rat β-cell line, RIN-38, was used in dose-response studies of hydrogen peroxide toxicity. Cells in multiwell dishes were cultured with hydrogen peroxide, in RPMI medium overnight; cell survival was determined as described in Example 32. The concentration at which hydrogen peroxide killed 50% of the cells (IC₅₀), was calculated from a fitted line of data derived from studies with 50-200 μM hydrogen peroxide. In the absence of pyruvate the hydrogen peroxide IC₅₀ was 14 μM. Pyruvate concentrations of 100-500 μM provided a modest shift in IC₅₀ to about 40 μM, with 1 mM pyruvate shifting to an apparent IC₅₀ of 530 μM. No indication of cytotoxicity was seen with 5 mM pyruvate. These studies provided the basis for adding pyruvate to the
cryopreservation solutions (in addition to the culture medium) to provide cells some protection from free radicals during the recovery of cells from the cryopreserved state.

Frozen vials were thawed and plated (see below) to test insulin secretory responsiveness and growth. The vials were quickly transferred from the liquid nitrogen and placed in a 37°C water bath for rapid thawing (120–135 seconds with gentle shaking). Dilution of the cryoprotectants was begun as soon as only a small piece of ice remained in the cryovial, by adding 0.5 ml of cold (4°C) BetaGene ChillSolution to the cryovial. The cells were allowed to equilibrate for 5 minutes at room temperature. A second 0.5 ml of cold BetaGene ChillSolution was added, and the contents were transferred to a sterile 15 ml tube and equilibrated 5 additional minutes at room temperature. The cryovial was rinsed with 1 ml of BetaGene ChillSolution and the rinse was added to the same 15 ml tube and the cells were equilibrated the final 5 minutes. An equal volume of growth medium was then added to the thawed cells and the contents were spun at ≈700 rpm (100 x g) in a bench top centrifuge for 2 minutes. The supernatant containing cryoprotectants was removed and fresh growth media added. The cells were dispensed into the appropriate culture vessels for growth.

The cells were plated in 24 well plates at 5-7x10⁴ cells/well (equivalent to a 1:16 split ratio) to determine doubling times or 1 x10⁵ cells/well to assess secretory performance. Doubling times were determined by assaying cell mass (Neutral Red dye uptake method) after 7-10 days of culture. Response to a mixed cocktail of secretagogues in medium (see Example 29) was assayed after 2-10 days of culture. The secretory response of pre-bulk, post bulk/harvest, and serum-free frozen and thawed cells is shown in FIG. 45. Repetitive bulk productions and cryopreservations (2 rounds) have not altered the doubling time; 46.3±0.7 h in plate cultures after 1 bulk run, and 46.8±0.7 h after a sequential bulk of these cells. The results demonstrate that each of the processes described; bulk production, harvest, freeze and thaw, has no appreciable effect on the secretory response of BG18/3E1 cells. The secretory response of BG498/45 cells was similarly preserved through bulk production and freeze-thaw.

In addition to serum-free cryopreservation, neuroendocrine cells were also bulk expanded in serum-free BetaGene Medium. The cells were initially seeded in FBS-
supplemented BetaGene Medium to provide attachment factors, then the cells were fed with serum-free medium. The serum-free supplement provides 0.1% serum albumin (human or bovine), transferrin (10 mg/liter), ethanolamine (additional 50 µM), phosphoethanolamine (additional 50 µM), additional amino acids (additional 1x MEM essential and nonessential amino acids; JRH Biosciences), ascorbate-2 phosphate (additional 0.2 mM), lipoic acid (additional 10 µM), myo inositol (additional 100 µM), tocopherol (additional 0.25 µM), vitamins K₁ and K₂ (≈5 nM), isobutyl methylxanthine (1 µM), cobalt (50 nM), copper (0.5 µM), molybdic acid (50 nM), manganese (1 µM), nickel (1 nM), selenium (30 nM); silicate (5 µM), Sn (50 nM), vanadate (5 nM), and zinc (additional 10 µM).

The results demonstrate that the process developed for neuroendocrine cells provide a well controlled method for the bulk production of neuroendocrine cells. Glucose consumption rates and lactate production rates indicated that with BG18/3E1 cells growth proceeded at a uniform rate. Glucose concentrations did not decrease below 5 mM, and lactate did not exceed 8 mM with either FBS- or SF-supplemented cultures. It was found that ammonia concentrations that are > 1 mM begin to inhibit insulin secretion. Bulk cultures supplemented with either FBS or SF did not have concentrations of ammonia > 1 mM. Ammonia with SF cultures tended to be higher than FBS cultures; the former typically had ammonia concentrations of 0.5–0.8 mM, and FBS cultures 0.25-0.45 mM.

Doubling times were similar between plate cultures at bench scale and in the bulk production. Doubling times of BG18/3E1 cells were 2-2.5 days with plate cultures, compared to 40 and 37 hours when bulk produced with FBS- or serum-free supplements, respectively. Doubling times with BG498/45 cells were 39 hours in plate cultures and 24 hours when bulk produced. The procedure and the medium provide nutrients to sustain cell growth and maintain biosynthesis of a secreted product. Peak insulin production with bulk BG498/45 cells was about 75 µg/h. Both FBS- and SF-cultures of BG18/3E1 cells peaked at insulin productions of 80 µg/h, however, insulin output/cell declined in the plateau phase of growth. The apparent fall of insulin output may reflect decreased proteolytic processing rates at plateau phase of growth. Serum-supplemented cultures exhibited normal processing from proinsulin to insulin with cells in log phase of growth. However, the amount of proinsulin increased in early plateau
phase of serum-supplemented bulk cultures. Unprocessed insulin in mid-log phase with BG18/3E1 cells represented 5% of the insulin produced, while in early plateau 13% was unprocessed insulin. The current serum-free formulation (see above formulation) resulted in insulin outputs indistinguishable from serum-supplemented bulk cultures. This indicates that the current serum-free formulation maintains the synthetic rate of bulk cultures as well as serum-supplemented media. The most significant difference observed between serum-free and serum-supplemented cultures was in the completeness of insulin processing. High-density phase bulk cultures with serum-free supplementation did not process insulin as well as serum-supplemented cultures at high-density. Serum-free cultures had 32% unprocessed insulin, compared to 13% with high-density cultures with serum. This represents about 2.5-fold increase in unprocessed insulin with serum-free cultures. It should be noted that glucose concentrations of the serum-free cultures were the lowest of the run for the high-density sample. It is possible that some amino acids had become rate-limiting at this time and contributed to the inadequate processing.

The late phase reduction in processing efficiency may indicate that further formulation improvements will be needed to provide normal insulin processing in serum-free cultures, alternatively higher flow rates may achieve a similar result. The finding that even serum-supplemented cultures exhibit processing difficulties with high-density cultures emphasizes that optimized bench-scale methods will not always translate to bulk scale procedures. The inventors consider that increased medium replacement rates may be required to sustain normal proteolytic processing in the high density setting of plateau phase with bulk neuroendocrine-cell cultures.

Overall, these results demonstrate that the current medium, not only was designed to provide an appropriate culture environment for neuroendocrine cell function, but also is robust enough to do so both at bench and production scales. In combination with the serum-free freezing solutions, serum-free BetaGene Medium provides an approach for growth and cryopreservation of cells with minimal exogenous protein.
Example 31

Utilization of amino acids and other components in bulk-scale cultures.

Methods

Cells were seeded into a Babygen™ (New Brunswick Scientific, NJ) bioreactor. The Babygen™ is a small-scale perfusion bioreactor designed to mimic the larger bench-scale Celligen™ bioreactor. Anchorage dependent cells are immobilized in a bed of non-woven polystyrene mesh-disks called FibraCel™ (New Brunswick Scientific, NJ). The bed constitutes a 50 ml volume and will accommodate $10^7$ cells per ml, and the reactor holds 450 ml of medium. The reactor fits in a regular tissue culture CO₂ incubator and is set up to be perfused; fresh medium is added while ‘spent’ medium is removed at the same rate. The medium is circulated in the reactor by means of a magnetic stir bar. Perfusion and medium circulation is performed while retaining the cell population undisturbed. The reactor is oxygenated and medium pH controlled by sparging the incubator atmosphere into the medium of the vessel.

The cell line BGI/17, a rat insulinoma engineered to express human insulin (Diabetes 46:958-967, 1997), was used for this study. The medium used was RPMI supplemented with 5% fetal bovine serum (FBS), and 2 g/L of glucose. The reactor was seeded with $10^5$ cell per ml of bed and the cells were allowed to settle in the bed over night. The culture was fed approx. 1 ml of RPMI medium supplemented with 5% FBS per $10^6$ cell per day while being propagated and was allowed to reach approx. $10^6$ cells per ml of bed in 4 days. At that point, time "0 hours", the entire medium volume was exchanged with fresh, pre-heated medium, and medium perfusion was stopped. A sample of the fresh medium was retained. After 4 hours, a sample of spent medium was collected from the reactor, and again at 8 hours a last sample was collected. The samples were sent to a clinical laboratory (Roche Laboratories) for analysis of "amines" (amino acids and other amines).

Results

Asparagine was the only amino acid supplied by the medium that remained essentially unchanged over the 8 hour period. The alanine provided by the serum supplement was also unchanged. The remaining amino acids were reduced by 30-40%, with the exception of
aspartic acid, serine, and tryptophan which were completely utilized. To compensate for this rate of consumption, amino acids should either be supplemented or the feed rate doubled to ≥ 2 ml of medium/million cells-24h. Routinely, the feed volume /million cells-24h has been maintained at ≥ 2 ml of medium/million cells-24h for cultures beyond early log phase of growth. In addition, most amino acids were increased 150-300% in the newly developed formulation.

The disappearance of some serum-derived components were also analyzed. The components that were measured and detected in the initial medium include phosphoserine, taurine, phosphoethanolamine, citrulline, L-amino-n-butyric acid, aminobutyric acid, hydroxylysine, ornithine, L-3- & L-1- methylhistidine. carnosine, and anserine. Of these components, phosphoethanolamine, taurine, phosphoethanolamine, aminobutyric acid, hydroxylysine, methylhistidines, carnosine and anserine were apparently utilized at a rapid rate, such that these components would be depleted in less than 24 h. Medium supply at ≥ 2 ml of medium/million cells-24h would potentially prevent this depletion. These components would not be available under serum-free conditions. These components represent candidates for inclusion in serum-free formulations. Phosphoethanolamine can impact on phospholipases, lecithin, choline and other associated pathways, as such this is the only candidate that has been tested as a supplement (see examples 29 and 32). The beneficial effects of phosphoethanolamine indicate that other candidates from this list should be examined for beneficial effects with serum-free cultures.

In the 8 hour period 45% of the glucose was consumed, doubling the feed/cell may prevent glucose from being a rate-limiting substrate. Notably, when several amino acids were depleted, less than half of the available glucose had been used, while other amino acids would be depleted at times concordant with glucose depletion. This indicates that with insulinoma cells control of bulk cultures can be conveniently effected by measuring glucose concentrations, and preventing >40% decreases in glucose concentrations. Reductions of glucose that exceed 40% are likely to be associated with depletions or rate-limiting concentrations of some amino acids.
EXAMPLE 32

Beneficial Effects of Ethanolamine/Phosphoethanolamine

Methods

**Cell Culture.** Cells were plated in 24 well plates (≈4 x 10^5/well), allowed to attach in BetaGene Medium supplemented with 2% FBS one day, then were changed to serum-free conditions. The cell lines used were βG18/3E1 and βG498/45, a rat insulinoma and a human lung carcinoma, respectively, engineered to synthesize and secrete human insulin. The serum-free media were RPMI- or BetaGene medium with supplements. RPMI serum-free was supplemented with 1mg/ml BSA and 10 µg/ml transferrin, and either with or without ethanolamine (EA) and phosphoethanolamine (PEA) at 50 µM. BetaGene Medium was supplemented with 1mg/ml BSA, 10 µg/ml transferrin, and EA and PEA at 50 µM. Doubling times of cells in these serum-free media were determined. In addition, dose-responsive cytotoxicity of linoleic acid (Sigma Chemical, BSA complexed or free acid with cyclodextrin as a carrier) was compared in these media by determining the effect of linoleic acid on cell doubling time with 4 log doses of linoleic acid (0.03 µM to 30 µM). Serum was not used in these studies because it contains undefined amounts of EA, PEA, and linoleic acid.

**Assay of Cell Growth: Neutral Red Uptake Assay.** A neutral red uptake assay was used for quantification of viable cell mass to allow rapid determinations of cell growth, and for calculation of cell doubling times. Neutral red diffuses across cell membranes, while protonated neutral red does not. Neutral red accumulation in metabolically active cells is dependent on an acidic compartment (maintained by)H+/ATPase. Accumulation is time and concentration dependent, and with conditions appropriate to cells of interest, uptake is linearly related to viable cell number. The assay is initiated by adding neutral red (from 1 mg/ml stock in acetic acid) to cells to provide a final concentration of 25-50 µg/ml (a minimum of 2 ml medium/cm² culture surface in each well is required). The cells are then incubated with neutral red for 0.5-1 h at 37°C. The medium with neutral red is then aspirated, the cells washed once with medium and the neutral red is extracted from the cells. Neutral red is extracted with a solution containing 50% ethanol and 0.1 M NaH₂PO₄ (pH 5.1-5.5). The soluble neutral red is quantified by determining absorbance at 540 nm in a plate reading spectrophotometer, with a standard curve of neutral red (1-40 µg/ml) dissolved in the same extraction solution.
Results

The two serum-free RPMI formulations differed in the content of EA and PEA. RPMI does not contain EA, PEA or linoleic acid. BetaGene medium contains EA, PEA and linoleic acid. Addition of EA and PEA to serum-free RPMI medium enhanced the growth rate of the cells. The doubling time of cells in RPMI was 3.89±0.12 and 2.11±0.05 days for those without and with EA and PEA, respectively. The addition of linoleic acid to cells in RPMI enhanced growth at the lowest concentration of 0.03 µM (doubling time = 3.27±0.47 days vs 1.73±0.03 with EA/PEA). A two log higher concentration of linoleic acid was toxic in the absence of EA/PEA; apparent doubling times were shifted to 23.7±0.8 days. The supplements of EA/PEA virtually prevented this cytotoxic effect; doubling time was increased to 2.31±0.08 days. Doubling times of cells in BetaGene medium with serum-free supplements, (at these plating densities) were slightly more than 1 day (1.04 to 1.09 days), with no deleterious effect of the 3 µM linoleic acid dose on doubling times; (30 µM was cytotoxic).

These results demonstrate that supplementation of media with EA/PEA exerts both a growth-promoting effect and a protective action on neuroendocrine cells. The use of PEA offers a greater concentration range for supplementation, as millimolar PEA (unlike EA) had no cytotoxic effects (see Example 29).

EXAMPLE 33

Development of BetaGene Medium

The development of a specific medium for neuroendocrine cells proceeded along the lines suggested by Ham and colleagues (Ham and McKeenan 1979. Media and growth requirements. Methods in Enzymology 58:44-93). Commercially available media were compared for performance with a human insulin-engineered beta-cell line, BG18/3E1. The better media were then compared in mixtures of two media. An objective of the medium development was to have a medium that supports neuroendocrine cells, particularly in serum-free conditions. To that end a screening methods was implemented to shorten the time needed to compare cell performance in different media.
The method involves encapsulating cells in alginate beads and culturing the cells in media being tested, in the absence of any supplements. Many cell types do not attach to culture dishes in the absence of serum; alginate encapsulation provides a stable format for studying cells without attachment, without loss in changes (as would occur with suspension cultures). Cells were recovered at the end of the study and viable cell mass quantified (see example 32 for assay).

Methods

Cell Encapsulation. The cells were removed from culture flasks with trypsin-EDTA, collected in medium with FBS, then pelleted by centrifugation. The pelleted cells were resuspended in a 1.5% sodium alginate solution (50% high viscosity and 50% low viscosity sodium alginate made up in serumless medium) at a concentration of 5 million cells per 1 milliliter of alginate. The suspension was transferred to a syringe and allowed to sit at room temperature for 5 minutes to allow all air bubbles to rise to the surface. A 25 gauge needle was attached to the syringe and the cell/alginate slurry dispensed through the syringe into a 50 ml conical tube containing approximately 35 mls of 1.35% CaCl\textsubscript{2}/20 mM HEPES. Beads formed in the CaCl\textsubscript{2} solution and were polymerized after about 10 minutes. The CaCl\textsubscript{2} solution was removed carefully and the beads were washed with two volumes of serumless medium/20 mM HEPES.

Cell Culture. The encapsulated cells were then cultured with the different medium in multiwell dishes (12, 24, and 48 wells) without any additives or with FBS. Medium samples were collected at 2-3 days intervals, BSA was added to samples of serumless media (to give 0.1% BSA) to prevent insulin adsorption in freeze thaws. The samples were frozen for later assay or assayed immediately. Media that best maintained insulin output, and cell growth for a 4-7 days were used in further studies, and compared to 1:1 mixtures of the best media. The selected media were also studied for recovery of cell performance when FBS was added back to the cultures. Results of these studies were subsequently extended to human cells using human islets (see Example 22).
All media were purchased from JRH Biosciences (Lenexa, KS), except CMRL 1066 was purchased from Life Technologies (Grand Island, NY).

**Assay of Insulin.** Insulin in the medium was quantified with a commercial radioimmunoassay (DPC, Los Angeles CA), that is referenced to USP human insulin in the inventors' laboratory (lot G; US Pharmacopeia, Rockville MD). The reference USP human insulin that is included in each assay, is validated by HPLC in the inventors' laboratory (see Example 22 for HPLC methods).

**Results**

The following commercial media were compared: CMRL 1066, M199E, alpha MEM, RPMI, F12, and DMEM (only in mixtures with F12). Ham recommends M199E, alpha MEM, and RPMI for culture with human cells. F12 has been used for pituitary cells (see Bottenstein et al. The growth of cells in serum-free hormone-supplemented media. *Methods in Enzymology* 58:94-109), and CMRL 1066 is used for human islets.

CMRL 1066 medium performed the poorest, with insulin output declining >80% in 3 days of culture without serum. Cell performance in CMRL 1066 with FBS was hardly discernible from cells in other media with FBS. Cells in M199E or RPMI decreased insulin output at a slower rate than with CMRL, but they performed more poorly than MEM or F12. Cells in F12 usually retained a higher insulin output than cells in MEM. Mixtures of F12 and M199 or F12 and MEM were found to perform the best, although DMEM-F12 provided similar cell performance. On the basis of these studies components of these media were included in the BetaGene Medium formulation, with additions of higher myoinositol, of phosphoethanolamine, and of ascorbate 2-phosphate (see Table 1). This medium was then custom manufactured by JRH Biosciences and cell performance was compared with cells in BetaGene Medium, MEM, and F12-MEM with and without FBS. Cells in BetaGene Medium and F12-MEM with FBS exhibited the best performance; growth in the two media was indistinguishable, while insulin output was highest with BetaGene Medium (FIG. 46). Growth of cells in BetaGene Medium without serum did not significantly differ from BetaGene Medium with FBS, but insulin output without FBS was reduced 30%; although it was not significantly lower than F12-MEM with
FBS. Performance of cells in BetaGene Medium without serum surpassed that of cells MEM with or without FBS, and of cells in F12-MEM without FBS (FIG. 46). Furthermore, switching cells grown in F12-MEM without FBS to BetaGene Medium without FBS resulted in increased insulin output, although not normalization of insulin (FIG. 47). These findings demonstrate that the BetaGene Medium formulation provides optimal culture conditions for this type of neuroendocrine cell- and that this cell type can be grown in defined Betagene Medium.

EXAMPLE 34

Altering responsiveness of human neuroendocrine cell lines to modulators of secretion.

βG 498/20 cells secrete insulin from a regulated secretory pathway as evidenced by an approximate 12-fold increase in basal insulin secretion versus that stimulated by PMA, carbachol, or a stimulatory cocktail (Swiss). Also, there is a lack of responsiveness to glucose and glucose plus IBMX. A preferred embodiment for the cell-based delivery of insulin includes the capacity to modulate release of the peptide in response to post-prandial (such as glucose) and/or hypoglycemic signals. The pancreatic beta-cell senses a variety of extracellular molecules through metabolism, receptors, and ion channels. Each of these sensing mechanisms impacts intracellular calcium levels, with increases in this ion stimulating the release of insulin.

Two lines a experimental evidence implicate Ca2+ in regulated insulin secretion from βG 498/20 cells: firstly, PMA and carbachol each exert effects on secretion via the stimulation of protein kinase C, where "C" is an abbreviation for "calcium"; and secondly, verapamil partially inhibits stimulated secretion from βG 498/20 cells. Verapamil antagonizes the uptake of extracellular Ca2+. Efforts are underway to exploit the role of Ca2+-regulated secretion in βG 498/20 cells, and to engineer these cell lines to respond to a variety of secretory modulators that are known to be involved in the physiological regulation of insulin secretion from the pancreatic beta cell. Table 13 lists potential candidates for engineering and the molecules to which they respond.
### TABLE 13
CANDIDATE TRANSGENES FOR ALTERING SECRETORY RESPONSES
OF NEUROENDOCRINE CELL LINES

<table>
<thead>
<tr>
<th>Candidate Transgenic Protein</th>
<th>Responsive to:</th>
</tr>
</thead>
<tbody>
<tr>
<td>glucokinase</td>
<td>glucose</td>
</tr>
<tr>
<td>GLUT-2 transporter</td>
<td>glucose</td>
</tr>
<tr>
<td>SUR</td>
<td>ATP, diazoxide</td>
</tr>
<tr>
<td>Kir</td>
<td>ATP</td>
</tr>
<tr>
<td>late rectifying K channels</td>
<td>membrane polarity, potassium</td>
</tr>
<tr>
<td>calcium channels</td>
<td>membrane polarity, calcium</td>
</tr>
<tr>
<td>GLP-1 receptor</td>
<td>GLP-1</td>
</tr>
<tr>
<td>muscarinic receptor</td>
<td>acetyl choline</td>
</tr>
<tr>
<td>pancreatic polypeptide receptor</td>
<td>pancreatic polypeptide</td>
</tr>
<tr>
<td>somatostatin receptor</td>
<td>somatostatin</td>
</tr>
<tr>
<td>alpha 2 adrenergic receptor</td>
<td>epinephrine</td>
</tr>
<tr>
<td>leptin receptor</td>
<td>leptin</td>
</tr>
</tbody>
</table>

---

All of the compositions and/or methods disclosed and claimed herein can be made and executed without undue experimentation in light of the present disclosure. While the compositions and methods of this invention have been described in terms of preferred embodiments, it will be apparent to those of skill in the art that variations may be applied to the compositions and/or methods and in the steps or in the sequence of steps of the method described herein without departing from the concept, spirit and scope of the invention. More specifically, it will be apparent that certain agents which are both chemically and physiologically related may be substituted for the agents described herein while the same or similar results would be achieved. All such similar substitutes and modifications apparent to those skilled in the art are deemed to be within the spirit, scope and concept of the invention as defined by the appended claims.
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The following references, to the extent that they provide exemplary procedural or other details supplementary to those set forth herein, are specifically incorporated herein by reference.

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CLAIMS:

1. A method of identifying a modulator of secretory function comprising the steps of:

   (i) providing an immortalized cell having a stable secretory function;
   (ii) contacting said cell with a candidate substance;
   (iii) measuring the secretory function of said cell; and
   (iv) comparing the secretory function of the cell in step (iii) with the secretory function of the cell of step (i),

wherein an alteration in the secretory function indicates that said candidate substance is a modulator of said secretory function.

2. The method of claim 1, wherein said secretory function comprises the secretion of a polypeptide.

3. The method of claim 1, wherein said secretory function is dependent on a regulator wherein said regulator is selected from the group consisting of calcium ions, cAMP, calmodulin, phosphorylation, dephosphorylation, membrane polarization glucose, ATP, ADP, fatty acids and NADPH.

4. The method of claim 1, wherein said modulator inhibits said secretion.

5. The method of claim 1, wherein said modulator stimulates said secretion.

6. The method of claim 2, wherein said polypeptide is an amidated polypeptide, a glycosylated polypeptide, a hormone, an enzyme or a growth factor.

7. The method of claim 1, wherein said cell is encapsulated in a biocompatible matrix.

8. The method of claim 1, wherein said cell is in an animal.
9. The method of claim 1, wherein said cell is in an adherent culture.

10. The method of claim 1, wherein said cell is in a suspension culture.

11. The method of claim 1, wherein said cell is an immortalized cell.

12. The method of claim 1, wherein said cell is a fetal cell.

13. The method of claim 1, wherein said cell is a primary cell obtained from human tissue.

14. The method of claim 1, wherein said secretory cell is an endocrine cell.

15. The method of claim 1, wherein said secretory cell is a neuroendocrine cell.

16. The method of claim 15, wherein said cell is obtained from a human neuroendocrine tumor.

17. The method of claim 1, wherein said cell is an insulinoma cell.

18. The method of claim 1, wherein said cell is a human cell.

19. The method of claim 1, wherein said cell is a non-human cell.

20. The method of claim 1, wherein said cell is a secretory cell.

21. The method of claim 1, wherein said cell is a pancreatic beta cell.

22. The method of claim 1, wherein said cell is a pancreatic alpha cell.

23. The method of claim 1, wherein said cell is a pituitary cell.
24. The method of claim 1, wherein said cell is an adipocyte.

25. The method of claim 1, wherein said cell is a hepatocyte.

26. The method of claim 1, wherein said cell is a muscle cell.

27. The method of claim 1, wherein said cell is a lung cell.

28. The method of claim 1, wherein said cell is responsive to modulators of secretion.

29. The method of claim 1, wherein said cell is non-responsive to modulators of secretion.

30. The method of claim 6, wherein said glycosylated polypeptide is selected from the group consisting of amylin, luteinizing hormone, follicle stimulating hormone and chorionic gonadotrophin.

31. The method of claim 6, wherein said hormone is selected from the group consisting of growth hormone, prolactin, placental lactogen, luteinizing hormone, follicle-stimulating hormone, chorionic gonadotropin, thyroid-stimulating hormone, leptin, adrenocorticotropic (ACTH), angiotensin I, angiotensin II, β-endorphin, β-melanocyte stimulating hormone (β-MSH), cholecystokinin, endothelin I, galanin, gastric inhibitory peptide (GIP), glucagon, insulin, lipotropins, neurophysins and somatostatin.

32. The method of claim 6, wherein said amidated polypeptide is selected from the group consisting of calcitonin, calcitonin gene related peptide (CGRP), β-calcitonin gene related peptide, hypercalcemia of malignancy factor (1-40) (PTH-rP), parathyroid hormone-related protein (107-139) (PTH-rP), parathyroid hormone-related protein (107-111) (PTH-rP), cholecystokinin (27-33) (CCK), galanin message associated peptide, preprogalanin (65-105), gastrin I, gastrin releasing peptide, glucagon-like peptide (GLP-1), pancreastatin, pancreatic peptide, peptide YY, PHM, secretin, vasoactive intestinal peptide (VIP), oxytocin, vasopressin (AVP), vasotocin, enkephalins, enkephalinamide,
metorphinamide (adrenorphin), alpha melanocyte stimulating hormone (alpha-MSH), atrial natriuretic factor (5-28) (ANF), amylin, amyloid P component (SAP-1), corticotropin releasing hormone (CRH), growth hormone releasing factor (GHRH), luteinizing hormone-releasing hormone (LHRH), neuropeptide Y, substance K (neurokinin A), substance P and thyrotropin releasing hormone (TRH).

33. The method of claim 31, wherein said cell secretes insulin in response to a modulator.

34. The method of claim 6, wherein said growth factor is selected from the group consisting of epidermal growth factor, platelet-derived growth factor, fibroblast growth factor, hepatocyte growth factor and insulin-like growth factor 1.

35. The method of claim 28, wherein said non-responsive cell is engineered to modify the secretion of said polypeptide in response to said secretagogue.

36. The method of claim 35, wherein said cell secretes an amidated polypeptide, a glycosylated polypeptide, a hormone or a growth factor in response to said secretagogue.

37. The method of claim 2, wherein said cell is an engineered cell that expresses a recombinant GLUT-2 gene.

38. The method of claim 2, wherein said cell is an engineered cell that expresses a recombinant glucokinase (hexokinase IV) gene.

39. The method of claim 2, wherein said cell is an engineered cell that has a reduced hexokinase I activity relative to the cell from which it was prepared.

40. The method of claim 1, wherein said cell is derived from a BTC, RIN, HIT, BHC, CM, TRM, TRM6, AtT20, PC12, BG 49/206, BG40/110, BG-H03, BG 498/45, BG 498/20, NCI-H810 (CRL-5816), BON, NES2Y, NCI-H508 (CLL-253), HEPG2 or HAP5 cell.
41. The method of claim 1, wherein said cell is selected from the group consisting of βG H01, βG H02, βG H03, βG H04, βG H05, βG H06, βG H07, βG H08, βG H09, βG H10, βG H11, βG H12, βG H13, βG H14, βG H15, βG H16, βG H17, βG H18, βG H19, βG H20, βG H21, βG H22, βG H23 and βG H25.

42. The method of claim 1, wherein said cell is selected from the group consisting of βG/498/20, βG/498/44, βG/498/45, βG 636/17 and βG 636/11.

43. The method of claim 1, wherein said cell secretes an endogenous secretory polypeptide.

44. The method of claim 1, wherein said cell is engineered to increase secretion of an endogenous secretory polypeptide.

45. The method of claim 1, wherein said cell is engineered to modify the secretion of an endogenous secretory polypeptide in response to said modulator.

46. The method of claim 43, wherein said cell comprises an endogenous gene encoding said polypeptide and the expression of said gene is inhibited.

47. The method of claim 46, wherein said cell comprises an exogenous gene that encodes an exogenous secretory polypeptide, said cell secreting said exogenous secretory polypeptide.

48. The method of claim 1, wherein said cell is grown in defined media further supplemented with a growth factor specific for said cell.

49. The method of claim 48, wherein said cell is a human pancreatic β-cell and said growth factor is HGF, IGF-1, PDGF, NGF or growth hormone.

50. The method of claim 1, wherein said cell expresses an endogenous receptor.
51. The method of claim 1, wherein said cell is engineered to modify the expression of an endogenous receptor in response to said modulator.

52. The method of claim 51, wherein said modulator is a stimulator of said expression.

53. The method of claim 51, wherein said modulator is an inhibitor of said expression.

54. The method of claim 50, wherein said cell comprises an endogenous gene encoding said receptor and the expression of said gene is inhibited.

55. The method of claim 54, wherein said cell comprises an exogenous gene that encodes an exogenous receptor. said cell expressing said exogenous receptor.

56. The method of claim 50, wherein said receptor is selected from the group consisting of α-adrenergic receptor, β-adrenergic receptor, potassium inward rectifying channel, sulphonylurea receptor, GLP-1 receptor, growth hormone receptor, luteinizing hormone receptor, corticotrophin receptor, urocortin receptor, glucocorticoid receptor, pancreatic polypeptide receptor, somatostatin receptor, muscarinic receptor, BK channel and leptin receptor.

57. The method of claim 1, wherein said cell secretory function is responsive to a cell signaling molecule wherein said cell signaling molecule is a modulator of said secretion.

58. The method of claim 57, wherein said modulator is a stimulator of said secretion.

59. The method of claim 57, wherein said modulator is an inhibitor of said secretion.

60. The method of claim 57, wherein said cell signal is Ca2+ dependent.
61. The method of claim 57, wherein said cell signal is Ca\textsuperscript{2+} independent.

62. A modulator of secretory function identified according a method comprising the steps of:

(i) providing an immortalized cell having a stable secretory function;
(ii) contacting said cell with a candidate substance;
(iii) measuring the secretory function of said cell; and
(iv) comparing the secretory function of the cell in step (iii) with the secretory function of the cell of step (i),

wherein an alteration in the secretory function indicates that said candidate substance is a modulator said secretory function.

63. A modulator of polypeptide secretion identified according a method comprising the steps of:

(i) providing a stable, immortalized cell that secretes a polypeptide;
(ii) contacting said cell with a candidate substance;
(iii) incubating said cell;
(iv) measuring the secretion of said polypeptide; and
(v) comparing the secretion of said polypeptide in the cell of step (iii) with the secretion of said polypeptide in the cell of step (i),

wherein an alteration in the secretion of said polypeptide indicates that said candidate substance is a modulator said secretion.

64. A method of identifying a modulator of insulin secretion comprising the steps of:

(i) providing an engineered pancreatic \beta\ cell;
(ii) contacting said cell with a candidate substance;
(iii) measuring the insulin secretion of said cell; and
(iv) comparing the insulin secretion of the cell in step (iii) with the insulin secretion of the cell of step (i).

wherein an alteration in the insulin secretion indicates that said candidate substance is a modulator said secretion.

65. A method of identifying a modulator of insulin secretion comprising the steps of:

(i) providing an engineered pancreatic β cell;
(ii) contacting said cell with a candidate substance;
(iii) measuring the intracellular signal of said cell; and
(iv) comparing the intracellular signal of the cell in step (iii) with the intracellular signal of the cell of step (i);

wherein an alteration in the intracellular signal indicates that said candidate substance is a modulator said insulin secretion.

66. A method of identifying a modulator of insulin secretion comprising the steps of:

(i) providing an engineered pancreatic β cell;
(ii) contacting said cell with a candidate substance;
(iii) measuring the intracellular Ca$^{2+}$ of said cell; and
(iv) comparing the intracellular Ca$^{2+}$ of the cell in step (iii) with the intracellular Ca$^{2+}$ of the cell of step (i);

wherein an alteration in the intracellular Ca$^{2+}$ indicates that said candidate substance is a modulator said insulin secretion.
Effectors that signal...

via Metabolism:
- FFA
- Glucose
- Amino acids
- IBMX

via Cell Surface Receptors:
- Epinephrine
- Somatostatin
- Carbachol

via Ion Channels:
- Arginine
- Diazoide
- Sulf.-ureas
- Prandin
Figure 2

Compounds

Validation

Human Islets

Discovery

Targets

Primary Screen

Hits

Target Engineered Cell Line

Secondary Screen

Candidate Compounds

Animal "Model" with Human Target

Transplanted Cell Line
Figure 7

% Maximal Stimulation

Insulin Secretion

Clonidine, nM

100
10
1

18/3E1
265/1
265/2

160 140 120 100 80 60 40 20
Figure 9

Human Insulin, ng/ml

Rat C-Peptide II, ng/ml

Control

Yohimbine

Clonidine

n=3 rats
(20 minutes after Drug)
Figure 10B

Viability cells % Control (Lot 7E3183 + FBS)
Figure 11A

Insulin (ng/10^6 cells/24 hrs)

- (-)
- (+)  (βG603/7)
- (+)  (βG603/11)
- (+)
- (+)
- (+)
- Glucose
  - 50pM
  - 500pM
  - 5nM
  - 50nM
  - 500nM
  - SS-28
Fig. 13A

Fold Insulin Secretion
Figure 17B

% Total glycated hemoglobin

n=2

HO3

n=4

498/20

CELL TYPE
Human neuroendocrine tumor such as insulinoma → Cell-specific proliferation → Cell-specific immortalization → Human β-Cell Line

Primary tissue such as islets

Figure 22
Figure 28A

Proinsulin (ca. 700 ng detected)

FRACTION NUMBER

INSULIN IMMUNOREACTIVITY (ng/ml)

120
100
80
60
40
20
0

0

20
40
60
80
100
Figure 29

A.

B.

-205RIP
-415RIP
FF3/-205RIP
FF6/-205RIP
-415RIP/FFE2/-205RIP
FFE3/-415RIP
FFE6/-415RIP

hGH levels relative to -415RIP
Figure 30A

Insulin mRNA levels relative to RIP mRNA level

- RIP
- FFE3/RIP
- FFE6/RIP
- FFE6/RIP/RIPint.
- CMV
Figure 30B

Insulin Secretion Relative to RIP

RIP, FFE3/RIP, FFE6/RIP, FFE6/RIP/INT, CMV
Figure 34

Glucose only
+ 10nM IGF-1
+ 10nM rGH
+ rGH + IGF-1

Fold Increase in $[^{3}H]T hymidine $ incorporation

Above No Glucose / No rGH Control
Figure 39

![Graph showing the effect of different supplements on insulin production in βG18/3E1 Cells. The x-axis represents the different supplements: SF+Minerals, SF+min.+Amino acids, SF+Amino acids, and FBS. The y-axis represents insulin production in ng/well-h, with bars indicating basal and stimulated conditions.](image-url)
Figure 43

The diagram shows the amount of GLP-1 (ng/100ul assayed) in different media and supplements. The x-axis represents the media and supplements tested, while the y-axis represents the amount of GLP-1. The bars indicate the amount of non-amidated and amidated GLP-1 in each condition.
Fig. 47

Insulin Secretion of Cells Switched to βGM for 3 Days

![Graph showing insulin secretion in different mediums: BGM Unsuppl., F12/MEM Unsuppl., F12/MEM to βGM.]

- Insulin, ng/bead-d

Medium
SEQUENCE LISTING

110 THIGPEN, ANICE E.
QUAADE, CHRISTIAN
CLARK, SAMUEL A.

120 RECOMBINANT CELL LINES FOR DRUG SCREENING

130 BTGN:055/BTGN:055P

140 Unknown
141 1999-01-11

150 60/072,556
151 1998-01-12

150 60/087,848
151 1998-06-03

160 15

170 PatentIn Ver. 2.0

210 1
211 19
212 DNA
213 Artificial Sequence

220
223 Description of Artificial Sequence: synthetic primer

400 1
cagccctgcc tggagggac 19

210 2
211 23
212 DNA
213 Artificial Sequence

220
223 Description of Artificial Sequence: synthetic primer

400 2
ccgagaaggc cagcagtgtg tac 23

210 3
211 23
212 DNA
213 Artificial Sequence

220
223 Description of Artificial Sequence: synthetic primer
<400> 3
tggtggaatt cctgaactcc ccc 23

<210> 4
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: synthetic primer

<400> 4
gattggccac cggcctgca 20

<210> 5
<211> 57
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: synthetic primer

<400> 5
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<210> 6
<211> 57
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: synthetic primer

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<210> 7
<211> 57
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: synthetic primer

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<210> 8
<211> 57
<212> DNA
<213> Artificial Sequence
<220>
<223> Description of Artificial Sequence: synthetic primer

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<210> 9
<211> 27
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: synthetic primer

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<210> 10
<211> 28
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: synthetic primer

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gggcaaceta ggtctggac cttctatc  28
<210> 11
<211> 28
<212> PRT
<213> Mus musculus

<220>
<223> Description of Artificial Sequence: synthetic primer

<400> 11
Ser Ala Asn Ser Asn Pro Ala Met Ala Pro Arg Glu Arg Lys Ala Gly
 1     5    10   15
Cys Lys Asn Phe Phe Trp Lys Thr Phe Thr Ser Cys
 20   25
<210> 12
<211> 25
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: synthetic primer
<400> 12
gagaagga attccatcc aata

<210> 13
<211> 23
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: synthetic primer

<400> 13
ttcagatatcc aaggatcagc agg

<210> 14
<211> 27
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: synthetic primer

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<210> 15
<211> 27
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: synthetic primer

<400> 15
ctcagtaact cgagtaatg aagtcc