NOVEL ROLES OF GASTRIN AND CHOLECYSTOKININ IN ISLET BETA CELL PROLIFERATION

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DEDICATION

To my wife Jaime,

I could not be here today without your love and support.

NOVEL ROLES OF GASTRIN AND CHOLECYSTOKININ IN ISLET BETA CELL PROLIFERATION

By

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Type 1 diabetes and the latter stages of Type 2 diabetes share a common theme: an insufficient β cell mass to maintain glucose homeostasis. In Type 1 diabetes this deficiency arises from autoimmune destruction of the β cells. In the latter stages of Type 2 diabetes, there is a precipitous drop in β cell mass, resulting from the combination of several factors. In the last decade, islet transplantation has re-emerged as a viable option for the treatment of Type 1 diabetes, thanks to greatly improved islet isolation protocols and immunosuppressive regimens. Despite these advancements, the supply of available islet β cells for transplantation is greatly eclipsed by the demand. Consequently, the identification of genes or external factors that promote β cell proliferation and survival is a key step toward developing a replenishable

population of β cells that can be used for transplantation for Type 1 diabetes. Additionally, any method discovered to promote β cell growth or enhance β cell function is directly applicable in the treatment of Type 2 diabetes.

Through a broad-based microarray screen, the preprogastrin gene was found to be differentially expressed in our model of β cell cytokine resistance, the INS-1_{res} cell lines compared to unselected INS-1 cells. As a result of this finding, preprogastrin was initially evaluated for its involvement in cytokine resistance and β cell survival. During the course of this analysis, preprogastrin was determined to exhibit significant mitogenic properties when overexpressed in INS-1 cell lines and isolated rat islets. These results led to the discovery that the related protein, preprocholecystokinin, also promotes impressive β cell growth, in addition to enhanced β cell function, as measured by improved glucose-stimulated insulin secretion. Several additional experiments suggest that traditional plasma membrane receptors and signaling pathways for gastrin and cholecystokinin do not explain the effect of overexpression of these prohormones on islet replication, including a lack of effect of exogenously added gastrin and cholecystokinin peptides. These results could be attributable to an intracrine mode of signaling that will require further investigation as a possible therapeutic target for the treatment of Type 1 and Type 2 diabetes.

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AEC – 3-amino-9-ethylcarbazole
Akt1 – protein kinase B
Ambp – alpha 1 microglobulin
AMPK – adenosine 5’ monophosphate-activated protein kinase
AP – alkaline phosphatase
ATP – adenosine triphosphate
βgal – beta-galactosidase
BLAST – Basic Local Alignment Search Tool
BrdU – 5-bromo-2’-deoxyuridine
BSA – bovine serum albumin
c-myc – protooncogenic transcription factor
CCK – cholecystokinin
CCK-8 – cholecystokinin 8
CCK-22 – cholecystokinin 22
CCK-33 – cholecystokinin 33
CCK-58 – cholecystokinin 58
CCK1R – cholecystokinin 1 receptor
CCK2R – cholecystokinin 2 receptor
CD4+ – cluster of differentiation 4
CD8+ – cluster of differentiation 8
cdk2 – cyclin-dependent kinase 2
cdk4 – cyclin-dependent kinase 4
cDNA – complementary DNA
CIP – cyclin inhibitory protein
CMV – cytomegalovirus (promoter)
Cy3 – green fluorophore used in microarray cDNA labeling
Cy5 – red fluorophore used in microarray cDNA labeling
DAB – 3,3-diaminobenzidine-tetra-hydrochloride
DAG – diacylglycerol
DAPI – 4',6-diamidino-2-phenylindole
DMEM – Dulbecco's Modified Eagle's Medium
E1A – early region 1A of the adenoviral genome
E2F – family of transcription factors in higher eukaryotes
ECL – enterochromaffin-like
EDTA – ethylene diamine tetraacetate
EGF – epidermal growth factor
EGFR – epidermal growth factor receptor
eIF4F – eukaryotic translation initiation factor 4E
EMSA – electromobility shift assay
ER – endoplasmic reticulum
ERK – extracellular signal-regulated kinase
FCS – fetal calf serum
G_1 – cell cycle growth phase 1
G17 – gastrin-17

G17-Gly – glycine-extended gastrin-17
G34 – gastrin-34
G34-Gly – glycine-extended gastrin-34
G-cells – gastrin secreting stomach cells
G-Gly – glycine-extended gastrins
GFP – green fluorescent protein
GSIS – glucose-stimulated insulin secretion
HEK293 – human endothelial kidney cell line
Hepes – 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HRP – horseradish peroxidase
IAPP – islet amyloid polypeptide
IFN-γ – interferon gamma
IL-1β – interleukin 1-beta
IL-1R1 – interleukin 1 receptor subtype 1
iNOS – inducible nitric oxide synthase
INS-1 – insulinoma-derived beta cell line
$INS\text{-}1_{res}$ – cytokine resistant insulinoma-derived beta cell line
IP_3 – inositol triphosphate
IRF-1 – interferon regulatory factor 1
JAK2 – janus kinase 2
KIP – kinase inhibitory protein
LKB1 – serine/threonine kinase that phosphorylates AMPK
MHC – major histocompatibility class
MIAME – minimum information about a microarray experiment
mTOR – mammalian target of rapamycin
MTT – C,N diphenyl-*N*-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide
NF-κB – nuclear factor kappa B
NO – nitric oxide
NOD – non-obese diabetic
NOS – nitric oxide species
OD – optical density
p107/p130 – homologs of retinoblastoma protein
p38MAPK – mitogen-activated protein kinase
p53 – tumor protein 53, a tumor suppressor
PAM – peptidyl-glycine alpha-amidating monooxygenase
PBMC – peripheral blood mononuclear cell
PBS – phosphate buffed saline
PCR – polymerase chain reaction
PC2 / PC1/3 – prohormone convertases 2 and 1/3
PEG – polyethylene glycol
PI3K – phosphoinositide-3 kinase
PIP_2 – phosphatidylinositol
PKC – protein kinase C
PKD/PKD2 – protein kinase D / D2
PLC-β – phospholipase C beta
pRb – retinoblastoma protein

qPCR – quantitative (real-time) polymerase chain reaction
ras – regulatory GTP hydrolase
RAD – RNA Abundance Database
RIA – radioimmunoassay
ROS – reactive oxygen species
S – DNA synthesis phase of the cell cycle
SAB – secretion assay buffer
SAM – Statistical Analysis of Microarrays
siRNA – small interfering ribonucleic acid
STAT1α – signal transducer and activator of transcription 1 alpha
STAT3 – signal transducer and activator of transcription 3
TCA – trichloroacetic acid
TCA cycle – tricarboxylic acid cycle, Krebs cycle
TGFα – transforming growth factor alpha
TFF3 – trefoil factor 3
TNFα – tumor necrosis factor alpha
TZDs – thiazolidinedione derivatives
v-src – viral form of a protooncogenic tyrosine kinase
WT – wild-type

CHAPTER ONE

Introduction

Diabetes Mellitus - A Brief History

The principal symptoms of diabetes were described as early as 1552 B.C. when ancient Egyptian physicians alluded to a disease with excessive urination. Aretaeus the Cappadocian, around 300-400 B.C., described diabetes as "a melting down of the flesh and limbs into urine" during which patients were unable to quench their thirst (Notelovitz 1970). In 1924, these classical symptoms were linked to deficiency of the hormone insulin in the seminal work published by Frederick Banting and Charles Best (Banting 1924). In their experiments, Banting and Best were able to eliminate the symptoms in diabetic dogs by administering a pancreatic extract, the active component of which was later shown to be the peptide hormone insulin. To this day, exogenous insulin administration remains a mainstay treatment for the two diseases collectively known as diabetes.

Type 1 Diabetes Mellitus

Type 1 diabetes, formerly known as insulin dependent diabetes mellitus, results from the autoimmune destruction of the insulin-producing β cells in the Islets of Langerhans within the pancreas (Gepts 1965) by CD4+ and CD8+ T lymphocytes and macrophages (Foulis *et al.* 1991). This variant of diabetes accounts for approximately 10% of all cases, occurs most commonly in people of European descent, and has a global

incidence that is increasing at approximately 3% per year (Onkamo *et al.* 1999), indicating a strong environmental component in the etiology of the disease.

The primary function of insulin is to facilitate the uptake of glucose into muscle and adipose tissues (Daniel *et al.* 1975). Autoimmune destruction of the β cells results in an insulin deficient state that promotes high blood glucose. Ironically, patients with Type 1 diabetes succumb to a wasting phenomenon in which their cells literally starve because they cannot gain access to the glucose in their bloodstream. The persistent hyperglycemic state can lead acutely to ketoacidosis and chronically to a host of complications including neuropathy, kidney failure, cardiovascular disease, and blindness.

Shortly after its discovery, insulin injection therapy became the mainstay treatment for Type 1 diabetes. While this treatment immediately rescinded the death sentence that previously came with the diagnosis of diabetes, it is not without significant shortcomings. Proper glycemic control requires continual blood glucose monitoring to minimize the long-term complications of hyperglycemia (Havas and Donner 2006) and patients are never free from the dangers of hypoglycemia due to over administration of insulin or insufficient glucose monitoring (Cryer *et al.* 2003).

Recent advancements in immunosuppressive drugs and enhanced techniques for islet harvesting (Linetsky *et al.* 1997; Lakey *et al.* 1999) are making islet transplantation a viable therapy for the treatment of Type 1 diabetes (Shapiro *et al.* 2000; Froud *et al.* 2005). However, the shortage in cadaveric pancreata, the need for life-long immunosuppression (Ryan *et al.* 2002), and the progressive decline in transplanted islet

mass over time (Robertson 2004) severely restricts the broad-based application of islet transplantation to a small minority of diabetic patients.

Currently, the demand for islet transplantation ***far*** exceeds the number of available donors by several orders of magnitude. One report estimated that in the small country of Switzerland, which has only a fraction of the diabetic population of the United States (30,000), only 30 islet transplantation surgeries could be performed in a year (Oberholzer *et al.* 2001). In order to establish β cell transplantation as a mainstay therapy or even a *bona fide* cure for Type 1 diabetes, the pool of available β cells for transplantation needs to be expanded several thousand fold. Additionally, transplanted cells need to be more resistant to the immune system in order to survive long-term in Type 1 diabetics, as these individuals have a heightened immune response to β cell autoantigens (Evgenov *et al.* 2006). Consequently, the identification of genes or external factors that promote β cell proliferation and survival is a key step toward developing a replenishable population of β cells that can be used for transplantation therapy for Type 1 diabetes.

Type 2 Diabetes Mellitus

Type 2 diabetes, formerly known as non-insulin dependent diabetes mellitus, has a pathogenesis that is very distinct from that of Type 1 diabetes. Type 2 diabetes results from a combination of obesity-associated insulin resistance in peripheral tissues and β cell dysfunction progressing to eventual β cell failure. In the pre-diabetic state, the overabundance of nutrients common to the modern lifestyle, in concert with peripheral insulin resistance, induces compensatory insulin hypersecretion and expansion of β cell

mass in order to maintain normoglycemia (Martin *et al.* 1992; Chen *et al.* 1994; Steil *et al.* 2001).

β cell mass expansion is observed in response to a variety of stimuli, including pregnancy (Parsons *et al.* 1992), persistent hyperglycemia (Lipsett and Finegood 2002), obesity (Karam *et al.* 1963; Polonsky *et al.* 1988), and surgical injury such as partial pancreatectomy and ductal ligation (Pearson *et al.* 1977; Wang *et al.* 1995). In non-diabetic individuals, β cell mass is maintained by a balance of β cell neogenesis (+), replication (+), and cell death (-) (Bonner-Weir 2000). In Type 2 diabetic individuals, peripheral insulin resistance increases the demand for insulin production from the β cells. This demand is believed to be met by β cell hypertrophy (Gapp *et al.* 1983; Milburn *et al.* 1995; Bock *et al.* 2003) and replication (Bonner-Weir *et al.* 1989; Paris *et al.* 2003; Topp *et al.* 2004). These changes in β cell size and number are strikingly similar to those observed in pregnancy, where the fetus places increased metabolic demands on the mother that are not unlike those seen in obesity (Van Assche *et al.* 1980).

Although the triggering event is not clear, the transition from the pre-diabetic state to overt Type 2 diabetes is marked by a rapid decompensation, during which β cell mass reverses, usually rapidly, and glucose levels rise sharply (Weir and Bonner-Weir 2004). This period of decompensation is marked by not only a drop in β cell mass of ~50% (Maclean and Ogilvie 1955; Butler *et al.* 2003; Yoon *et al.* 2003), but by decreased efficiency of insulin secretion of the remaining β cells in response to a glucose challenge as well (Ward *et al.* 1984). One contributing factor to the demise of β cell mass in Type 2 diabetes appears to be the accumulation of lipids in the islets. Lipid accumulation has been demonstrated to cause heightened β cell sensitivity to inflammatory mediators such

as cytokines, hastening their death and negatively impacting β cell mass and glucose homeostasis (Milburn, Hirose *et al.* 1995; Shimabukuro *et al.* 1997; Shimabukuro *et al.* 1998; Shimabukuro *et al.* 1998).

Recent work on islet amyloid polypeptide (IAPP), a 37-amino acid protein co-secreted with insulin, has uncovered another possible mechanism of β cell destruction in Type 2 diabetes. Type 2 diabetics show large extracellular accumulation of IAPP in the islets and those individuals with the most severe symptoms demonstrated significantly larger IAPP deposits (Maloy *et al.* 1981; Narita *et al.* 1992). Furthermore, transgenic overexpression of IAPP has been shown to promote β cell apoptosis in a rat model of Type 2 diabetes (Butler *et al.* 2004; Matveyenko and Butler 2006; Matveyenko and Butler 2006).

The pre-diabetic state and the initial stages of Type 2 diabetes can be effectively treated with diet and exercise. Diminished nutrient intake reduces the demand for insulin placed on the β cells (Tuomilehto *et al.* 2001; Larson-Meyer *et al.* 2006). Additionally, exercise increases insulin sensitivity in peripheral tissues, further reducing hyperinsulinemia (Goodpaster *et al.* 1999).

For patients with more severe Type 2 diabetes, diet and exercise are insufficient to maintain normoglycemia. The pharmacological treatment of Type 2 diabetes uses a multifaceted approach, with classes of drugs that attempt to improve insulin sensitivity and/or potentiate insulin secretion. Metformin primarily targets the liver to impair gluconeogenesis and reduce hepatic glucose production. Recent evidence shows that metformin acts to activate adenosine 5’ monophosphate-activated protein kinase (AMPK), a regulator of cellular energy homeostasis, by inhibiting the serine/threonine

kinase LKB1 from phosphorylating AMPK. Activated AMPK acts peripherally in muscle to increase glucose uptake (Kola *et al.* 2006). One concern of prolonged metformin usage, however, is that it can reduce glucose-stimulated insulin secretion (GSIS) and may promote β cell apoptosis (Leclerc *et al.* 2004; Raile *et al.* 2005). A relatively new class of drugs, the thiazolidinedione derivatives (TZDs), also acts peripherally to improve the insulin sensitivity of target tissues. TZDs bind to peroxisome proliferator-activated γ receptors in the nucleus and enhance the transcription of a set of genes involved in regulation of glucose and lipid metabolism (Saltiel and Olefsky 1996; Koeffler 2003).

Two other classes of drugs, the sulfonylureas and the meglitinides, act principally on the β cells themselves. These drugs potentiate insulin secretion by binding to the regulatory subunit of the ATP-sensitive K^+ channel on the plasma membrane and inducing closure of the channel. The subsequent Ca^{2+} influx induces insulin secretion (Ribalet *et al.* 1996). Unfortunately, long-term use of these drugs results in diminished therapeutic value, as they lose their ability to potentiate insulin secretion and exhibit the unwanted side effect of weight gain (Kabadi and Kabadi 2003).

Pro-inflammatory Cytokine Involvement in Type 1 Diabetes

In Type 1 diabetes, pancreatic β cells are targeted by T lymphocytes and macrophages in an inflammatory reaction, insulitis, that results in the concomitant release of cytokines such as interleukin-1β (IL-1β) and interferon-γ (IFN-γ). The combined attack by T lymphocytes, macrophages, and cytokines results in the destruction of the vast majority of β cells (Itoh *et al.* 1993). In most affected individuals, loss of β cell mass

is not instantaneous, but is rather a progressive process that can take months to years. During the pre-clinical phase, aberrant islet function is marked by elevated proinsulin levels and the loss of first-phase insulin secretion in response to glucose (Srikanta *et al.* 1985; Roder *et al.* 1994).

The loss of first-phase insulin secretion appears to be attributable to multiple factors resulting from IL-1β signaling in the β cell. Many of the cytotoxic effects of IL-1β result from its stimulation of inducible nitric oxide synthase (iNOS) to produce the free radical nitric oxide (NO) (Eizirik *et al.* 1992; McDaniel *et al.* 1996; Tabatabaie *et al.* 2001). Aconitase, an enzyme in the tricarboxylic acid (TCA) cycle that catalyses the isomerization of citrate to isocitrate, is strongly inhibited by NO. NO inhibition of aconitase impairs TCA cycle flux, thereby significantly reducing adenosine triphosphate (ATP) levels (Eizirik *et al.* 1996). In the β cell, high ATP levels, generated by glucose oxidation through the TCA cycle, are critical to induce closure of the ATP-sensitive K^+ channels and stimulate plasma membrane depolarization, Ca^{2+} influx, and the eventual secretion of insulin from secretory granules (Misler *et al.* 1992). In addition to reducing glucose oxidation, and thereby insulin secretion, IL-1β has recently been shown to block the docking and fusion of insulin secretory granules to the β cell plasma membrane (Ohara-Imaizumi *et al.* 2004), thereby inhibiting insulin by both direct (secretory granule docking) and indirect (glucose oxidation) mechanisms.

Exposure of islets to IL-1β and IFN-γ not only impairs glucose-stimulated insulin secretion (GSIS) but also promotes β cell death. Early mechanistic studies investigating cytokine-induced β cell death pointed to a purely apoptotic mechanism driven by IL-1β-stimulated NO production (Delaney *et al.* 1993; Ankarcrona *et al.* 1994; Kaneto *et al.*

1995; Kavdia *et al.* 2000; Eizirik and Mandrup-Poulsen 2001; Oyadomari *et al.* 2001). In addition to the impairments on GSIS previously mentioned, NO has been shown to promote extensive DNA damage (Delaney, Green *et al.* 1993). Recent work from our lab and others, however, has demonstrated that the mechanism of β cell destruction by IL-1β and IFN-γ is a largely non-apoptotic one, as key caspases necessary to promote apoptosis are not activated by this combination of cytokines (Collier *et al.* 2006; Steer *et al.* 2006). In these studies, INS-1_{res} cell lines (to be discussed in detail in the next section) that have been selected for their resistance to IL-1β and IFN-γ cytotoxicity, partially through blockade of iNOS, were just as susceptible to apoptosis promoted by chemical agents such as camptothecin and etoposide as were cytokine sensitive cell lines. Also, overexpression of a constitutively active form of Protein Kinase B (Akt1) that has been shown to inhibit apoptosis (Fujio and Walsh 1999) had no protective effect against cytokine-mediated β cell killing. Furthermore, apoptosis promoted by camptothecin increases cellular ATP levels to drive the apoptotic machinery (Nicotera and Melino 2004). In contrast, cellular ATP levels in both β cell lines and isolated rat islets drop upon IL-1β and IFN-γ exposure, further discrediting the hypothesis that cytokines induce β cell death by apoptosis (Collier, Fueger *et al.* 2006).

In addition to being key players in the etiology of Type 1 diabetes, IL-1β and IFN-γ appear to play an important role in the post-transplantation destruction of β cell mass (Johansson *et al.* 2005; Yang *et al.* 2005; Amoli and Larijani 2006). Within a few days of transplantation, as much as 60% of the total transplanted islet mass can be destroyed (Amoli and Larijani 2006), further emphasizing the need for cytoprotection against cytokine-mediated β cell death.

INS-1_{res} – A Model for β Cell Survival

The parental INS-1 cell line was created from an x-ray induced rat insulinoma and consisted of a heterogeneous mixture of insulin-producing cells (Asfari *et al.* 1992). The human proinsulin gene was stably transfected into the parental INS-1 cell line and highly glucose responsive clones were selected in order to derive a model system that more closely mimicked β cells (Hohmeier *et al.* 2000). Our group has previously developed a subset of the highly glucose responsive INS-1 cell lines that are resistant to the cytotoxic effects of IL-1β and IFN-γ, termed INS-1_{res} (Chen *et al.* 2000). Development of the INS-1_{res} cell lines involved the iterative culture of highly glucose-responsive, cytokine sensitive INS-1 cells in increasing doses of IL-1β and IFN-γ over an eight week period. For each passage, the surviving cells were used to seed the subsequent generation that would be grown in a higher dose of cytokines. At the end of this selection procedure, INS-1_{res} cells were developed that were 78 ± 1.2% viable after five days of continuous culture in media with high dose cytokines (IL-1β at 10 ng/mL and IFN-γ at 100 U/mL). For the same five day treatment, unselected INS-1 cells were only 0.3 ± 0.03% viable. In addition to IL-1β and IFN-γ resistance, INS-1_{res} cells demonstrated tolerance to treatment with media harvested from activated peripheral blood mononuclear cells (PBMCs). Activated PBMCs secrete cytokines other than IL-1β and IFN-γ as well as toxic ROS and NOS species.

The mechanism of cytokine resistance in the INS-1_{res} cell lines appears to be partially mediated by the impaired activation of iNOS, resulting in complete blockade of NO production upon cytokine or PBMC stimulation (Chen, Hohmeier *et al.* 2000). By analyzing the IL-1β signaling pathway, failure of NF-κB nuclear translocation emerged

as a possible mechanism that could explain the lack of iNOS activation. Electrophoretic mobility shift assay (EMSA) of INS-1 and INS-1_{res} nuclear extracts demonstrated diminished protein binding to a consensus NF-κB binding site. However, this assay is not able to distinguish between impaired DNA binding, impaired nuclear translocation, or decreased NF-κB concentrations in the nucleus that could result from increased NF-κB shuttling due to hyperactive histone deacetylase activity. All of these mechanisms could result in a decreased signal in the EMSA. Regardless of the mechanism, IL-1β resistance proved to be permanent in the INS-1_{res} cell lines and did not require the continual presence of cytokines in the culture media.

When we first described the INS-1_{res} phenotype, the resistance to IFN-γ was transient and withdrawal of cytokines from the culture media could re-instate a partial sensitivity to acute IL-1β + IFN-γ treatment. The resistance mechanism for IFN-γ appeared to be tied to the expression of the transcription factor signal transducer and activator of transcription 1α (STAT1α). Continuous culture in IL-1β and IFN-γ maintained high endogenous expression of STAT1α and a phenotype that was fully resistant to cytokines**.** Genetic manipulation of STAT1α, using a recombinant adenovirus to overexpress the rat STAT1α gene, was able to induce partial resistance in the cytokine sensitive INS-1 cell line 834/40. Viability in response to IL-1β + IFN-γ exposure after STAT1α overexpression increased from 10 ± 2% to 50 ± 6% (Chen *et al.* 2001).

Since publication of the original INS-1_{res} work, a significant change in the resistant phenotype has been observed. The INS-1_{res} cell lines are now fully resistant to acute IFN-γ and IL-1β + IFN-γ treatments, even when cultured in the absence of cytokines for up to 6 months. Furthermore, STAT1α expression no longer correlates with

cytokine resistance in INS-1_{res} cell lines, and overexpression of STAT1α in the cytokine sensitive cell lines does not induce any significant change in viability. These findings coincided with our move from UT Southwestern Medical Center in Dallas, TX to Duke University in Durham, NC in 2002. Before the move, all of the INS-1 and INS-1_{res} cell lines underwent a rigorous protocol to remove latent mycoplasma infections. We now believe that the original selection protocol was successful in selecting cells permanently resistant to IFN-γ as well as IL-1β. The transient resistance previously reported, as well as the protection afforded by STAT1α overexpression, could have resulted from the latent mycoplasma infections observed in the cell lines. Unfortunately, there is no way to test this hypothesis without re-infecting the INS-1 and INS-1_{res} cell lines with mycoplasma.

As discussed earlier, in addition to their cytotoxic effects on β cells and INS-1 cell lines, cytokines also completely inhibit the process of GSIS (Mandrup-Poulsen 1996). INS-1_{res} cell lines, however, remained completely glucose responsive when treated with IL-1β alone and treatment with IFN-γ or IL-1β + IFN-γ resulted in only marginal decreases of 20 and 30%, respectively, in GSIS (Chen 2001).

The INS-1_{res} cell lines provide us with a unique model system in which to study the genes and factors that promote cytokine resistance, without interfering with the primary function of the β cell – secreting insulin in response to changes in extracellular glucose.

Gastrin

The gastrin peptide was first identified for its ability to promote acid secretion from gastric mucosal tissue (Edkins 1905). Gastrin is synthesized and secreted by G-cells in the antrum of the stomach, where it acts to regulate acid secretion by upregulating

histamine production (Kahlson *et al.* 1964). In adults, gastrin secretion is tightly regulated by both the enteric nervous system (Jafee *et al.* 1970; Lanciault *et al.* 1971) and post-prandial lumen stimuli (Dockray *et al.* 1975). In addition to promoting acid secretion, gastrin is critical to the proliferation, differentiation, and maturation of stomach and intestinal mucosa (Koh *et al.* 1997; Friis-Hansen *et al.* 1998). In these tissues, abnormal or excessive gastrin production can result in carcinogenesis and tumor formation (Johnson 1988; Thorburn *et al.* 1998).

Gastrin knockout mice are viable, fertile, and exhibit few extragastric effects (Friis-Hansen, Sundler *et al.* 1998; Lacourse *et al.* 1999). In these mice, basal gastric acid secretion was undetectable and could not be induced chemically. Histologically, there were no differences in these mice compared to wild-type mice except in the stomach mucosa, where enterochromaffin-like (ECL) and parietal cells were both diminished in number. Exogenous gastrin administration for six days reversed these deficiencies, inducing significant proliferation of ECL and parietal cells and a partial restoration of gastric acid secretion. Gastrin knockout mice had no discernible defects in pancreatic exocrine or endocrine histology; however the authors did not undertake detailed morphological measurements of the islets.

The human gastrin gene consists of three exons, the first of which is non-coding. Exons 2 and 3 encode for a 101 amino acid peptide (104 in the rat), preprogastrin, that contains an N-terminal endoplasmic reticulum (ER) translocation signal sequence (Ito *et al.* 1984). Once translocated into the ER, the 21 amino acid signal sequence is cleaved by signal peptidase and progastrin is shuttled to the Golgi network (see Figure 1-1), where it is sulfated at Tyr^{87} and phosphorylated at Ser^{96}, the latter an important event for

N-terminal amidation in the final steps of prohormone processing (Huttner 1988; Varro *et al.* 1990). The transport of progastrin intermediates through the Golgi network appears to be by a bulk flow mechanism, and not through vesicle-directed transport (Rothman and Orci 1992).

After exiting the trans-Golgi network, progastrin is concentrated in secretory granules, where prohormone convertases (PC2 and PC1/3) cleave the peptide at dibasic residues (Lys-Arg, Arg-Arg, Lys-Lys, or Arg-Lys) into the final processed peptides of varying lengths (Steiner *et al.* 1992). Cleavage at $Arg^{57}Arg^{58}$ and $Arg^{94}Arg^{95}$ results in the formation of Gastrin-34 (G34), whereas cleavage at $Lys^{74}Lys^{75}$ and $Arg^{94}Arg^{95}$ results in the formation of Gastrin-17 (G17) (Varro *et al.* 1995). The residual amino-terminal glutamine spontaneously converts to a pyrole ring in the presence of phosphate ions. In gastrin, this pyroglutamyl group has no influence in receptor binding but it does increase peptide stability to extend plasma half-life (Takahashi and Cohen 1969). The removal of $Arg^{94}Arg^{95}$ by carboxypeptidase E after processing by PC2 and PC1/3 results in the formation of Gly^{93}-extended gastrins (G-Gly).

In the final step of processing, glycine extended gastrins act as substrates for peptidyl-glycine α-amidating monooxygenase (PAM) (Varro, Nemeth *et al.* 1990). After processing, a mixture of peptides is present in the secretory vesicle including G17, G17-Gly, G34, G34-Gly, and sulfated variants of all four of these peptides. Gastrin processing, as well as the subcellular localizations for each processing event, are summarized in Figure 1-1.

Sulfated G17, and to a much lesser degree G34, binds to the heptahelical cholecystokinin 2 receptor (CCK2R) (Kopin *et al.* 1992). CCK2R binding can stimulate

up to four principle signaling pathways (Figure 1-2); the inositol triphosphate (IP_3) / protein kinase C (PKC) pathway, the PI3K/Akt/mTOR pathway, ERK/p38MAPK pathway, and the JAK2/STAT3 pathway. A summary of each of these pathways with its relevance to gastrin signaling will be presented here.

CCK2R activation stimulates phospholipase C β (PLC-β) through heterotrimeric G proteins of the $G\alpha_q$ family (Piiper *et al.* 1997; Yule *et al.* 1999). PLC-β cleaves phosphatidylinositol (PIP_2) in the plasma membrane to form IP_3 and diacyl glycerol (DAG). IP_3 serves to mobilize Ca^{2+} from intracellular stores and activate PKC and facilitate its anchoring to the plasma membrane through DAG (Sethi and Rozengurt 1992). PKC acts as a signaling hub, facilitating crosstalk between several signaling pathways by phosphorylating a host of different proteins, including but not limited to mitogen activated protein kinase (MAPK), NF-κB, epidermal growth factor receptor (EGFR), and protein kinases of the D subclass (PKD/PKD2), all of which have been shown to be activated by stimulation of CCK2R (Daulhac *et al.* 1997; Miyazaki *et al.* 1999; Chiu and Rozengurt 2001; Hiraoka *et al.* 2001; Sturany *et al.* 2002). In regards to gastrin signaling, the IP_3/PKC pathway appears to be important in facilitating the synthesis and secretion of histamine in enterochromaffin-like (ECL) cells in the stomach (Hocker 2004).

In addition to activation of the IP_3/PKC pathway, gastrin binding to CCK2R can activate the phosphatidylinositol 3-kinase (PI3K) signaling cascade. Activation of PI3K signaling appears to promote proliferation and protect against apoptosis (Kowalski-Chauvel *et al.* 1997; Daulhac *et al.* 1999; Ferrand *et al.* 2004). The cascade of phosphorylation events, briefly, includes phosphorylation of Protein Kinase B (Akt),

mammalian target of rapamycin (mTOR), p70S6kinase, ribosomal protein S6, and the initiation factor eIF4E, the latter two being components of the translational machinery.

CCK2R also appears to be able to signal through the janus kinase 2 (JAK2)/STAT3 pathway. JAK2 activation, like $G\alpha_q$ signaling, proceeds via the NPXXY motif in the seventh transmembrane domain of CCK2R. This motif appears to play a bifunctional role, as it activates both $G\alpha_q$ for IP_3/PKC and JAK2 for STAT3 signaling. Similar to PI3K signaling, STAT3 activation promotes many of the CCK2R-mediated growth effects seen in mucosal and tumor cell lines (Ferrand, Kowalski-Chauvel *et al.* 2004; Ferrand *et al.* 2005; Beales and Ogunwobi 2006; Ferrand *et al.* 2006). CCK2R signaling is summarized in Figure 1-2 (as well as CCK1R, to be covered in the next section).

Cholecystokinin

Cholecystokinin (CCK), like gastrin, is a peptide hormone involved in gut physiology. Unlike gastrin, CCK appears to have a significant role as one of the most prevalent neurotransmitters in the central nervous system (Crawley 1985). The CCK gene is divided into 3 exons, like gastrin, and also encodes a preprohormone, similar to the gastrin gene. At 115 amino acids, preproCCK is slightly longer than preprogastrin, but the post-translational processing of the protein is almost identical to preprogastrin (see Figure 1-1 for gastrin processing). The notable exception in preproCCK processing is that there are more di- and monobasic sites for cleavage by prohormone convertases, leading to more bioactive final processed forms (CCK-8, CCK-22, CCK-33, and CCK-58) (Rehfeld 1978; Rehfeld and Hansen 1986). PreproCCK processing is cell-specific,

with endocrine cells producing a mixture of mid-length CCKs (CCK-22, CCK-23, and CCK-58) and neurons producing almost exclusively CCK-8.

The "classical" functions of CCK include gallbladder contraction and pancreatic enzyme secretion from the pancreas into the duodenum of the small intestine (Ivy and Oldberg 1928; Harper and Raper 1943; Jorpes and Mutt 1966). The neurological function of CCK in the central nervous system appears to be linked to satiety, nociception, anxiety, and depression (Cesselin 1995; van Megen *et al.* 1996; Shlik *et al.* 1997; Wiesenfeld-Hallin *et al.* 1999).

PreproCCK knockout mice, like preprogastrin knockout mice, are viable, fertile, and exhibit no gross physiological changes or significant differences in pancreatic weight or morphology when compared to wild type animals (Lacourse, Swanberg *et al.* 1999). These finding are somewhat peculiar, because exogenous CCK stimulates the growth of the exocrine pancreas in both neonate and adult rodents (Werlin *et al.* 1988; Zucker *et al.* 1989; Wisner *et al.* 1990). These results indicate that CCK is involved in pancreatic hyperplasia secondary to environmental stimuli and is not required for the normal growth and development of the pancreas.

CCK peptides can bind to both the CCK1 and CCK2 receptors. Sulfated isoforms of CCK-8, CCK-22, and CCK-33 bind to CCK1R at a 500 fold higher affinity than their non-sulfated cohorts, and gastrin has virtually no affinity for this receptor (Huang *et al.* 1989). By contrast, sulfated gastrin and CCK appear to have equal affinity for CCK2R, with non-sulfated analogs of the two peptides demonstrating 3-10 fold lower binding affinity. Due to the abundance of sulfated CCK-8 in the central nervous system, this isoform is presumed to be the dominant ligand for neuronal CCK2R activation. Outside

of the central nervous system, sulfated G17 levels are 5-10 fold higher than CCK-8 and G17 is presumed to be the dominant ligand. Studies undertaken to localize the specific receptor subtypes within the pancreas have yielded significantly different results, with the most controversy concerning CCK2R localization. Different studies have reported that the CCK2R is expressed over the entire human pancreas (Tang *et al.* 1996), co-localizes with only glucagon cells (Saillan-Barreau *et al.* 1999; Rooman *et al.* 2001), or co-localizes with only somatostatin producing cells (Morisset *et al.* 2000). These perplexing results have been clarified more recently via a study that tested the specificity of all the antibodies used in the aforementioned co-localization studies, resulting in the conclusion that CCK1R expression is restricted to glucagon (α) and insulin (β) positive cells, whereas CCK2R expression is limited to the somatostatin-secreting δ cells of the islet (Morisset *et al.* 2003).

CCK1R signaling modalities are similar to those for CCK2R, with IP_3/PKC activation of NF-κB being the predominant pathway (Gukovsky *et al.* 1998). The known signaling modalities of CCK1R and CCK2R are summarized in Figure 1-2.

CCK1 and CCK2 receptor knockout mice, like gastrin and CCK knockout mice, are viable, fertile, and exhibit no gross or histological abnormalities outside of the gastrointestinal tract (Nagata *et al.* 1996; Kopin *et al.* 1999). CCK2R knockout mice show similar problems with gastric mucosal growth and acid secretion as gastrin knockout animals, but there are no overt neurological deficits in the animals, which is a surprising finding considering the abundant expression of CCK2 receptors in the cerebral cortex of WT animals.

By comparison, CCK1R knockout mice displayed an interesting feeding phenotype. Intraperitoneal injection of CCK failed to decrease short-term food intake in mice lacking CCK1 receptors but could reduce food intake in CCK2R knockout or WT animals by 90% (Kopin, Mathes *et al.* 1999). Long-term measurements of body weight in the three genotypes, however, showed no difference in the CCK1R knockout animals, demonstrating that while CCK signaling through CCK1R can modulate immediate food intake it, does not regulate long-term weight gain.

Gastrin and Cholecystokinin in the β Cell

Mature β cells do not express gastrin. However, during development, gastrin is robustly expressed in fetal islets at a time when they are undergoing maximal growth (Brand and Fuller 1988). Although gastrin expression in fetal islets was first observed more than twenty years ago, its function there is still undefined. Several studies have investigated gastrin for the induction of islet growth, despite the absence of gastrin expression in mature islets. The first reports of β cell mass expansion secondary to gastrin exposure came from patients with Zollinger-Ellison Syndrome, a disease in which one or more tumors in the pancreas or duodenum secrete supraphysiologic doses of gastrin (Zollinger and Ellison 1955; Larsson *et al.* 1973; Creutzfeldt *et al.* 1975; Meier *et al.* 2006).

In one study, exogenous gastrin enhanced β cell regeneration from pancreatic ductal ligation (Rooman *et al.* 2002), a commonly used surgical manipulation that promotes pancreatic regeneration (Hultquist *et al.* 1979). Interestingly, in the same study, gastrin administration to non-ligated control pancreata was unable to induce any

measurable proliferation, indicating that ligation induces a physiological change that is necessary to allow gastrin to potentiate islet growth. Furthermore, work done in transgenic mice overexpressing transforming growth factor α (TGFα), an EGFR ligand, and/or gastrin under the control of the insulin promoter (INS-Gast) showed an approximate doubling of β cell mass in the combined TGFα/INS-Gast transgenics (Wang *et al.* 1993). Like gastrin knockout mice, the INS-Gast transgenic mice have normal pancreatic ductal tissue and islet mass. TGFα transgenic mice show increased numbers of insulin-expressing pancreatic ductal cells but normal islet mass. The importance of this finding is further emphasized by work showing that EGF receptor activation can modulate gastrin expression through an EGF-responsive element in the gastrin promoter (Godley and Brand 1989; Ford *et al.* 1997; Chupreta *et al.* 2000). The impressive islet growth seen in the TGFα/INS-Gast double transgenics, but not the INS-Gast mice alone, reiterates the idea that a "permissive" state is required for gastrin-mediated increases in β cell mass.

In alloxan- or streptozotocin-treated diabetic rats, both model systems for Type 1 diabetes, combination therapy with G17 and EGF can increase β cell mass and reduce hyperglycemia (Brand *et al.* 2002; Rooman and Bouwens 2004). These results have been replicated in non-obese diabetic (NOD) mice, another model for Type 1 diabetes in which the β cells are destroyed by the immune system at an early age (Kano *et al.* 1986; Shimada *et al.* 1996; Suarez-Pinzon *et al.* 2005). Furthermore, two infusion studies using G17 and EGF have duplicated this work *in vivo*, demonstrating that G17 plus EGF can stimulate islet regeneration from pancreatic ductal cells (Rooman and Bouwens 2004; Suarez-Pinzon, Yan *et al.* 2005). These studies are significant not only because they

showed a substantial increase in β cell mass, but more importantly they also demonstrated that the newly-produced islets function properly and are able to restore normoglycemia.

In all these studies, neither G17 nor EGF alone were sufficient to increase β cell mass. Only combination therapy of G17 plus EGF could induce significant increases in β cell mass. Furthermore, it is important to note that in all of these experiments, β cell proliferation required pre-existing damage of the islets, either chemically (alloxan or streptozotocin) or through immune-mediated destruction (NOD); again indicating the necessity of a significant shift in islet homeostasis before islet growth is possible through a gastrin-mediated mechanism.

Unlike gastrin, CCK expression in the fetal pancreas is questionable and has not been thoroughly investigated. To date, only one study has reported CCK expression in fetal islets, demonstrating co-localization with glucagon-producing α cells (Liu *et al.* 2001). CCK is robustly expressed in adult pancreatic tissue (Brand and Fuller 1988; Rivard *et al.* 1991; Yoshinaga *et al.* 1996). Mature, processed CCK is released from pancreatic acinar cells in response to the presence of nutrients in the lumen of the duodenum. Its involvement in gallbladder contraction, induction of satiety, and regulation of pancreatic enzyme secretion are well documented. Repeated exposure to CCK-8 has been shown to stimulate increases in pancreas size and weight (Pfeiffer *et al.* 1982), as well as insulin secretion from β cells via stimulation of CCK1R and activation of PLC-β (Ahren and Lundquist 1981; Fridolf *et al.* 1992). Recently, one group has investigated the potential of recombinant CCK-8 to stimulate islet β cell proliferation (Kuntz *et al.* 2004). Administration of 4 μg/kg CCK-8 to streptozotocin-induced Type 1 diabetic rats was able to induce noticeable BrdU incorporation into insulin-positive cells.

The same treatment for control and nicotinamide-induced Type 2 diabetic rats showed no increase over vehicle-controls. This result, like that seen with G17 or G17 plus EGF administration, also seems to indicate that β cell damage could be a pre-requisite for islet proliferation.

The Cell Cycle in the β Cell

Despite evidence indicating that β cell turnover can be promoted in islets by physiological stimuli such as pregnancy and obesity (Swenne 1985), research on the replication of mature β cells has been eclipsed in recent years by the emphasis on stem cell to β cell maturation (Brolen *et al.* 2005; Yang 2006) and ductal to β cell neogenesis (Bonner-Weir *et al.* 1993; Rosenberg 1995; Wang, Kloppel *et al.* 1995). While the evidence for the formation of β cells from ductal cell precursors is extensive, and the efforts to differentiate stem cells into fully functional β cells is making significant headway, the potential for increasing β cell mass from already existing populations of β cells represents a large opportunity that should not be ignored. To pursue this goal, it is critical to understand the underlying cell cycle control mechanisms of the β cell so that targeted strategies can be developed to expand the available β cell mass for therapeutic application; specifically, islet transplantation for the treatment of Type 1 diabetes and expansion of existing β cell mass for the treatment of Type 2 diabetes.

Current knowledge of the molecular mechanisms that control β cell replication are poorly understood and lag those of other fields (Cozar-Castellano *et al.* 2006). As with many other cell types, the retinoblastoma protein (pRb) appears to be one of the genes critical to cell cycle progression in the β cell. pRb, and the homologs p107 and p130,

preferentially bind to and inactivate the E2F family of transcription factors, the proteins principally involved in mediating the progression through growth phase 1 (G_1) into the DNA synthesis phase (S) of the cell cycle (Cam and Dynlacht 2003; Chau and Wang 2003; Munger 2003). pRb knockouts are early embryonic lethal, however knockouts of two E2F isoforms are viable and do not exhibit generalized whole-body disruption of cell cycle control, but have phenotypes limited to the pancreas. E2F1 knockouts have small islets with reduced β cell mass (Fajas *et al.* 2004), while E2F1 + E2F2 double knockouts show complete ablation of the pancreas (Iglesias *et al.* 2004). These findings underscore the importance of these E2F isoforms in β cell cycle control.

pRb, p107, and p130 are regulated very tightly at the post-transcriptional level, with at least 16 different phosphorylation sites (Hernando *et al.* 2004). During normal cell cycle progression phosphorylation of pRb dissociates it from E2F proteins, which are locked on to various cell cycle promoter elements. With pRb inhibition now removed, E2F proteins initiate transcription and progression out of the quiescent state (G_0) in which β cells normally exist. There are a large number of cell cycle proteins that phosphorylate pRb to influence changes in the cell cycle. Among these, cyclin dependent kinase 4 (cdk4) which partners with the D-type cyclins appears to be specifically involved in the β cell cycle. Whole animal knockout of cdk4, surprisingly, causes a very specific β cell phenotype in which islets are barely detectable and the animals have severe hyperglycemia (Rane *et al.* 1999). Conversely, knock-in of constitutively active cdk4 showed impressive islet hyperplasia (Hino *et al.* 2004). Similarly, adenoviral overexpression of cyclin D1, alone or in combination with cdk4, induces significant β cell replication (Cozar-Castellano *et al.* 2004). Surprisingly, a β cell specific knockout of

pRb has no overt phenotype, indicating either lack of involvement of pRb in the β cell cycle or compensation by p107 and/or p130 (Vasavada *et al.* 2007).

In addition to the D cyclins, the E cyclins, which pair with cyclin dependent kinase 2 (cdk2), are also able to phosphorylate pRb and act as key regulators of progression through the G_1 phase of the cell cycle (Hwang and Clurman 2005). Genetic manipulation studies of these two proteins in the β cell have not yet been performed. Furthermore, there are dozens of other proteins such as p53, cyclin inhibitory proteins (CIPs), and kinase inhibitory proteins (KIPs), to name a few, that actively modulate the activity of many of the aforementioned proteins. Studies to define the function of these proteins in the β cell are just beginning.

To date, many of the manipulations shown to increase β cell mass cause significant impairment of the primary function of the β cell, secretion of insulin in response to glucose. These manipulations include transfection of SV40 large T antigen, ras, c-myc, or v-src into primary β cells (Hanahan 1985; Welsh *et al.* 1988). More recently it has been demonstrated that it is possible to convert immortalized non-insulin secreting pancreatic cell lines into functional β cells using virally transduced PDX-1, a β cell specific homeobox transcription factor (de la Tour *et al.* 2001). Upregulation of PDX-1 in these cell lines promotes a more β cell-like phenotype that can secrete insulin in response to a glucose challenge. Using a slightly different approach, another group demonstrated that immortalized hepatic FH-hTERT cells could be transdifferentiated into insulin-producing cells via overexpression of PDX-1 (Zalzman *et al.* 2003). However, these approaches currently have limited therapeutic value because of the tumorigenic concerns associated with transplantation of tissue created from transformed cell lines.

Promising work on human islets, not mediated by the overexpression of protooncogenes, has recently showed that it is possible to induce β cell expansion by applying a cocktail of growth factors to islets which have been suspended in a 3-dimensional fibrin growth matrix (Beattie *et al.* 2002). While this work holds significant promise for β cell mass expansion, the rate of de-differentiation of cultured islets and the loss of the β cell phenotype currently prevents its widespread application.

Statement of the Research Problem and Goals of the Dissertation

Both Type 1 and 2 diabetes result in the loss of β cell mass and function. Therefore, discovery of any genes or pathways that can promote β cell proliferation and survival will be directly applicable to the development of new therapeutic approaches for the treatment of these diseases. Microarray profiling of the INS-1_{res} cell lines compared to cytokine sensitive lines was used as an unbiased approach for the identification of genes involved in cytokine resistance. From this analysis, preprogastrin was identified as one of the most upregulated genes in the INS-1_{res} phenotype, relative to unselected cytokine sensitive INS-1 cells. This discovery led to a detailed analysis of the effects of preprogastrin and the related protein preprocholecystokinin in mature β cell biology. The results of these studies are summarized in the following three chapters of this dissertation.

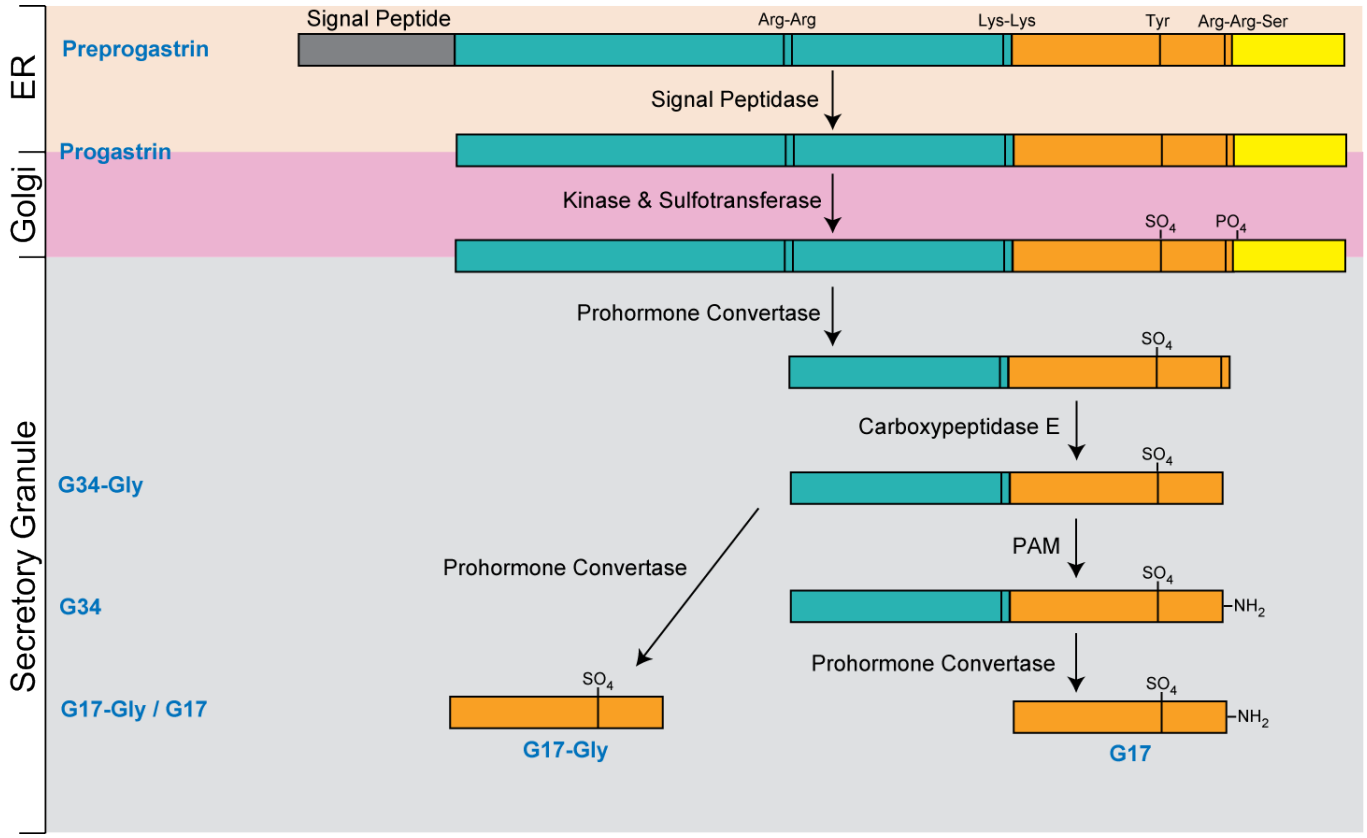


Figure 1-1. Post-translational processing of preprogastrin. The 21 amino acid signal peptide directs full length preprogastrin into the endoplasmic reticulum (ER). The resulting product, progastrin, is shuttled to the Golgi network where Tyr^{87} is variably sulfated and Ser^{96} is phosphorylated. Prohormone convertases cleave off N- and C-terminal flanking peptides at dibasic residues followed by carboxypeptidase E removal of the $Arg^{94}Arg^{95}$ N-terminal dibasic residues to form Gastrin-34-Gly (G34-Gly). G34-Gly can be optionally amidated by peptidyl-glycine α-amidating monooxygenase (PAM) to form G34. Both G34 and G34-Gly can undergo an additional cleavage at an internal dibasic site to form G17 and G17-Gly, respectively.

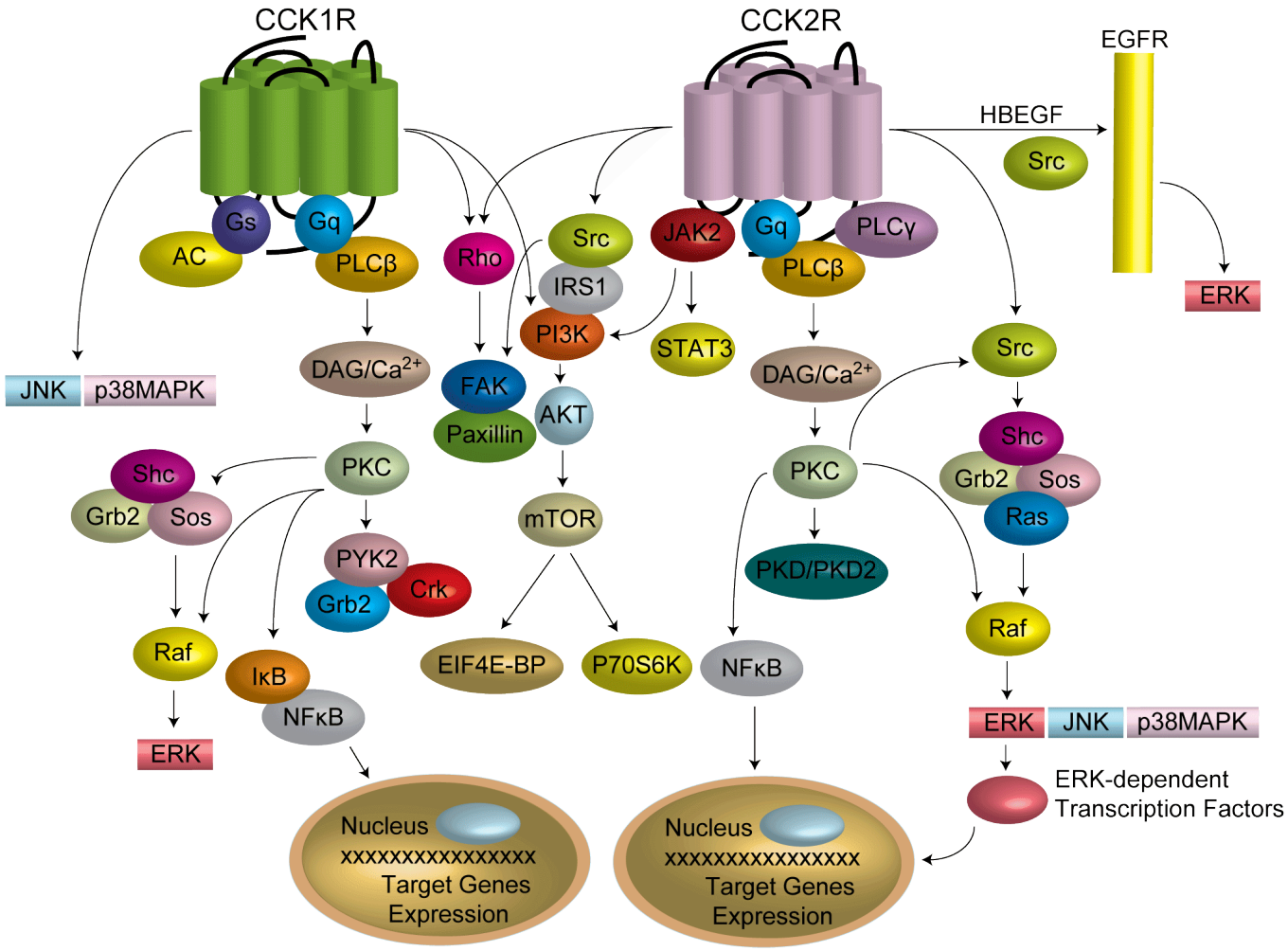


Figure 1-2. Summary of the known signaling modalities of CCK1R and CCK2R. CCK1R and CCK2R are the classical receptors for cholecystokinin and gastrin peptides. Gastrin-17 and Gastrin-17S preferentially bind to CCK2R (1000 times more potently than to CCK1R). CCK-8 shows equal affinity for CCK1R and CCK2R.

CHAPTER TWO

DIFFERENTIAL GENE EXPRESSION IN THE INS-1_{RES} PHENOTYPE

Introduction

Type 1 diabetes is a direct consequence of autoimmune destruction of the β cells of the islets of Langerhans within the pancreas. Initiation of the disease is characterized histologically by macrophage and T lymphocyte infiltration of the islets (Foulis, McGill *et al.* 1991; Itoh, Hanafusa *et al.* 1993), followed by progressive β cell destruction, which results in the inability to produce and secrete insulin in response to glucose. The secretion of pro-inflammatory cytokines from the infiltrating immune components is a key step in this destructive process (Rabinovitch and Suarez-Pinzon 1998).

Over the past five years, islet transplantation has emerged as a viable alternative to insulin injection therapy for the treatment of Type 1 diabetes (Shapiro, Lakey *et al.* 2000; Froud, Ricordi *et al.* 2005). However, broad-based application of this technique has been slowed by the limited supply of viable cadaveric pancreata as well as transplantation protocols that fail to fully protect the transplanted islets from immune attack (Hohmeier and Newgard 2005). Discoveries that can be used to enhance β cell survivability or promote β cell growth and thereby increase the supply of available insulin-producing cells would be a significant advancement.

Toward these goals, our lab has previously developed subsets of the parental rat insulinoma cell line (INS-1) that are resistant to the cytotoxic effects of the pro-inflammatory cytokines interleukin 1β (IL-1β) and interferon-γ (IFN-γ), the two cytokines primarily responsible for β cell destruction (Chen, Hohmeier *et al.* 2000).

These INS-1_{res} cell lines form the cornerstone of the experiments presented in this chapter, providing us with cells that are both glucose responsive and cytokine resistant. Our previous characterizations of these cell lines employed a candidate gene approach and focused on evaluating various molecules in the receptor signaling pathways for the two cytokines IL-1β and IFN-γ (Chen, Hohmeier *et al.* 2000; Chen, Hohmeier *et al.* 2001; Tran *et al.* 2003). This strategy was effective in uncovering significant modifications in the IL-1β signaling mechanism that prevent the activation of iNOS and thereby block production of the free radical species nitric oxide (NO).

This chapter describes studies in which microarray technology was used to evaluate the differential gene expression patterns between the INS-1_{res} cell lines and the cytokine sensitive, unselected INS-1 cell lines. From these experiments, multiple candidate genes were identified, some of which have been shown previously to be implicated in the regulation of islet biology. The methods employed in this analysis and the statistical analysis of the microarray data are discussed in this chapter.

Materials and Methods

Cells and Reagents

832/13, 834/40, 833/15, and 833/117 clonal derivatives of the rat insulinoma cell line INS-1 (Chen, Hohmeier *et al.* 2000; Hohmeier, Mulder *et al.* 2000) were cultured in filter-sterilized RPMI-1640 (Sigma R8758) media containing 11.1 mM glucose, 10% fetal calf serum, 10 mM Hepes, 2 mM L-glutamine, 1 mM sodium pyruvate, 50 μM β-mercaptoethanol, 100 units/mL penicillin, and 100 μg/mL streptomycin (Sigma) at 37 °C and 5% CO_2. Recombinant rat IL-1β (Pierce Endogen) was used at 10 ng/mL and recombinant rat IFN-γ (Cell Sciences) was used at 100 U/mL in all experiments, unless otherwise noted.

MTT Viability Assay

INS-1 cells were trypsinized, counted, and seeded at a density of 50-100,000 cells/well in flat-bottom 96-well tissue culture plates (Corning) containing 100 μL of RPMI-1640. After 24 hours, 100 μL of additional media containing the treatment (e.g. cytokines) was added to each well. 48 hours later, the media was decanted, and the plates were gently blotted on paper towels. 115 μL/well of media containing 75 μg/mL of C,N diphenyl-*N*-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) was added, and the plates were incubated for 90 minutes at 37 °C and 5% CO_2. 115 μL of 0.04 N HCl in isopropanol was added to each well, and the optical density of the solubilized formazan was read at 575 nm and 650 nm using a SpectraMax 340 plate reader (Molecular Devices). Changes in optical density correlate to mitochondrial

viability, a measure of cell viability. Cells incubated in control media are considered 100% viable and all other treatment groups are normalized to these samples.

Isolation and Purification of Total RNA for Microarrays

INS-1 cells were grown to 90-95% confluence in 25 cm^2 filter flasks (Corning). Total RNA was isolated from cells by TRIzol (Gibco BRL, Grand Island, NY) according to the manufacturer's protocol. Before purification, 20 μg glycogen (Ambion) was added directly to the isolates to aid in visualization of the RNA pellet. RNA was purified by a procedure involving chloroform and ethanol extraction, passage of the extracted material through a Qiagen RNeasy mini-column, treatment with DNase, and elution of the column with RNase-free H_2O. The purified RNA samples were then quantified spectrophotometrically at 260 nm and 280 nm. Only samples with a 260/280 ratio of 1.6-2.0 and a minimum concentration of 10 μg were submitted for microarray analysis.

Expression Profiling using Microarrays

Gene expression profiling was performed on the two-state model of β cell cytokine resistance using the INS-1 model system. Two types of microarrays were used in these analyses. The first, the PancChip 5.0 (Beta Cell Biology Consortium), was used on total RNA isolated from three INS-1 clonal lines: 834/40 (cytokine sensitive), and 833/15 and 833/117 (cytokine resistant) (Chen, Hohmeier *et al.* 2000). The second microarray set, the Qiagen Rat Operon Set version 1.1, was used on four INS-1 clonal lines: 832/13 and 834/40 (cytokine sensitive), and 833/15 and 833/117 (cytokine resistant).

For each microarray experiment, INS-1 cells were cultured and harvested for total RNA as previously described. Replicate RNA samples (5 per cell line for the PancChip 5.0 array, and 3-4 for the Qiagen Rat Operon 1.1 array) were collected from cytokine sensitive and cytokine resistant INS-1 cell lines. For each RNA harvest, a parallel group of INS-1 cells were assayed for acute (18 hours) cytokine sensitivity using the MTT assay, to assure consistency of the two phenotypes.

PancChip 5.0 Microarray Expression Profiling (Hardy et al. 2006): Over 100 two-channel hybridization assays for the five independent paradigms were performed, collecting more than 10 million data points. All studies, including MIAME compliant (www.mged.org) detailed biomaterial and protocol annotation, raw and processed data, have been deposited into the RAD database (Manduchi *et al.* 2004) and are available for querying and downloading at http://www.cbil.upenn.edu/RAD. Further information on the PancChip array is available at http://www.cbil.upenn.edu/EPConDB/Chips/pancChip.shtml.

All arrays were scanned using an Agilent DNA Microarray Scanner Model #G256BA and the images were quantified using GenePix Pro, version 5 (http://www.axon.com/GN-GenePixSoftware.html). Scanning and quantification parameters used for each assay are available at the RAD website described above. The GenePix foreground mean intensities were used for each spot and each channel. No background subtraction was performed because these methods add to data variability and lead to the occurrence of negative values (Yang *et al.* 2002). For each assay performed, Cy3 anchors, blanks, yeast and dilution controls, and Stratagene PCR controls were

removed from the analyses (thus the total number of spots analyzed was 13,008 for each PancChip 5.0 experiment). For each assay, *M* values were computed for each spot considered, where the *M* value for two conditions C1 and C2 was defined as $\log_2$(C1)-$\log_2$(C2). The *M* values were normalized on each assay with the print-tip LOWESS approach (Yang, Dudoit *et al.* 2002) using the implementation provided by the R marrayNorm package (http://cran.r-project.org) from Bioconductor (http://www.bioconductor.org), with default parameter settings (R version 1.8.1, marrayNorm version 1.1.6). After normalization, the *M* values for each available pair of dye-swaps were combined ((M_1-M_2)/2). All spots were ranked according to lod scores (Lönnstedt and Speed 2002) and statistical significance (Statistical Analysis of Microarrays – SAM http://www-stat.stanford.edu/~tibs/SAM).

Qiagen Rat Operon 1.1 Microarray Expression Profiling: This study was performed using the Qiagen Rat Operon Version 1.1 Spotted Arrays (5,705 unique 70mers) on a GeneMachines OmniGrid 100 arrayer. The slides were printed on Corning UltraGaps (amino-silane coated). 10 μg total RNA from each sample was reverse-transcribed to cDNA and labeled with the Cy3 fluorophore (GE Healthcare, CyScribe First-Strand cDNA Labeling Kit). 10 μg rat reference RNA was reverse-transcribed according to the manufacturer's directions (Qiagen) by the Duke Microarray Core facility and labeled with the Cy5 fluorophore (GE Healthcare, CyScribe First-Strand cDNA Labeling Kit). Labeled probes were hybridized to the array overnight at 42 °C and then scanned using an Axon Genepix 4000B Scanner with Axon Genepix 4.0 software.

Raw Genepix data files consisting of Cy3 and Cy5 intensities were imported into GeneSpring 6.1.1 microarray analysis software. Genes for which a minimum signal of 100 (arbitrary optical value) were not detected in at least 2 of the 4 samples per INS-1 cell line were eliminated from the analysis. Statistical analysis included a LOWESS (per spot and per chip) normalization, removal of spots flagged as absent or marginal by the Axon Genepix 4000B Scanner, a 1-way ANOVA statistical test, and a fold-change calculation. Gene lists were generated with *p*-value cutoffs set ≤ 0.05 for each gene.

Statistical Methods

A one-way ANOVA was used in statistical analysis of the microarray data sets to calculate *p* values. Unequal variances were assumed in all calculations. $P \leq 0.05$ were considered significant.

Results

RNA expression profiling in INS-1_{res} clones

The development of cytokine resistant INS-1-derived cell lines (generally termed INS-1_{res} lines) has been previously described (Chen, Hohmeier *et al.* 2000; Chen, Hohmeier *et al.* 2001; Tran, Chen *et al.* 2003). The INS-1_{res} 833/15 and 833/117 cell lines exhibit a permanent resistance to the cytotoxic effects of the cytokines IL-1β and IFN-γ, even when cultured for months in their absence (Figure 2-1). By contrast, the cytokine sensitive 832/13 and 834/40 cell lines demonstrated a 41±2% and 45±4% decrease in viability after 1 day of IL-1β exposure. There was no statistically significant viability change with IFN-γ treatment, however, the combination treatment of IL-1β and IFN-γ resulted in a 67±1% and 74±2% drop in 823/13 and 834/40 cell viability, respectively. For all treatment conditions, there was no statistically significant decrease in 833/15 and 833/117 (INS-1_{res}) viability in any of the treatment groups. In an effort to gain insight into genes involved in conferring resistance to cytokine-induced cytotoxicity, we performed two independent sets of microarray studies comparing INS-1_{res} lines to unselected, cytokine sensitive INS-1 cells.

PancChip 5.0 microarray analysis

The first set of microarray experiments were performed on the PancChip 5.0, a spotted microarray containing 13,008 mouse cDNA sequences prepared from endocrine pancreas at various stages of development (Kaestner *et al.* 2003). This microarray analysis is one of several experiments on "two-state" models of β cell function, dysfunction, replication, and survival that is being conducted in our laboratory, with our

collaborators in the laboratory of Dr. Klaus Kaestner at the University of Pennsylvania School of Medicine as part of the Beta Cell Biology Consortium (Schisler *et al.* 2005; Hardy, Hohmeier *et al.* 2006).

In this two-state model analysis, cytokine sensitive INS-1 834/40 cells and INS-1_{res} 833/15 and 833/117 cells were grown in RPMI-1640 media lacking cytokines. Five serial passages of each of these cell lines were harvested for total mRNA isolation as described in Methods and Materials. Each mRNA sample was measured spectrophotometrically, and use for microarray analysis was only allowed if an optical density (OD) 260/280 ratio cutoff of 1.6 was met. Additionally, each sample was run on a 1% agarose gel to further assay for sample purity (Figure 2-2).

Parallel cultures for each of the five serial passages were treated with IL-1β (10 ng/mL), IFN-γ (100 U/mL), or IL-1β + IFN-γ (10 ng/mL and 100 U/mL, respectively) for 48 hours and subjected to MTT viability assays to verify that the cells were appropriately cytokine resistant or sensitive. As shown in Figure 2-3a, all five serial passages of 833/15 and 833/117 cells were completely resistant to the cytotoxic effects of IL-1β + IFN-γ, whereas viability of 834/40 cells was consistently decreased for all five serial passages by 63±2%, 31±6%, and 82±1% for IL-1β, IFN-γ, or IL-1β + IFN-γ respectively (Figure 2-3b). Data for both 833/15 and 833/117 cells grown continuously in IL-1β + IFN-γ (10 ng/mL and 100 U/mL, respectively) for 3 months is also included in the above data sets. There was no significant deviation in acute cytokine susceptibility for INS-1_{res} cells grown long-term (3 months) in the presence or absence of cytokines.

RNA samples were reverse-transcribed with nucleotides coupled with either Cy3 or Cy5 fluorophores and the cDNA hybridized to the PancChip 5.0 array. Each slide was

normalized using the Cy3 anchors and Stratagene PCR controls. These anchors and controls were removed from the analyses after this step and before any further data manipulation. Paired comparisons were made of resistant versus sensitive lines by hybridizing one reverse-transcribed RNA sample of each phenotype, cytokine resistant and cytokine sensitive, to the same array slide. Each comparison was repeated on a second slide with samples labeled with the opposite fluorophore. This type of comparison is known as a "dye swap" and improves the reproducibility of the microarray results. Dye swap pairings can be found in Table 2-1.

Table 2-1. Dye swap pairings for PancChip 5.0 analysis.

833/15 vs. 834/40		833/117 vs. 834/40	
Cy3	Cy5	Cy3	Cy5
833/15-62	834/40-61	833/117-73	834/40-61
834/40-61	833/15-62	834/40-61	833/117-73
833/15-60-1:3	834/40-59	833/117-71	834/40-60/40
834/40-59	833/15-60-1:3	834/40-60/40	833/117-71
833/15-60-1:4	834/40-60/40	833/117-69	834/40-59
834/40-60/40	833/15-60-1:4	834/40-59	833/117-69
833/15-64	834/40-58		
834/40-58	833/15-64		
834/40-56	833/15-68		
(no dye swap available)			

Cell passage is designated after the cell line for each RNA sample (i.e. 833/15-XX).

Insufficient RNA was available from two of the five 833/117 samples. Therefore, only three dye swap pairings could be performed and this reduction in sample number had a significant impact on the statistical power of the study. For this reason, the primary analysis of the cytokine resistance phenotype was reduced to the 833/15 vs. 834/40 pairing, which had the greatest statistical power.

Once collected, the microarray data was analyzed using the Excel plug-in Significance of Microarrays (SAM) 1.21. From this analysis, 855 genes with a *p*-value cutoff of ≤ 0.05 were determined to be differentially expressed between the 833/15 INS-1_{res} cell line relative to the unselected 834/40 INS-1 cell line. 601 of these genes were upregulated, while 154 genes were downregulated. This list of genes was ranked by statistical power, with the *M* value calculated by SAM 1.21. This list is included in Appendix A.

Qiagen Rat Operon 1.1 microarray analysis

The second set of microarray experiments were run on Qiagen Rat Operon Set version 1.1 arrays at the Duke University Microarray Core facility (Dr. Holly Dressman, Director). These arrays were spotted oligonucleotide arrays consisting of 5,705 unique 70mers. In these experiments, an additional cytokine sensitive cell line, 832/13 (Hohmeier, Mulder *et al.* 2000), was included to improve the statistical power of the study since fewer replicates were available for each phenotype. Serial collections of RNA were harvested as described in the PancChip 5.0 array experiment, but this time by two independent operators. As operator 1, I collected 4 serial passages and a lab technician (Lisa Poppe – operator 2) collected 3 serial passages under slightly different culture conditions. RNA was hybridized to the arrays and scanned as described in Methods and Materials.

Once collected, the array data was analyzed using GeneSpring 6.1.1 with the following parameters, in order: LOWESS (per spot and per chip) normalization, removal of spots flagged as absent or marginal, 1-way ANOVA statistical test, and a fold-change

calculation. From these experiments, two lists of 158 and 312 differentially expressed genes were generated from the sample sets provided by the two operators, using a *p*-value cutoff of ≤ 0.05 for each gene. These gene lists are included in Appendix B.

Meta-analysis of microarray data sets

From the three microarray data sets, a detailed meta-analysis was performed to cull the number of significant candidate genes down to a more manageable size. The concept schema for this meta-analysis is laid out in Figure 2-4. In all three data sets, control genes such as STAT1α, IL-1R1, and iNOS were checked to verify previously reported changes (Chen, Hohmeier *et al.* 2001).

Data meta-analysis began with the generation of an overlap list between the two gene lists produced from the Qiagen Rat Operon 1.1 microarrays (Figure 2-5). This first pass was chosen to include only the Qiagen gene lists because they were fully annotated and the overlap operation could be completed efficiently using the GeneSpring software package. The overlap list contained 19 upregulated and 11 downregulated genes. These 30 genes were subjected to sequence BLAST against the PancChip 5.0 and, if a positive BLAST match occurred, checked against the 855 genes found to be differentially regulated in that experiment. 9 genes were found to be in all three lists (highlighted in blue in Figure 2-5), although due to sequence disparity and annotation gaps of the PancChip 5.0, there is the possibility that additional genes may also overlap between the 3 data sets. Although not a rigorous statistical analysis, each of the 30 genes listed in Figure 2-5 was checked to see if it was in the differentially regulated list of 833/117 vs. 834/40 genes from the PancChip 5.0 experiment that was discarded early in the analyses

due to lack of statistical power. Six of the nine genes that overlap all three lists were in the 833/117 vs. 834/40 comparison and are noted in Figure 2-5.

Discussion

Our previously reported model of cytokine resistance, derived by selection of resistant INS-1 clones through culture in increasing doses of IL-1β and IFN-γ (Chen, Hohmeier *et al.* 2000), was used as the cornerstone of these experiments. This model system provides a potentially powerful tool for identifying cell survival and replication genes, with possible implication for the treatment of Type 1 diabetes mellitus. As previously noted, the phenotype observed in these experiments (Figure 2-1) is an enhancement of the original INS-1_{res} phenotype described in our lab (Chen, Hohmeier *et al.* 2000). In current experiments, the 833/15 and 833/117 cytokine resistant cell lines demonstrated a permanent resistance to both IL-1β and IFN-γ, even when cultured successively for 3 months without cytokines prior to acute cytokine treatment. The original INS-1_{res} phenotype exhibited a permanent resistance to only IL-1β, which was mediated by failure to activate iNOS, possibly through disruption of the NF-κB signaling pathway (Chen, Hohmeier *et al.* 2000). Resistance to IFN-γ, however, was transient and required continual culture in cytokine-enriched media. The degree of IFN-γ resistance correlated with the level of expression of the transcription factor STAT1α (Chen, Hohmeier *et al.* 2001). In our most recent experiments, IFN-γ resistance was permanent, did not require the presence of IFN-γ in the culture media, and was no longer correlated with STAT1α expression. The cause of this phenotypic shift is currently unknown, but is suspected to be related to prior mycoplasma infections in the INS-1 cell lines. Rather than investigating this issue further, our efforts centered on understanding genes that might confer permanent resistance in the INS-1_{res} cell lines.

Our previous attempts to characterize the INS-1_{res} cytokine resistance mechanism involved evaluating molecules and signaling events in the known IL-1β and IFN-γ signal cascades (Chen, Hohmeier *et al.* 2000; Chen, Hohmeier *et al.* 2001). In the experiments described in this dissertation, a more unbiased approach was undertaken, involving application of microarray technology in an attempt to uncover novel candidate genes that contribute to the resistance phenotype. This chapter summarizes the experiments used to generate a set of gene lists encompassing the differentially regulated genes in the INS-1_{res} phenotype.

Through the Beta Cell Biology Consortium, we were provided the opportunity to employ microarray analysis of mRNA expression patterns in the INS-1 cell lines using the recently developed PancChip 5.0 on several two-state model systems, before the release of this technology to the field. The PancChip 5.0 is a particularly attractive microarray set for our purposes because it is enriched for genes expressed in the endocrine pancreas, enhancing the probability of identifying β cell specific genes involved in the resistance phenotype that might not be present on other arrays.

In our experiment, total RNA samples were isolated from 5 serial passages of 3 treatment groups: INS-1 834/40 cells grown in normal RPMI-1640, INS-1_{res} 833/15 and 833/117 cells grown in normal RPMI-1640, and INS-1_{res} 833/15 and 833/117 cell lines grown continuously in RPMI-1640 containing the cytokines IL-1β and IFN-γ for up to 3 months. These RNA samples were reverse-transcribed to cDNA containing either Cy3 or Cy5 fluorophores and hybridized pairwise to the arrays. Sample pairings consisted of one INS-1_{res} cell line, either the 833/15 or 833/117 cell line (cytokine-free growth media), against the 834/40 INS-1 cell line. Pairings were also done with 833/15 and 833/117

cells grown in the presence versus absence of cytokines. This +/- cytokine comparison was used as an internal control to check for up or downregulation of genes known to be modified by cytokine exposure, such as STAT1α, interferon regulatory factor 1 (IRF-1), and multiple major histocompatibility class (MHC) I and II genes.

Sample pairs used for array hybridization are listed in Table 2-1. Unfortunately, insufficient 833/117 RNA prevented completion of the 5 comparisons using this resistant cell line. This reduction in sample number had a significant impact in the statistical power of the 833/117 versus 834/40 comparison, to the point that it was discarded from the subsequent data meta-analysis. The complete list of differentially expressed genes between the 833/15 and 834/40 cell lines are listed in Appendix A, with the genes ranked by highest statistical power first (lod score). This list consists of 601 upregulated and 154 downregulated genes in the resistant state. All of these 855 genes have a *p*-value of 0.05 or less and represent 6.57% of the total genes on the PancChip 5.0.

At 855 genes, the sheer number of resistance candidates that could be culled from the PancChip 5.0 analysis was overwhelming. Fortunately, we had already begun a secondary analysis using the Duke Microarray Core facility. This analysis was performed using rat microarrays (Qiagen Rat Operon 1.1) and instead of running INS-1_{res} against ISN-1 samples on the same array, both were run against a common rat reference. This methodology has both advantages and disadvantages. One significant advantage is that it allows us to examine the two states independently and compare them to a standardized norm. The most significant disadvantage to this method is that it requires additional mathematical manipulations to compare INS-1_{res} gene expression to INS-1 gene expression, thereby reducing the statistical power of the data set.

To improve data reproducibility, two modifications were made to the experimental design. The first involved adding an additional set of RNA samples, isolated by a technician (Lisa Poppe), under slightly different culture conditions (media change 48 hours versus 24 hours pre-harvest) to generate a secondary data set. This additional data set was significant because it helped reduce the number of false positive genes caused by operator-induced biases. The second experimental modification was the inclusion of an additional cytokine sensitive cell line, the 832/13 cell line, in the analysis. Inclusion of this cell line strengthened the data by removing genes that might be up or downregulated in the 834/40 cell line that have nothing to do with cytokine resistance.

Microarray data collected from the Qiagen Oligo 1.1 experiments was analyzed using the microarray analysis software GeneSpring 6.1.1. This software package offers several advantages over traditional analysis techniques such as SAM; specifically, the ability to define experimental groups consisting of multiple cell lines, baseline normalization of data to pre-defined control spots on the arrays and between array slides, and data filtering (removal of genes flagged by the array scanner as marginal or not present). Our analysis of the two Qiagen data sets included a LOWESS per spot and per chip normalization, filtering of spots flagged as absent in the array, and a 1-way ANOVA statistical test which included a multiple testing correction using the Benjamini and Hochberg False Discovery Rate method included in the GeneSpring package. From this analysis, two lists of 158 and 312 differentially expressed genes were generated, with each gene in the two lists having a *p*-value cutoff of ≤ 0.05 (Appendix B).

Screening candidate genes for possible biological relevance is a time-consuming process. For this reason, we decided to focus on genes overlapping all three lists. Our

rationale was that genes common to all three analyses had the greatest probability of being significant to the cytokine resistance phenotype. To generate this overlap list, a meta-analysis was performed in which the two Qiagen data sets were overlapped and genes common to the two lists were pulled out (Figure 2-5). The rat sequence for the resulting 30 genes was then BLASTed against the PancChip 5.0 using the EpconDB database search engine (Beta Cell Biology Consortium, www.betacell.org). This method allowed us to determine which genes in our consensus list of 30 were on the PancChip 5.0 and then to cross-reference our PancChip 5.0 dataset to see if those genes were differentially expressed in that experiment as well. Using this technique, nine genes were found to be common to all three lists. These genes are listed in blue in Figure 2-5.

While the 833/117 vs. 834/40 PancChip 5.0 comparison was not statistically stringent enough for us to generate a gene list, the comparison did generate fold change values that we could use qualitatively to compare to the list of 30 genes generated from the overlap of the two Qiagen Oligo 1.1 array experiments. The results of this screening are listed in Figure 2-5 under the column titled "Match 117 v 40?" Of the 30 genes in the overlap list, 10 were differentially regulated in the 833/117 vs. 834/40 data set. This result is very encouraging. If our list of 30 genes were pulled from the arrays at random, then according to statistical probability we would expect only one or two genes to be on the 833/117 vs. 834/40 list. Additionally, of the 9 genes found in common to all 3 array experiments, 6 of those were on the 833/117 vs. 834/40 list.

Examination of the gene lists in Figure 2-5 and Appendices A and B reveals several interesting genes that could logically be involved in the cytokine resistant state. Among some of the more interesting candidates are growth-inducing and anti-apoptotic

genes such as alpha 1 microglobulin (Ambp), Trefoil factor 3, Bnip3, gastrin, and Cyclin D3. In addition to growth-inducing and anti-apoptotic genes, we also see genes from just about every classification of cellular function: structural, metabolic, housekeeping, receptors, transporters, and transcription factors. The large variance of gene type in these findings is common to microarray analyses and demonstrates the inherent difficultly of trying to measure finite biological differences of a single variable, such as cytokine resistance, using a broad-based technique like microarray analysis. In the following chapters, the rationale for choosing one of these genes, gastrin, will be explored and a detailed analysis of this gene's involvement in the β cell and INS-1_{res} phenotype undertaken.

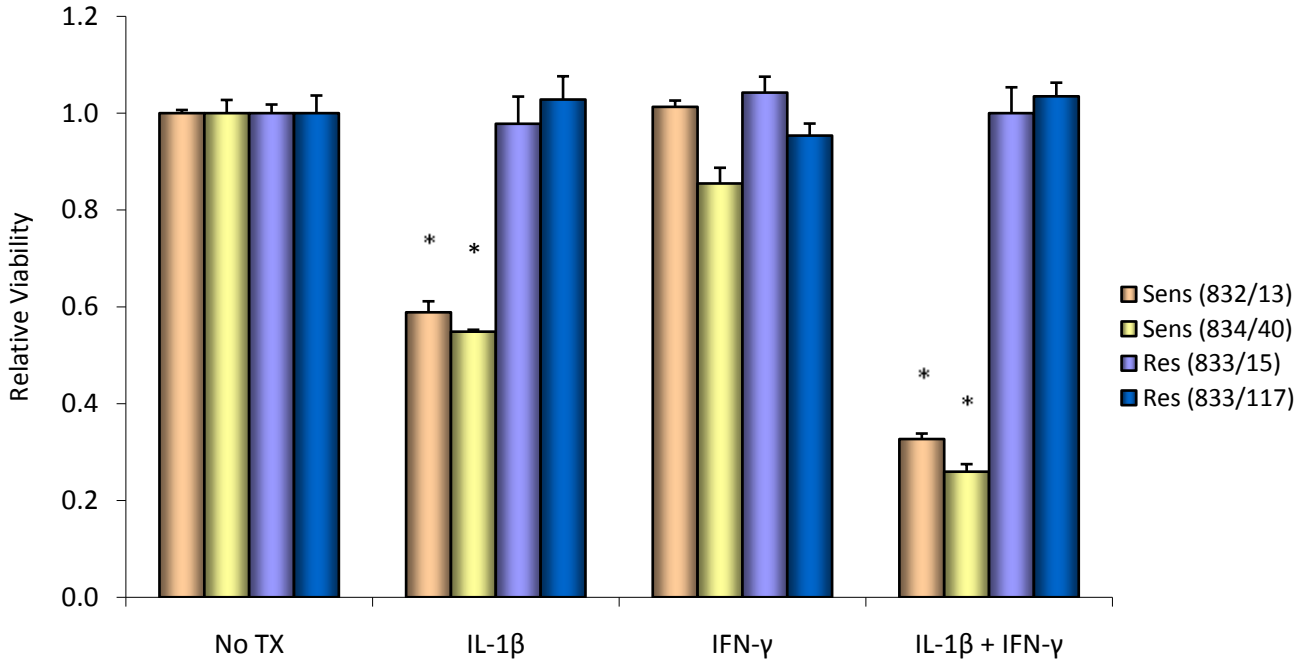


Figure 2-1. Cytokine resistance phenotype. INS-1-derived (832/13, 834/40) and INS-1_{res}-derived (833/15, 833/117) clonal cell lines were plated out in cytokine-free media (high glucose RPMI-1640) in 96-well plates at 50,000 cells/well and grown for 24 hours. Cytokines were added to the media as indicated above (100 U/mL IL-1β and 10 ng/mL IFN-γ) and cells grown for 48 hours. MTT viability assay performed as described in Materials and Methods 72 hours post-seeding with viability measured spectrophotometrically. Treatment groups are normalized to each cell line's no treatment group. Data represent mean ± SEM for 3 independent experiments, each performed in triplicate. The symbol * indicates $p \leq 0.05$.

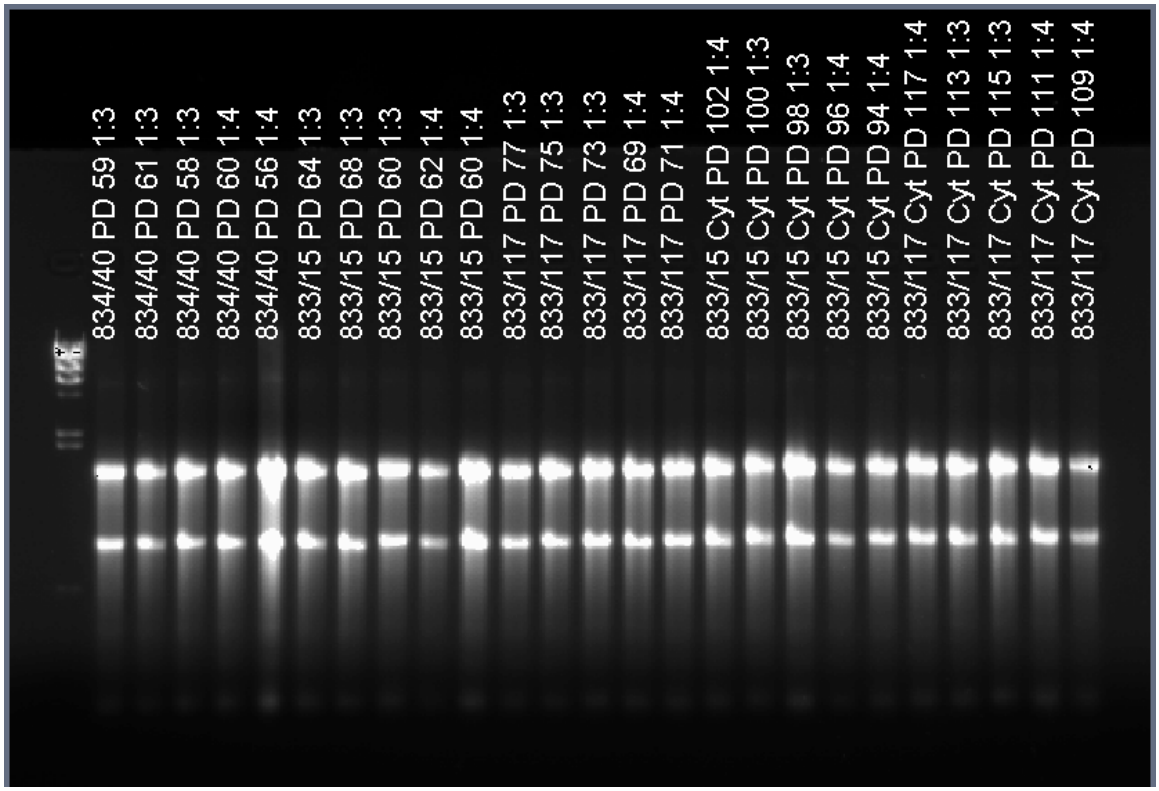


Figure 2-2. Purity of total RNA isolated from INS-1 clonal cell lines for microarray analysis on the PancChip 5.0. Subsequent to RNA hybridization on the PancChip 5.0, aliquots of the total RNA isolates from INS-1 834/40 and INS-1_{res} 833/15 and 833/117 were run on a 1% agarose gel doped with ethidium bromide to assay for potential RNA degradation. Distinct 18S rRNA bands present in all the samples indicate no exposure to RNases during the purification process and both high concentration and quality of RNA.

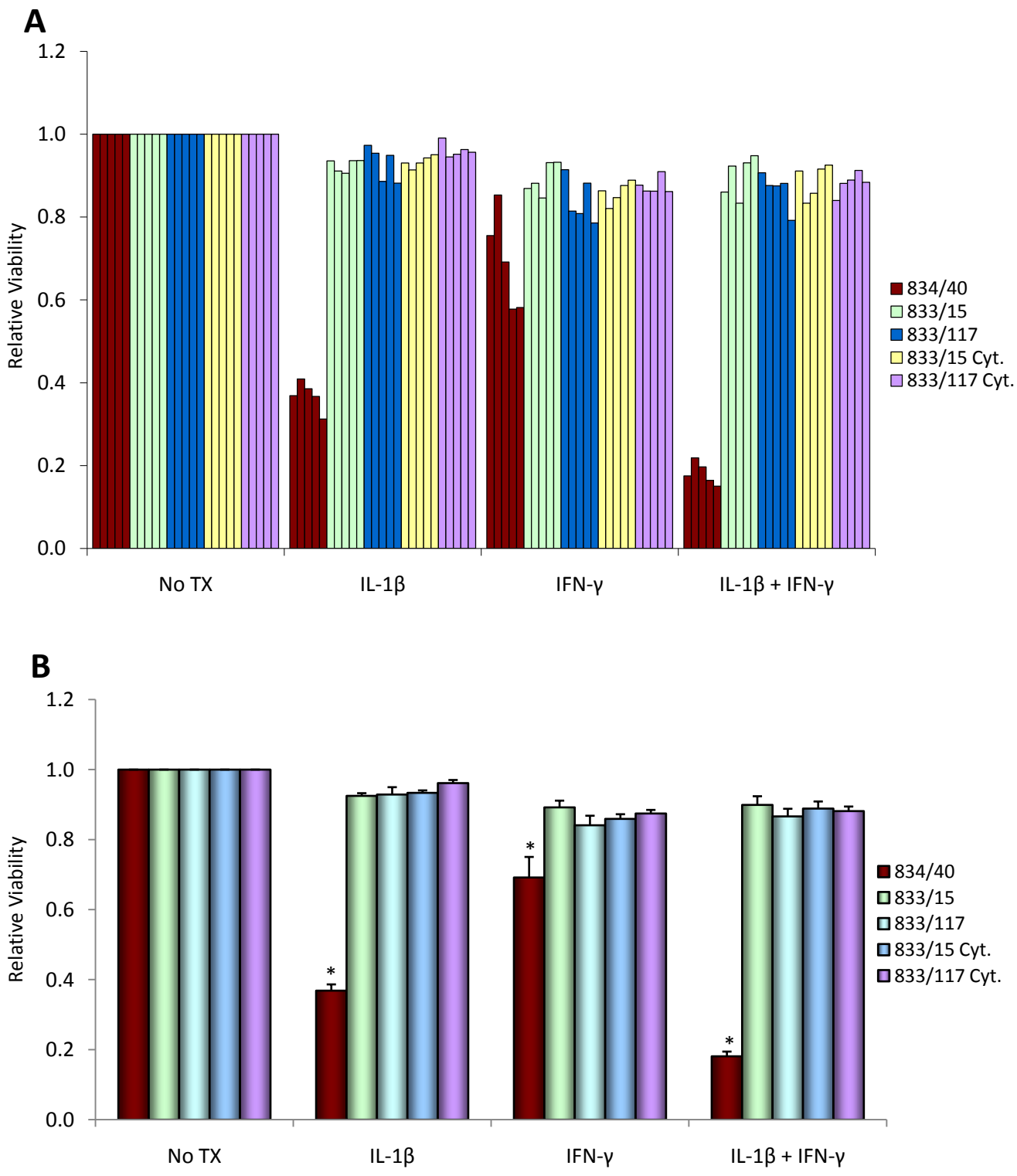


Figure 2-3. Consistency of the INS-1$_{res}$ phenotype. A. MTT Viability assay performed on INS-1 and INS-1$_{res}$ clonal cell lines as in Figure 2-1. **B.** Composite of data in A. Cyt. denotes cells grown continuously in IL-1β and IFN-γ. The symbol * indicates $p \leq 0.05$.

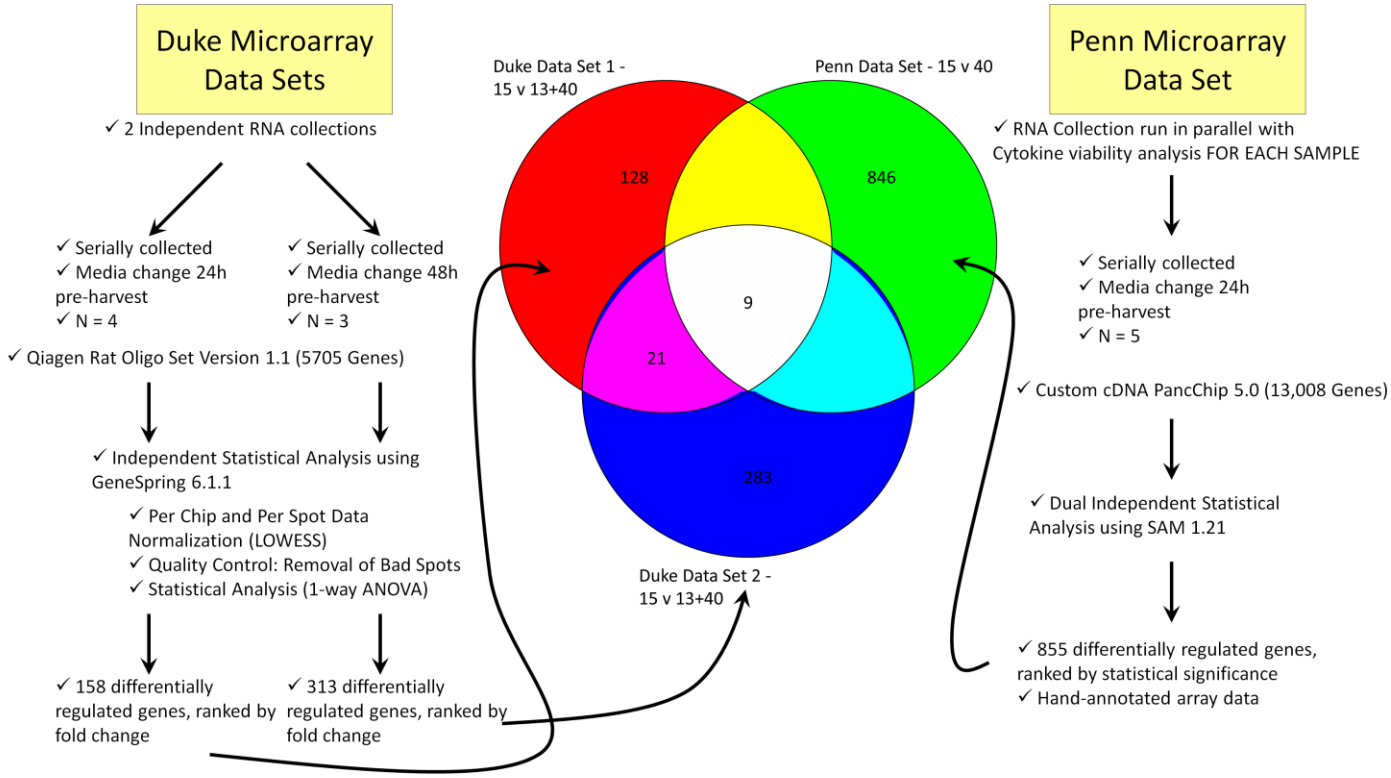


Figure 2-4. Meta-analysis of microarray data. Three independent microarray experiments were conducted. RNA collections were performed by two different operators. Two sample sets were run on rat microarray chips at the Duke Microarray Core facility; the third at the University of Pennsylvania on a mouse microarray enriched for genes expressed in the endocrine pancreas. A *p*-value cutoff of ≤ 0.05 was used to determine statistically significant differences in expression of genes in the INS-1 and INS-1_{res} cell lines. Control genes STAT1α, IL-1R1, and iNOS all match previously reported changes (Chen, Hohmeier *et al.* 2001). Meta-analysis involved independent statistical analyses of each data set, then an overlap analysis of the three data sets.

Genebank ID (rat)	Description	Fold Change 15 v 13	Fold Change 15 v 40	Match 117 v 40?	Penn 15 v 40 SAM Score	Notes
AF370882	TORID	5.53	7.59	No	Not on list.	Matches w/ DT.55258311 and DT.101275698 (riken cDNA 1810009M01Rik)
NM_017207	Vanilloid receptor-like protein 1	2.46	2.36	YES	No BLAST match.	Capsaicin-receptor homolog.
AB062758 (XM_213769)	carbonyl reductase	2.46	2.56	**YES**	↑ 1.31x	carbonyl reductase/NADP-retinol dehydrogenase (match on Penn array w/ hypothetical protein MGC25614)
M83143	Sialyltransferase 1 (β-galacto side α-2,6-sialytransferase)	9.32	22.32	No	Not on list.	
NM_012849	Gastrin	7.99	9.42	**YES**	↑ 2.03x	Anti-apoptotic (with CCK-2 receptor) in Barrett's esophagus. Stimulated beta-cell neogenesis and increases islet mass.
NM_019622	Jerker, deafness locus	5.04	29.46	YES	Not on list.	65% identity to 50% of mouse espin (Penn). Involved in cross-linking actin to promote elongation of microvillus-type parallel actin bundles.
NM_012578 (BC061842)	Histone H1-0	1.69	1.4	No	↑ 1.69x	Accumulates in terminally differentiated cells.
NM_031645	Receptor (calcitonin) activity modifying protein 1	2.31	1.8	YES	No high match.	Interesting b/c may be adrenomedullin or involved in that pathway. Expression of adrenomedullin and its receptor promotes proliferation and inhibits apoptosis.
J05132	UDP glycosyltransferase 1 family, polypeptide A6	1.88	1.59	No	Not on list.	
AF378332	BCL2-related protein A1	1.64	1.4	No	No high match.	Activated through NF-κB pathway, known anti-apoptotic effects.
AF063890	Focal adhesion kinase-related protein (PYK2)	2.51	2.72	No	Not on list.	Activated by TNF-alpha and UV. Involved in cytoskeletal changes, but one paper showed inhibition doesn't affect apoptosis.
NM_012489	Acetyl-CoA acyltransferase, 3-oxo acyl-CoA thiolase A	3.46	4.34	No	Not on list.	
NM_017136	Squalene epoxidase	2.35	1.57	No	Not on list.	
U41803 (BC070880)	Mitofusin 2	1.31	1.59	**YES**	↑ 1.25x	Mitochondrial protein that helps regulate fusion, essential for embryonic development, and repression causes reduced glucose oxidation.
AW143206	ESTs, Moderately similar to RIKEN cDNA 1110029G07	1.35	1.21	No	Not on list.	Unknown protein with unknown function.
BF567456	ESTs, Moderately similar to RIKEN cDNA 2810036K01	1.42	1.82	No	No BLAST match.	Unknown protein with unknown function.
NM_013224 (BC061561)	Ribosomal protein S26	1.47	1.37	**YES**	↑ 1.37x	Storage of pre-mRNAs inactive in splicing.
NM_017160 (BC058149)	Ribosomal protein S6	1.42	1.49	No	↑ 1.37x	AKA Transcription Factor CP2, located in mRNA binding site of 40S subunit.
NM_022510	Ribosomal protein L4	1.41	1.66	No	Not on list.	Involved in antibiotic resistance in bacteria.
NM_019331	Proprotein convertase subtilisin/kexin type 3 (paired basic amino acid cleaving enzyme, furin, membr	0.68	0.56	No	Not on list.	AKA Furin. Converts growth-related precursor proteins to their active products. Expression in islet-based cell lines may induce production of growth factors and increase islet mass via autocrine/paracrine mechanisms.
AY122323	protein phosphatase 1, regulatory (inhibitor) 5 subunit 14B	0.55	0.55	No	↓ 0.59x	PKC-potentiated PP1 inhibitor. Integrates PKC and PKA signaling.
NM_013067	Ribophorin I	0.75	0.57	No	Not on list.	Rough endoplasmic reticulum (RER) specific membrane protein which is a subunit of the oligosaccharyltransferase.
AW918154	ESTs, Highly similar w/ mitoch. ribosomal protein 64	0.81	0.83	No	Not on list.	Mitochondrial ribosomal protein L51.
NM_145084	hypothetical protein RMT-7	0.51	0.63	No	No BLAST match.	
NM_022538	Phosphatidate phosphohydrolase type 2a	0.38	0.27	No	Not on list.	
AI602689	ESTs, Highly similar to RIKEN cDNA 1500031O19	0.52	0.75	No	No BLAST match.	
NM_031711	ADP-ribosylation-like 2	0.96	0.72	No	Not on list.	
NM_031967 (AY217032)	Development-related protein	0.17	0.1	**YES**	↓ 0.65 – 0.54x	Sara (1600019H17Rik); Wdr5 – brain and heart protein NDRG4-C1.
NM_022715	Major vault protein	0.53	0.56	YES	Not on list.	Involved in drug resistance. 13-MDa ribonucleoprotein particles composed largely of a 104-kDa protein.
NM_022669 (BC061549)	Secretogranin II	0.45	0.47	**YES**	↓ 6x	Found in secretory granules, regulates protein sorting.

Figure 2-5. Candidate genes. The overlap in the two Duke Microarray Core data sets was 30 genes. Of these, 19 were upregulated and 11 downregulated. This list was sequence BLASTed against the Penn PancChip 5.0 and, if a positive BLAST match occurred, checked against the 855 differentially expressed genes in that experiment. Genes differentially regulated in all three data sets are in blue (9 genes).

CHAPTER THREE

CANDIDATE GENE ANALYSIS – THE ROLE OF GASTRIN IN INS-1 CYTOKINE RESISTANCE

Introduction

We chose gastrin as a first-pass candidate gene for further analysis based not only its high statistical significance among the genes in all three of our microarray analyses, but also because it is a gene with potential, yet unclear biological function in the islet β cell. Previous studies have shown that gastrin is robustly expressed in the fetal pancreas during islet development just prior to birth (Greider and McGuigan 1971; Larsson *et al.* 1976; Brand *et al.* 1984). The pattern of gastrin expression corresponds with the expansion phase of islet β cell development, and shortly after birth gastrin expression decreases sharply. Within one week of birth, neither gastrin mRNA nor protein are detectable in the pancreas (Larsson, Rehfeld *et al.* 1976). Aside from these studies, and a few others that have attempted to elicit islet growth with exogenous gastrin or transgenic overexpression (Wang, Bonner-Weir *et al.* 1993; Brand, Tagerud *et al.* 2002; Rooman and Bouwens 2004; Suarez-Pinzon *et al.* 2005; Suarez-Pinzon, Yan *et al.* 2005), little is known about gastrin biology with respect to the mature β cell.

Based on the findings described in Chapter 2, wherein we demonstrated upregulation of the preprogastrin gene in our INS-1_{res} cell lines, we sought to further investigate the effects of the preprogastrin gene in β cell function, survival, and proliferation. Previous work in gastric mucosa and colonic adenocarcinomas has shown that gastrin production is stimulated by the cytokines tumor necrosis factor α (TNFα) and

interleukin 1β (IL-1β) through a mitogen-activated protein kinase (MAPK) and protein kinase C (PKC)-dependent mechanism (Suzuki *et al.* 2001). Secreted gastrin peptides are thought to then have an autocrine/paracrine effect through the cholecystokinin 2 receptor (CCK2R) to induce progression through the cell cycle and promote tumor growth (Smith and Watson 2000).

Interestingly, one group has shown that gastrin stimulation of CCK2R modulates NF-κB activity (Hiraoka, Miyazaki *et al.* 2001; Ogasa *et al.* 2003), a pathway we have already shown to be modified in the INS-1_{res} cell lines (Chen, Hohmeier *et al.* 2000). This indicates a possible direct involvement of gastrin in modulating cytokine sensitivity; however, gastrin signaling through NF- κB has yet to be established in β cells. In the present studies, our goal was to determine if manipulation of either extracellular or intracellular gastrin levels could modulate the cytokine sensitivity of our various INS-1 cell lines.

Materials and Methods

Cells and Reagents

832/13, 834/40, 833/15, and 833/117 clonal derivatives of the rat insulinoma cell line INS-1 (Chen, Hohmeier *et al.* 2000; Hohmeier, Mulder *et al.* 2000) were cultured in filter-sterilized RPMI-1640 (Sigma R8758) media containing 11.1 mM D-glucose, 10% fetal calf serum, 10 mM Hepes, 2 mM L-glutamine, 1 mM sodium pyruvate, 50 μM β-mercaptoethanol, 100 units/mL penicillin, and 100 μg/mL streptomycin (Sigma) at 37 °C and 5% CO_2. Recombinant rat IL-1β (Pierce Endogen) was used at 10 ng/mL and recombinant rat IFN-γ (Cell Sciences) was used at 100 U/mL in all experiments, unless otherwise noted. Recombinant gastrin-17 (BaChem) was reconstituted in 0.1% NaOH and used at 10 ng/mL in all experiments unless otherwise noted.

MTT Viability Assay

INS-1 cells were trypsinized, counted, and seeded at a density of 50-100,000 cells/well in flat-bottom 96-well tissue culture plates (Corning) containing 100 μL of RPMI-1640. After 24 hours, 100 μL of additional media containing other additives (e.g. cytokines) was added to each well. 48 hours later the media was decanted, and the plates were gently blotted on paper towels. 115 μL/well of media containing 75 μg/mL of C,N diphenyl-*N*-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) was added, and the plates were incubated for 90 minutes at 37 °C and 5% CO_2. 115 μL of 0.04 N HCl in isopropanol was added to each well, and the optical density of the solublized formazan was read at 575 nm and 650 nm using a SpectraMax 340 plate reader (Molecular Devices). Changes in optical density correlate to mitochondrial

viability, a measure of cell viability. Cells incubated in control media are considered 100% viable and all other treatment groups were normalized to these samples.

Gastrin and CCK Radioimmunoassays

Secreted gastrin-17/34 and CCK-8/22/33 were measured using radioimmunoassays (ALPCO MD 302 and RB 302, respectively) according to the manufacturer's directions on a Perkin Elmer 1470 automatic gamma radiation counter. The sensitivity of the gastrin assay was 5 pmol/L, while that for CCK was 0.3 pmol/L. These values are both well below the 100-1000 pmol/L of gastrin and the 20-50 pmol/L of CCK that is secreted from INS-1 cells and isolated islets treated with recombinant adenoviruses expressing the respective propeptides. Coefficients of variation for the two kits were 2.2-3.0% (gastrin-17) and 2.0-5.5% (CCK-8) with detection ranges of 5-500 pmol/L and 0.3-20 pmol/L, respectively.

siRNA Nucleofection

The AMAXA^{TM} Biosystems nucleofection system was used with pre-validated small interfering RNA (siRNA) oligonucleotides for CCK2R and gastrin (Ambion Inc.). The AMAXA^{TM} siRNA gene silencing technique uses a combination of electroporation and oligofectamine technologies, collectively termed nucleofection, to deliver siRNA oligonucleotides for specific genes into cells. Cytokine resistant and cytokine sensitive INS-1 cells were grown to 80% confluence, fed, trypsinized, harvested, and treated according to the AMAXA^{TM} protocol. This involved suspension of 1 x 10^6 cells in 100 μL Nucleofector T Solution treated with 2 μg siRNA duplex for the gene of interest

(CCK2R or gastrin), followed by electroporation using the T-20 program setting. Cells were re-plated in 24-well plates, allowed to grow for 24 hours, and then exposed to cytokines for the MTT assays.

Construction of Recombinant Gastrin Adenovirus

Rat gastrin cDNA was cloned from 833/15 cytokine resistant INS-1 cells (Chen, Hohmeier *et al.* 2000) grown to 95% confluence in RPMI-1640 media containing IL-1β and IFN-γ. RNA was isolated using the TRIzol RNA isolation technique and preprogastrin cDNA was reverse-transcribed using Invitrogen Platinum® *Taq* DNA Polymerase High Fidelity per manufacturer's instructions. Primers were designed from the published rat preprogastrin cDNA sequence (GenBank accession number NM_012849) and included the addition of EcoRI (5') and BamHI (3') restriction enzyme recognition sequences to facilitate cloning into the pACCMV.pLpA (Becker *et al.* 1994) shuttle vector to generate a new vector, pACCMV-rGast. Primer sequences were: 5'-GATGAATTCGCTCTGCAGACAAGATGCC (sense), encompassing nucleotides -19 to 5 relative to the ATG start codon, and 5'-GATGGATCCGGGCTCTGGAAGAGCATTG, encompassing nucleotides 305 to 323, encompassing the entire rat preprogastin sequence (Genbank NM_012849).

Sequence-verified plasmid minipreps were used to construct a recombinant adenovirus by co-transfecting pACCMV-rGast and pJM17, a plasmid containing the adenovirus genome lacking the E1A gene required for viral replication (Berkner 1988), into human embryonic kidney cells (HEK293) as previously described (Becker, Noel *et al.* 1994). HEK293 cells were seeded into 6-well plates at a density of 1×10^6 cells/well

in DMEM (Sigma D5671) and 10% fetal calf serum and allowed to grow to 50% confluence. The cells were co-transfected with 0.5 μg each of pACCMV-rGast and pJM17 using FuGene 6 transfection reagent (Roche). Any samples demonstrating lysis 6-10 days post-transfection were harvested for viral amplification. Crude lysates were screened for overexpression of the gastrin gene via quantitative (qPCR) and radioimmunoassay of gastrin secreted into the culture medium. The crude virus, AdCMV-rGast, was purified by the CsCl gradient method described below, and the product used to treat INS-1 cell lines at multiplicities of infection (MOI) ranging from 10-2500 for 4 hours and islets at concentrations ranging from 5×10^6 to 2.5×10^8 particles/mL media for 18 hours. Assays were performed 48 hours post-treatment (cell lines), or 72 hours post-treatment (islets). Viruses containing either the bacterial β-galactosidase gene (AdCMV-βGal) (Herz and Gerard 1993) or green fluorescent protein (AdCMV-GFP) (Lehman *et al.* 2000) were used as a controls.

Construction of Recombinant siRNA Adenoviruses

Adenoviruses expressing small interfering RNA sequences (siRNAs) were constructed according to previously described methods (Bain *et al.* 2004; Schisler, Jensen *et al.* 2005) with some modification to the protocol. In brief, steps requiring ligation of siRNA oligonucleotides into pSUPER and then a subsequent excision and re-ligation into the expression vector (EH006) were streamlined into one step using the new expression vectors FF805 and FF701. Oligonucleotides containing siRNAs corresponding to rat gastrin (accession number NM_012849; GGAAGAAGAGGAAGCATAC) and rat TGFα (accession number NM_012671; GGTTTTTGGTGCAGGAAGA) were annealed into

linearized expression plasmids (FF805 and FF701, respectively) between the promoter and T5 transcriptional terminator. FF805 is driven by the human H1 RNA pol III promoter and FF701 is driven by the rat insulin pol II promoter. It was necessary to drive expression of the siRNA for TGFα with pol II because there is a TTTTT sequence in the siRNA hairpin loop that is read by pol III as a stop codon. Co-transfections for the adenoviruses expressing siGastin and siTGFα, Ad-siGast and Ad-siTGFα respectively, were performed as described above. qPCR and radioimmunoassay were used to measure Ad-siGast effectiveness at suppressing gastrin expression and secretion in the INS-1 cell lines 833/15 and 833/117, both of which express high levels of endogenous gastrin. Ad-siTGFα effectiveness was measured with qPCR in 833/15 and 833/117 cell lines.

Ad-siScrambled, a virus constructed to contain a random siRNA sequence (GAGACCCTATCCGTGATTA) with no known gene homology, was used as a control (Bain, Schisler *et al.* 2004; Schisler, Jensen *et al.* 2005; Ronnebaum *et al.* 2006). All assays performed with the siRNA adenoviruses were performed 96 hours post-treatment.

Purification of Recombinant Adenoviruses

Crude viral stocks of all viruses were produced by amplifying the primary co-transfection lysate twice in HEK293 cells. Final crude stocks to be used for CsCl purification were prepared using the following method. Sixteen 150 mm tissue culture plates of HEK293 cells were grown in 20 mL DMEM containing 10% fetal bovine serum. When cells reached 80% confluence, the media was changed and the cells were treated with the respective viruses. Approximately 2-3 days, later the total cell mass was

harvested and frozen at -80 °C. This freeze-thaw cycle method was used to increase the yield of mature viral particles by lysing any cells that were assembling viral particles.

Crude viral stocks were then purified by overnight precipitation in 2.5 M NaCl/20% PEG 8000. Precipitated viruses were collected and resuspended in a minimal volume of PBS, cellular debris was removed by centrifugation, and CsCl was added to the final supernatant for a final density of 1.34 g/mL. After 3 hours of ultracentrifugation at 438,000 G, viral bands were collected, pooled, desalted with a Sephadex G-25 column (Pharmacia Biotech), and stored in 25% glycerol. Optical density (OD) of the purified viral stocks was measured at 260 nm, and viral concentrations were calculated based on the formula 1 OD = 1 x 10^{12} particles/mL. Typical concentrations ranged from 5 x 10^{12} to 2 x 10^{13} particles/mL. Stocks were subdivided into 100 μL aliquots and stored at -80 °C.

Quantitative PCR Measurements of RNA Levels

Quantitative PCR (qPCR) reactions were performed using the ABI PRISM® 7000 sequence detection system and Applied Biosystems Taqman® primers and probes (An *et al.* 2004). Pre-validated Taqman® primer and probe sets used are listed in Appendix C. Triplicate reactions from independent RNA samples were carried out in a final volume of 25 μL containing 20 ng of cDNA template. Samples were normalized to 18S rRNA expression levels and compared to values obtained for cells treated with appropriate control adenoviruses (AdCMV-βGal, AdCMV-GFP, or Ad-siScrambled).

^{3}H-Thymidine Incorporation Assay

Differences in INS-1 growth rate were determined by measuring the differences in ^{3}H-thymidine incorporation into genomic DNA (Frodin *et al.* 1995; Hugl *et al.* 1998; Dickson *et al.* 2001; Cozar-Castellano, Takane *et al.* 2004). INS-1 cells at approximately 60% confluence were incubated with ^{3}H-thymidine (GE Healthcare) to a final concentration of 1 μCi/mL media and grown for 4 hours prior to harvest. For the assay, INS-1 cells cultured in 24-well tissue culture plates (Corning) were placed on ice for 10 minutes and washed with ice-cold serum-free media three times. DNA was precipitated by three washes with 10% trichloroacetic acid. The resulting precipitate from the acid washes was solubilized in 0.3 M NaOH and the radioactivity measured using liquid scintillation counting in a Beckman Coulter LS 6500 scintillation counter. A fraction of the solubilized product was kept to measure protein content (Bio-Rad Protein reagent). Sample counts were individually normalized to protein, and an average for each treatment group was determined. All treatment groups were normalized to either a no treatment or control virus (AdCMV-βGal, AdCMV-GFP, or Ad-siScrambled) treatment group within each experiment.

Statistical Methods

The two-tailed Student's t-test was used in statistical analysis of the data to calculate *p* values. Unequal variances were assumed in all calculations. $P \leq 0.05$ were considered significant.

Results

Verification of gastrin expression in INS-1_{res} cell lines

Two independent methods were used to verify the microarray expression data for gastrin. Quantitative-PCR (qPCR) was used to evaluate mRNA expression of the preprogastrin transcript in INS-1 and INS-1_{res} cell lines, while a radioimmunoassay (RIA) was used to measure secreted gastrin-17 and gastrin-34 – the predominate isoforms of gastrin resulting from post-translational processing through the trans-Golgi network (Daugherty and Yamada 1989; Daugherty *et al.* 1991).

For the qPCR analysis, total mRNA was isolated from two cytokine sensitive INS-1 cell lines, 834/40 and 832/13, and from two cytokine resistant INS-1_{res} cell lines, 833/15 and 833/117, in a manner identical to that described for the microarray analyses in Chapter 2. INS-1_{res} cell lines were subdivided into groups grown with and without the cytokines IL-1β and IFN-γ (10 ng/mL and 100 U/mL, respectively). qPCR analysis of gastrin expression confirmed the microarray results, showing a 6-10 fold increase in the preprogastrin transcript in INS-1_{res} cell lines compared to the two cytokine sensitive INS-1 cell lines (Figure 3-1). This increase in preprogastrin expression did not require the presence of cytokines in the culture media, and is thus a stable feature of the INS-1_{res} cell lines.

Secreted gastrin was measured by RIA of gastrin-17/34 accumulation in RPMI-1640 media over a 24 hour time-frame (Figure 3-2). Secreted gastrin was 4.8 to 20-fold higher in INS-1_{res} cells compared to 834/40 INS-1 cells. Culture of INS-1_{res} cell lines in cytokines had a significant impact on the levels of secreted gastrin measured over 24 hours, despite almost identical levels of preprogastrin mRNA expression. Interestingly,

despite similar levels of mRNA expression in the two INS-1_{res} cell lines, the INS-1_{res} 833/15 cell line showed significantly higher levels of gastrin accumulation in the media, but no difference in cell viability (Figure 2-1). Gastrin was not detectable in media collected from 832/13 INS-1 cells.

Exogenous gastrin-17 has no effect on INS-1 or INS-1_{res} viability

Recombinant rat gastrin-17 (G17) and gastrin-17 sulfated (G17S) were tested for mitogenic capacity in the INS-1 and INS-1_{res} cell lines. These peptides have previously demonstrated mitogenic capacity in gastric parietal cells, however their role in proliferation is believed to be secondary to their ability to induce gastric acid secretion (Kopin, Lee *et al.* 1992).

To measure the short-term effects of exogenous gastrin on viability, cells were incubated in RPMI-1640 culture media containing recombinant rat G17 at 10 ng/mL for 24 hours. This concentration closely matches that secreted by the INS-1_{res} 833/15 cell line, as measured by RIA (Figure 3-2). After the initial 24 hour incubation period, cytokines were added to the culture media for 48 hours followed by an MTT viability assay. During the 48 hour acute cytokine exposure, G17 levels were maintained at 10 ng/mL, resulting in a total exposure time of 72 hours. Short term incubation of INS-1 cell lines in G17 had no effect on viability or acute cytokine sensitivity (Figure 3-3). Longer incubations up to 2 weeks and at supraphysiologic concentrations (up to 100 ng/mL) also had no measurable effect in protecting INS-1 834/40 cells from the cytotoxic effects of acute cytokine exposure (data not shown). Incubation with G17S also had no measurable effects on INS-1 viability (data not shown).

One previous study demonstrated that co-overexpression of gastrin and transforming growth factor alpha (TGFα) could induce a significant change in islet mass, possibly through co-stimulation of the gastrin receptor, CCK2R, and the epidermal growth factor (EGF) receptor (Wang, Bonner-Weir *et al.* 1993). To test this finding in our model system, INS-1 cells were co-incubated in recombinant rat G17 + rat TGFα (100 ng/mL each) for three serial passages. During the final passage, cytokines were added to the media for the final 48 hours, as per previous experiments, and an MTT viability assay performed (Figure 3-4). There were no statistically significant differences in cell viability of the G17 + TGFα treatment groups compared to untreated controls.

A conditioned media exchange was performed with media from 834/40 INS-1 and 833/15 INS-1_{res} cells to assay for possible extracellular co-factors besides TGFα that might mediate cytokine resistance (Figure 3-5). For this experiment, media was harvested from cells at 80-90% confluence, absent cytokine treatment. These harvests were centrifuged at 1000 G for 5 minutes, to remove any cellular debris, and then applied to freshly-split 834/40 INS-1 or 833/15 INS-1_{res} cells. An MTT viability assay was performed 72 hours post-seeding. There were no statistically significant differences in the media exchanged groups. These studies suggest that if upregulation of progastrin is important for the enhanced survival of INS-1_{res} cells, enhanced survival may not be mediated by the mature gastrin peptides that are secreted into the cell culture media.

CCK2R expression in islets is controversial, with some groups claiming to have definitively measured both mRNA and protein expression via immunohistochemistry and others claiming a complete absence of CCK2R expression (Helander *et al.* 1997; Bourassa *et al.* 1999; Morisset, Wong *et al.* 2000). We attempted to verify CCK2R

expression in islets using a Taqman® primer + probe set specific for rat CCK2R. As Figure 3-6A demonstrates, this receptor was barely detected in INS-1 cells. Based upon the assumption that some CCK2R mRNA was expressed in the INS-1 cells, we performed a siRNA knockdown of CCK2R and measured cytokine sensitivity of the treated cells (Figure 3-6B). No change in cytokine sensitivity was noted.

Suppression of gastrin expression reduces INS-1 but not INS-1_{res} viability and proliferation

We used the AMAXA^{TM} Biosystems nucleofection system to ascertain if modulation of gastrin expression could affect INS-1 viability and proliferation. Pre-validated siRNA oligonucleotides specific for the rat preprogastrin transcript were purchased from Ambion. Nucleofection was performed according to the manufacturer's specifications, as outlined in Materials and Methods. Using this system, we were able suppress gastrin mRNA expression by 90±2% in INS-1 834/40 cells and 95±4% in INS-1_{res} 833/15 cells, as measured by qPCR (Figure 3-7A). 48 hours after nucleofection, the level of preprogastrin mRNA in 833/15 cells was suppressed to 36±2% of basal. It is important to note that the absolute Ct value for preprogastin in suppressed 833/15 cells (Ct 29-30) falls between the unsuppressed (Ct 28) and suppressed 834/40 cells (Ct 32). These data show that while we are able to suppress preprogastrin expression below the basal 834/40 expression levels, we are not able reach the same absolute level of suppression.

Gastrin suppression in the INS-1 834/40 cell line results in a 38±7% decrease in basal viability, absent any additional treatments such as cytokines, as measured by the

MTT assay (Figure 3-7B). Cytokine-mediated cytotoxicity was enhanced by suppression of gastrin expression in 834/40 cells. The 90±2% decrease in gastrin expression corresponds to a 80±2% drop in viability as measured by the MTT assay, compared to a 61±6% drop in viability for siScrambled treated controls ($p \leq 0.05$ for siGastrin vs. siScrambled in both untreated and cytokine-treated 834/40s).

siGastrin treatment of 833/15 cells did not result in a statistically significant reduction in viability in either the presence or absence of cytokines. This finding is important because siGastrin suppression resulted in gastrin mRNA levels 64±2% below basal INS-1 834/40 expression; however, at these levels of gastrin expression, the INS-1_{res} 833/15 cell line was still cytokine resistant.

^{3}H-thymidine incorporation was used to measure the rate of proliferation of the INS-1 and INS-1_{res} cell lines after siGastrin treatment. 48 hours after nucleofection, the media was changed in the cell lines to introduce ^{3}H-thymidine. INS-1 834/40 cells treated with siGastrin showed a 44±7% reduction in ^{3}H-thymidine incorporation compared to siScrambled-treated control cells (Figure 3-8). There was no significant change in INS-1_{res} 833/15 cell proliferation, as measured by ^{3}H-thymidine incorporation, despite the 64±2% decrease in gastrin expression relative to INS-1 834/40 cells, as measured by qPCR.

Gastrin overexpression increases INS-1 cell proliferation as measured by ^{3}H-thymidine incorporation

An adenovirus overexpressing rat preprogastrin under control of the CMV promoter (AdCMV-rGast) was constructed as described in Materials and Methods.

Adenovirus-mediated gastrin expression was measured via qPCR and RIA in INS-1 834/40 cells (Figure 3-9). 834/40 cells were treated with AdCMV-rGast for 4 hours. 48 hours after treatment, media and total RNA were collected. AdCMV-rGast doses of up to 50 μL crude lysate induced gastrin mRNA expression up to ~130 fold above baseline, a level 15 – 20 times that seen in INS-1_{res} 833/15 cells (Figure 3-9). RIA demonstrated that gastrin accumulated in the media of treated 834/40 cells in a dose-dependent manner.

We next tested whether overexpression of preprogastrin in the INS-1 834/40 cell line could provide protection against the cytotoxic effects of acute IL-1β and IFN-γ exposure. In this experiment, 834/40 cells were seeded, grown for 24 hours, and then treated with AdCMV-rGast for 4 hours as per previous experiments. Cytokine incubation was initiated 24 hours post-viral treatment and lasted for 48 hours, at the end of which an MTT viability assay was performed (Figure 3-10). Pre-treatment with AdCMV-rGast had no measurable effect on acute cytokine sensitivity in the INS-1 834/40 cell line. Similar observations were made in another cytokine sensitive cell line, the 832/13 cell line (data not shown).

Proliferation was measured in AdCMV-rGast treated 834/40 cells using the ^{3}H-thymidine assay. 24 hours post-seeding, 834/40 cells were treated with AdCMV-rGast for 4 hours. 48 hours later, media was changed with that containing 1 μCi/mL ^{3}H-thymidine for 4 hours. Treatment with 5 or 20 μL crude viral extract resulted in almost a doubling of proliferation versus AdCMV-GFP control treated cells (Figure 3-11).

Discussion

The previous chapter described the identification of candidate genes that might be involved in the unique cytokine resistance phenotype that we see in the INS-1_{res} cell lines. Those microarray experiments generated a large data set that required metadata analysis to select out a manageable number of candidate genes for further study. Due to the features of its biology summarized in Chapter 1, and its rank as one of the most differentially expressed genes in INS-1_{res} cell lines, my work has focused on the potential role of gastrin in the cytokine resistance phenotype.

Two independent methods were used to verify differential expression of gastrin in the INS-1_{res} cell lines. First, quantitative PCR (qPCR) was performed on total RNA from INS-1 and INS-1_{res} cell lines. This analysis verified a robust 6-10 fold upregulation of gastrin expression in the INS-1_{res} cell lines. Second, processed gastrin peptide was detected in culture media using a RIA cross-reactive with gastrin-17 and gastrin-34, but not the precursor peptide, progastrin. Detection of secreted gastrin in the media demonstrated that INS-1 cell lines were capable of full post-translational processing of the progastrin peptide and secretion of mature gastrin. One interesting observation from these experiments is that INS-1_{res} cells cultured for several weeks with cytokines demonstrated a slight increase in expression of preprogastrin mRNA, but a marked decrease in secreted gastrin. The reason for this observation is unknown at this time but could be the result of the prolonged cytokine exposure down regulating the secretory machinery of the INS-1_{res} cell lines.

Much of our knowledge about the biological functions of gastrin has been discovered through studies about gastric adenocarcinomas. In these studies, both cancerous cells and normal stomach mucosa exhibit significant hyperplasia when exposed to mature, processed gastrin peptide (Willems *et al.* 1972; Sirinek *et al.* 1985). In these systems, gastrin binding to the G protein-coupled seven transmembrane cholecystokinin 2 receptor (CCK2R) activates phosphoinositide-3 kinase, phospholipase Cβ, and STAT3, inducing progression through the cell cycle (Nagata, Ito *et al.* 1996; Akagi *et al.* 1999; Paulssen *et al.* 2000).

In our experiments, exogenous gastrin administration had no measurable effect on the acute cytotoxic effects of IL-1β and IFN-γ on INS-1 834/40 cells (Figure 3-3). Short-term gastrin exposure involved culture of freshly-split cells in both 10 ng/mL (physiologic) and 100 ng/mL (supraphysiologic) gastrin-17-enriched RPMI-1640 media. Under both culture conditions, neither the INS-1 nor INS-1_{res} cells exhibited a change in cytokine sensitivity in response to gastrin-17 treatment. Because gastrin sulfation has been shown as a necessary prerequisite in some systems for biological activity (Huang, Yu *et al.* 1989), the gastrin-enriched media experiment was conducted a second time with gastrin-17S. Again, there was no measurable change in cytokine sensitivity.

Some studies have shown that gastrin signaling through CCK2R can be potentiated by co-stimulation of the EGF receptor (Durrant *et al.* 1991; Wang, Bonner-Weir *et al.* 1993). To determine if this applied to β cells, we co-administered recombinant gastrin-17 and TGFα at 100 ng/mL each to 834/40 INS-1 and 833/15 INS-1_{res} cells. This treatment, nor the substitution of sulfated gastrin-17, had any measurable effect on cytokine sensitivity (data not shown). Therefore we conclude that exogenous

gastrin, either alone or co-administered with TGFα, has no effect on cytokine sensitivity in INS-1 cells.

While gastrin-17 is the predominant form of secreted gastrin, we must consider the possibility that a different processed form of gastrin, possibly gastrin-34 or even progastrin itself, could be responsible for protecting INS-1_{res} cells against cytokines. Instead of individually testing a myriad of isoforms of processed gastrin that include amidated, sulfated, and glycine extended variants of all the major species, we decided that a conditioned media exchange between the 834/40 INS-1 and 833/15 INS-1_{res} cell lines would best serve our purposes. This method had the added advantage that if other factors, aside from secreted gastrin, are important in cytoprotection they would be harvested from INS-1_{res} media and be made available to the necessary receptors on INS-1 cytokine sensitive cells. 48-hour media harvests from 833/15 INS-1_{res} and 834/40 INS-1 cells were used to treat freshly-split cells which were then allowed to grow for an additional 72 hours (Figure 3-5). No differences in cytokine sensitivity were induced by this treatment, indicating that the mechanism of cytokine resistance is either an intracellular one, or involves an autocrine or paracrine loop with local concentrations that are higher than can be achieved in conditioned media.

Our studies with exogenous gastrin and TGFα, as well as the conditioned media exchange, indicate that if gastrin is involved in cytokine sensitivity, it is most likely through a mechanism that does not involve the traditional gastrin receptor, CCK2R. Measurements of CCK2R using qPCR indicagted the gene was at the limit of detection (> 35 cycles) in the INS-1 cell lines (Figure 3-6A). We attempted to suppress CCK2R expression further using a siRNA delivery technique developed by AMAXA^{TM}

Biosystems. We have found this system to be rapid and robust enough to be used as a first-pass screening protocol for candidate gene analysis (Collier, Fueger *et al.* 2006; Jensen *et al.* 2006; Ronnebaum, Ilkayeva *et al.* 2006). The AMAXA^{TM} nucleofection system is very effective at gene silencing; however, overexpression of genes with this system has a very low efficiency in the INS-1 cell lines (unpublished observations). Delivery of siCCK2R oligonucleotides to INS-1 and INS-1_{res} cell lines had no measurable effect on the cytokine viability of these cells (Figure 3-6B). Unfortunately, because CCK2R mRNA was already at the threshold of qPCR detection, we were not able to reliably verify if 1) the CCK2R mRNA was expressed in siScrambled controls and 2) if further reduced by siCCK2R treatment.

Collectively, our experiments using exogenously administered gastrin, gastrin + TGFα, and conditioned media indicate that β cell exposure to extracellular gastrin is not sufficient for cytokine resistance. These results, however, do not preclude the possibility of an intracellular mechanism of cytokine resistance that is either driven by or requires gastrin. To investigate this possibility, we decided to evaluate whether manipulation of gastrin expression could affect cytokine sensitivity.

siRNA nucleofection with the AMAXA^{TM} system was used to suppress preprogastrin gene expression in the INS-1 and INS-1_{res} cell lines. Preprogastrin suppression was very effective, with 90-95% silencing relative to basal expression (Figure 3-7A). Corresponding changes in gastrin protein was measured using the RIA previously described and secreted gastrin was reduced by approximately 50% in INS-1_{res} 833/15 cells. Secreted gastrin in siGastrin-treated INS-1 834/40 cells was below the level of detection of the RIA. These levels of suppression fall in line with our observations for

other genes suppressed using the AMAXA^{TM} nucleofection system (Collier, Fueger *et al.* 2006; Jensen, Joseph *et al.* 2006; Ronnebaum, Ilkayeva *et al.* 2006). In general, in these short-term experiments the suppression of target genes is greater at the mRNA level than at the protein level, indicating slow turnover of protein (Wu *et al.* 2004).

We hypothesized that preprogastrin suppression would cause a partial or full restoration of cytokine sensitivity in the INS-1_{res} 833/15 cells. To our surprise, this was not observed. As seen in Figure 3-7B, preprogastrin suppression in the INS-1_{res} 833/15 cells had minimal impact on cytokine sensitivity. Although there was a trend towards slightly increased cytokine sensitivity in the 833/15 cells, it was not statistically significant ($p = 0.27$). However, preprogastrin suppression in the cytokine sensitive 834/40 INS-1 cell line caused a marked decrease in cell viability in both untreated and cytokine treated groups, relative to siScrambled controls. A recombinant adenovirus expressing the same siRNA hairpin for gastrin as that used in the AMAXA^{TM} experiments was constructed per Materials and Methods and used to suppress preprogastrin expression in the INS-1 and INS-1_{res} cell lines. The results for those experiments paralleled that seen with the AMAXA^{TM} oligo suppression method (data not shown). A recombinant adenovirus expressing a siRNA hairpin against TGFα was also constructed and tested on the INS-1 cell lines. While qPCR data demonstrated successful knockdown of TGFα expression, there was no change in INS-1 viability (data not shown).

These observations seem somewhat counter-intuitive unless we consider that gastrin could be involved in β cell proliferation rather than cytokine resistance. Since the MTT assay does not distinguish between cell number differences resulting from altered

growth rates instead of cytokine killing, we decided to employ a ^{3}H-thymidine assay to measure cell division as a function of DNA synthesis (Figure 3-8). The data from this experiment clearly demonstrates that knockdown of preprogastrin in the INS-1 834/40 cell line results in a reduced rate of cellular proliferation. Preprogastrin knockdown in the INS-1_{res} 833/15 cell line had no significant effect on proliferation, but it should be noted that the final suppressed levels of preprogastrin mRNA in these cells were still approximately 4-fold higher than those in siGastrin-treated 834/40 cells (Figure 3-7A). Attempts to further suppress preprogastrin were unsuccessful as higher concentrations of siRNA gastrin oligonucleotides proved toxic to the cells. These experiments were replicated using a recombinant adenovirus to express siGastrin, rather than nucleofection of siGastrin oligonucleotides, and the results matched the nucleofection data (data not shown).

Our results demonstrating that suppression of gastrin expression on unselected 834/40 INS-1 cells impaired cell proliferation led us to the idea that overexpression of the preprogastrin gene could stimulate islet β cell proliferation. To this end, a recombinant adenovirus expressing the full-length rat preprogastrin gene was constructed. Preprogastrin was PCR amplified from 833/15 INS-1_{res} mRNA and sequenced, verifying that the gene upregulated in the INS-1_{res} cells is the wild-type gastrin gene. 834/40 INS-1 cells treated with the recombinant preprogastrin adenovirus (AdCMV-rGast) showed a dose-dependent increase in both preprogastrin mRNA and secreted gastrin protein (Figure 3-9).

From the titration curve, two doses of AdCMV-rGast were chosen (5 and 20 μL crude lysate). These doses were chosen because 5 μL AdCMV-rGast induces gastrin

expression levels almost 3 times that seen in the INS-1_{res} 833/15 cells, with no visible toxicity, while 20 μL is the highest dose that can be used without the INS-1 cells showing signs of viral toxicity, measured qualitatively as cells losing their ability to adhere to plastic tissue culture dishes. AdCMV-rGast treatment had no measurable effect on the cytokine sensitivity of 834/40 INS-1 cells (Figure 3-10), but did have a marked and significant impact on the rate of ^{3}H-thymidine incorporation (Figure 3-11). Cells treated at both 5 and 20 μL doses had almost a doubling of ^{3}H-thymidine incorporation. There was no significant change in 833/15 INS-1_{res} cytokine sensitivity or ^{3}H-thymidine incorporation after AdCMV-rGast treatment (data not shown).

While gastrin may not be involved in cytokine sensitivity, it does appear to be involved in islet β cell proliferation. Futhermore, the fact that our experiments demonstrate the importance of intracellular, rather than extracellular, gastrin on proliferation rate is a unique finding that points to gastrin signaling through a non-traditional mechanism that does not involve the traditional gastrin receptor, CCK2R. In the next chapter, I describe studies aimed at determining whether the results shown here translate to primary islets, and further investigate the mechanism of gastrin-mediated stimulation of β cell replication.

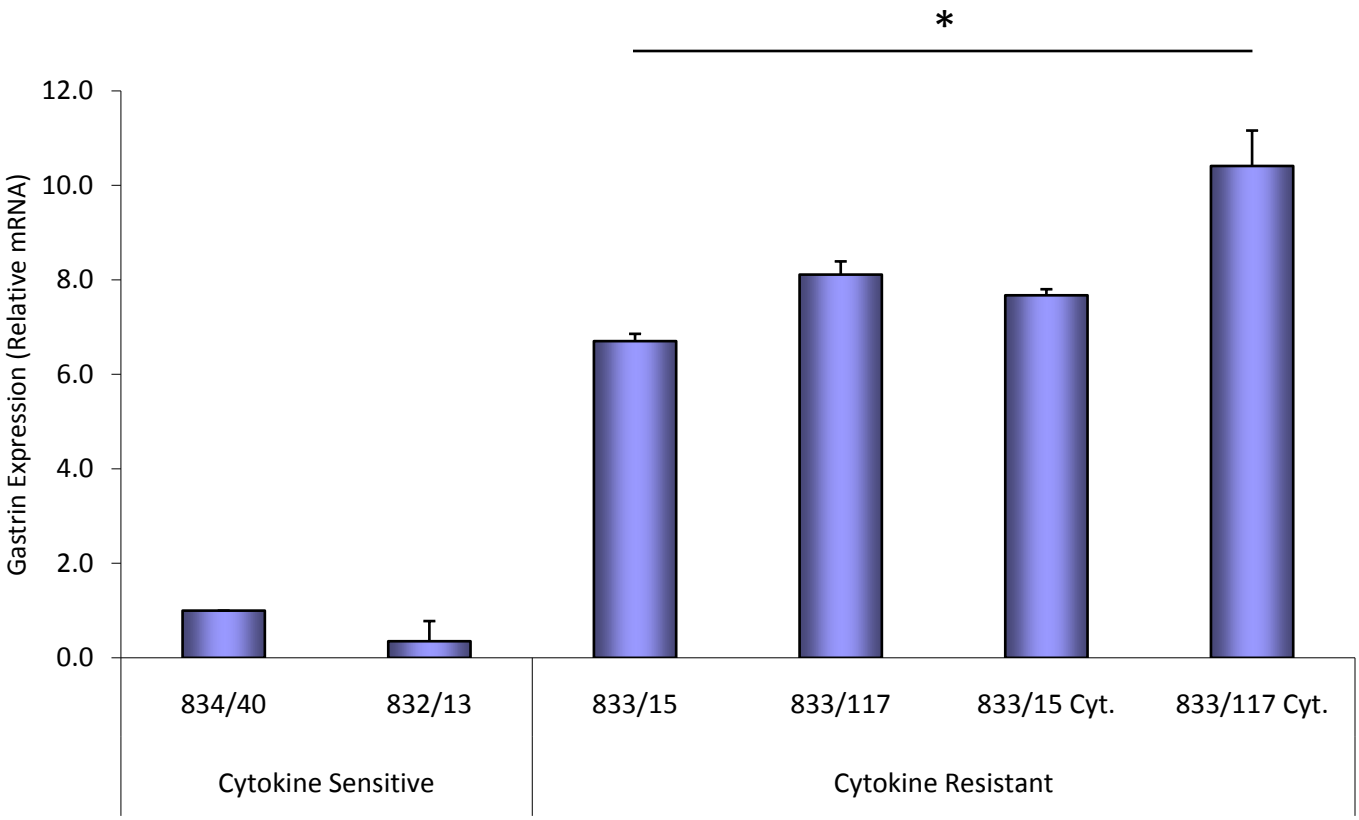


Figure 3-1. Quantitative-PCR analysis of gastrin expression in INS-1 and INS-1_{res} cell lines. mRNA isolated from the indicated cell lines was reverse-transcribed to cDNA and subjected to quantitative analysis using Taqman® rat-specific gastrin oligonucleotides. Cell lines designated with "Cyt." are resistant lines grown perpetually in cytokines. All cytokine resistant cell lines demonstrate a robust upregulation of gastrin, independent of cytokine culture conditions. Data represent mean ± SEM for 3 independent experiments. * denotes $p \leq 0.05$ versus 834/40 cytokine sensitive cell line.

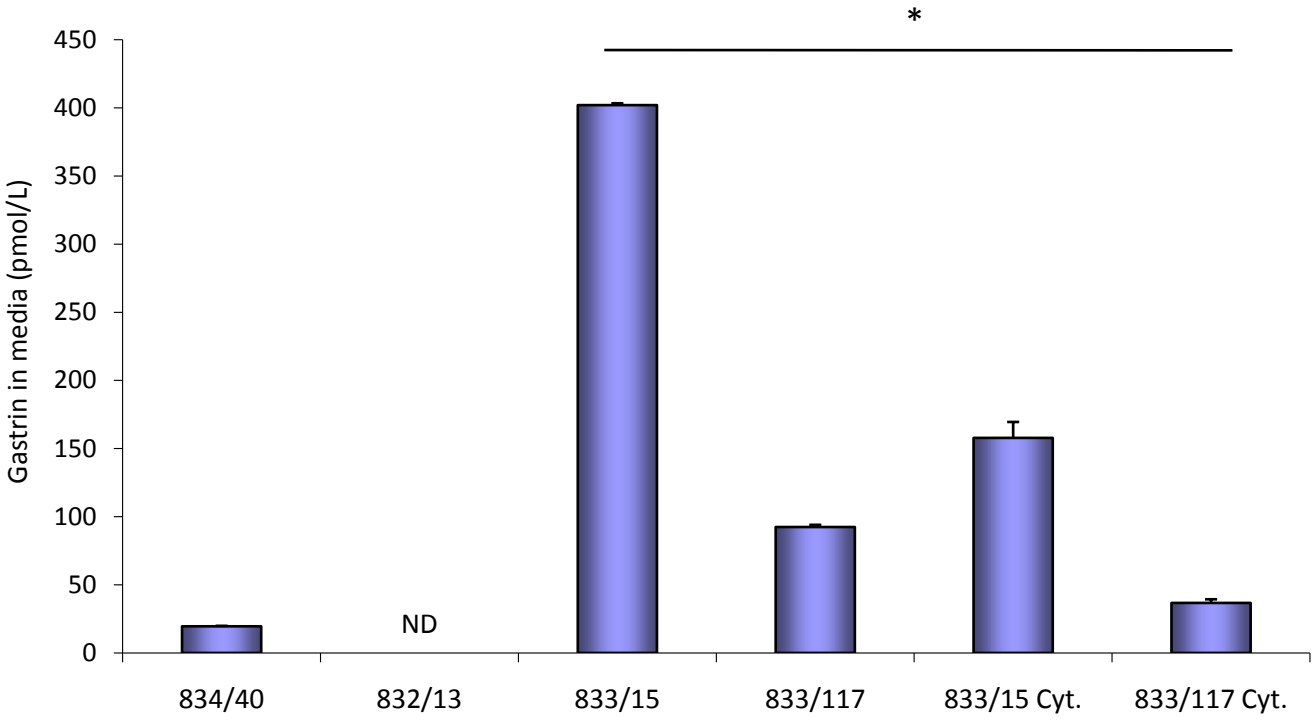


Figure 3-2. Gastrin secretion from INS-1 and INS-1_{res} cell lines. Cells were cultured in RPMI-1640 media with 10% fetal calf serum for 96 hours without media change. Media was collected and centrifuged at 300 x G to remove cells and debris. Secreted gastrin concentrations were measured with a radioimmunoassay specific to human gastrin, but cross-reactive with rat and mouse gastrin (ALPCO). ND = not detectable. * denotes $p \leq 0.05$ versus 834/40 cytokine sensitive cell line.

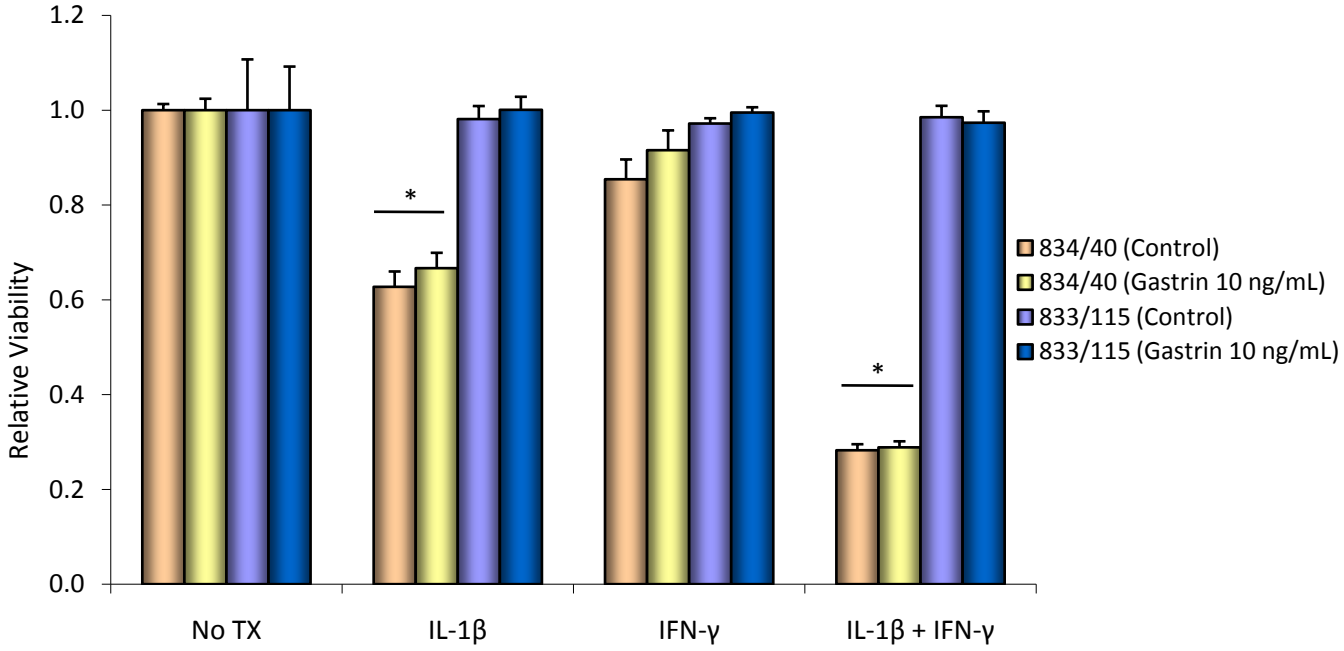


Figure 3-3. Exogenous administration of recombinant gastrin to INS-1 and INS-1_{res} cell lines. Cells were plated in cytokine-free media (high glucose RPMI-1640 media) in 96-well plates with or without recombinant rat gastrin-17 (Sigma G1276) at 50,000 cells/well and grown for 24 hours. Cytokines were added to the media as indicated above (100 U/mL IL-1β and/or 10 ng/mL IFN-γ) and cells grown for 48 hours. MTT viability assays were performed as described in Materials and Methods 72 hours post-seeding. Treatment groups are normalized to each cell line's no cytokine treatment group (No TX). Data represent mean ± SEM for 3 independent experiments, each performed in triplicate. There was no statistically significant difference in cytokine resistance in gastrin-treated cell lines. * indicates $p \leq 0.05$ vs. No TX.

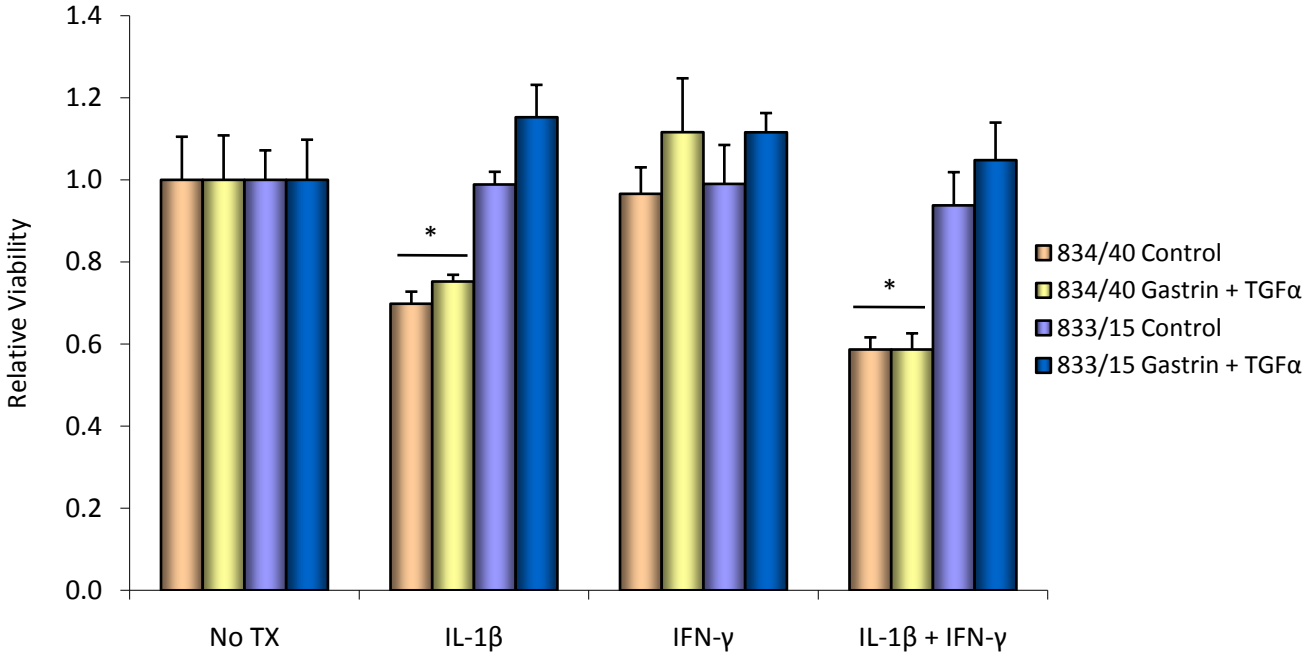


Figure 3-4. Exogenous administration of recombinant gastrin and TGFα to INS-1 and INS-1$_{res}$ cell lines. Cells were plated in cytokine-free media per conditions in Figure 3-3, and then grown in media containing 100 ng/mL recombinant rat gastrin-17 (Sigma G1276) and 100 ng/mL TGFα for 3 serial passages. After the final passage, cytokines were added to the media as indicated above (100 U/mL IL-1β and/or 10 ng/mL IFN-γ) and cells grown for 48 hours. MTT viability assay was performed as described in Materials and Methods at 72 hours post-seeding. Data for the three treatments are normalized to each cell line's no cytokine treatment (No TX) group and represent the mean ± SEM for 3 independent experiments, each performed in triplicate. There was no statistically significant difference in cytokine resistance in gastrin-17 + TGFα –treated cell lines compared to No TX controls. * indicates $p \leq 0.05$ vs. No TX.

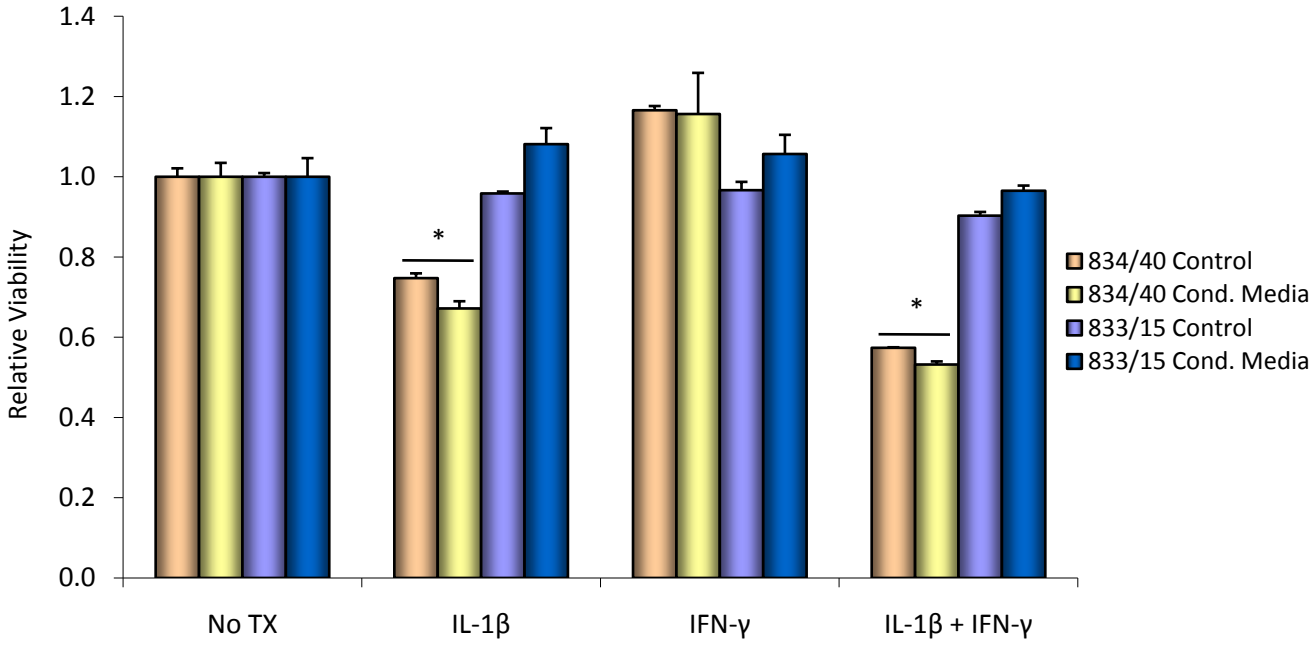


Figure 3-5. Conditioned media exchange between INS-1 and INS-1_{res} cell lines. Cells were plated in cytokine-free media per conditions in Figure 3-3. After 48 hours, media was collected from each cell line and centrifuged at 1000G for 5 minutes to remove cells and cellular debris. Cell lines were then split and re-exposed to media as listed in the figure legend and allowed to grow for 72 hours with cytokine treatments (100 U/mL IL-1β and/or 10 ng/mL IFN-γ). MTT viability assay was performed as described in Materials and Methods at 72 hours post-seeding. Data for the three treatments are normalized to each cell line's no cytokine treatment group (No TX) and represent the mean ± SEM for one experiment. There was no statistically significant difference in cytokine resistance in any of the media exchange groups. * indicates $p \leq 0.05$ vs. No TX.

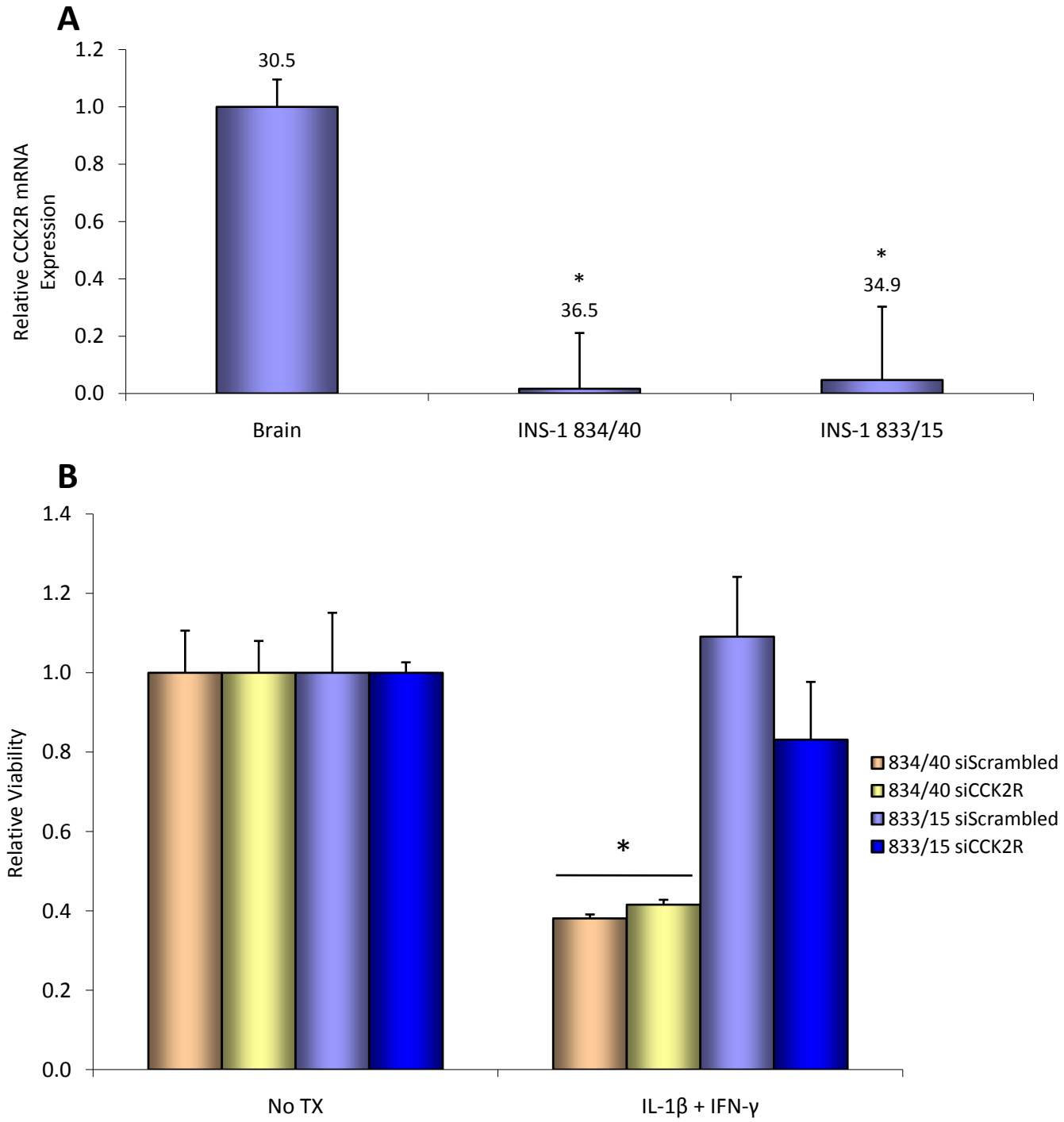


Figure 3-6. CCK2R expression in INS-1 cell lines and viability of cells treated with siCCK2R. A. Relative CCK2R expression. Of all tissues, brain expresses the highest levels of CCK2R. The two INS-1 cell lines have levels of CCK2R expression that are at the limits of detection for qPCR, bringing into doubt whether the receptor is expressed in the INS-1 model system. Ct values are listed above the graph for each group. **B. MTT viability assay.** siCCK2R-treated cells showed no statistically significant change in viability in the presence of cytokines. * indicates $p \leq 0.05$ vs. no cytokine treatment (No TX).

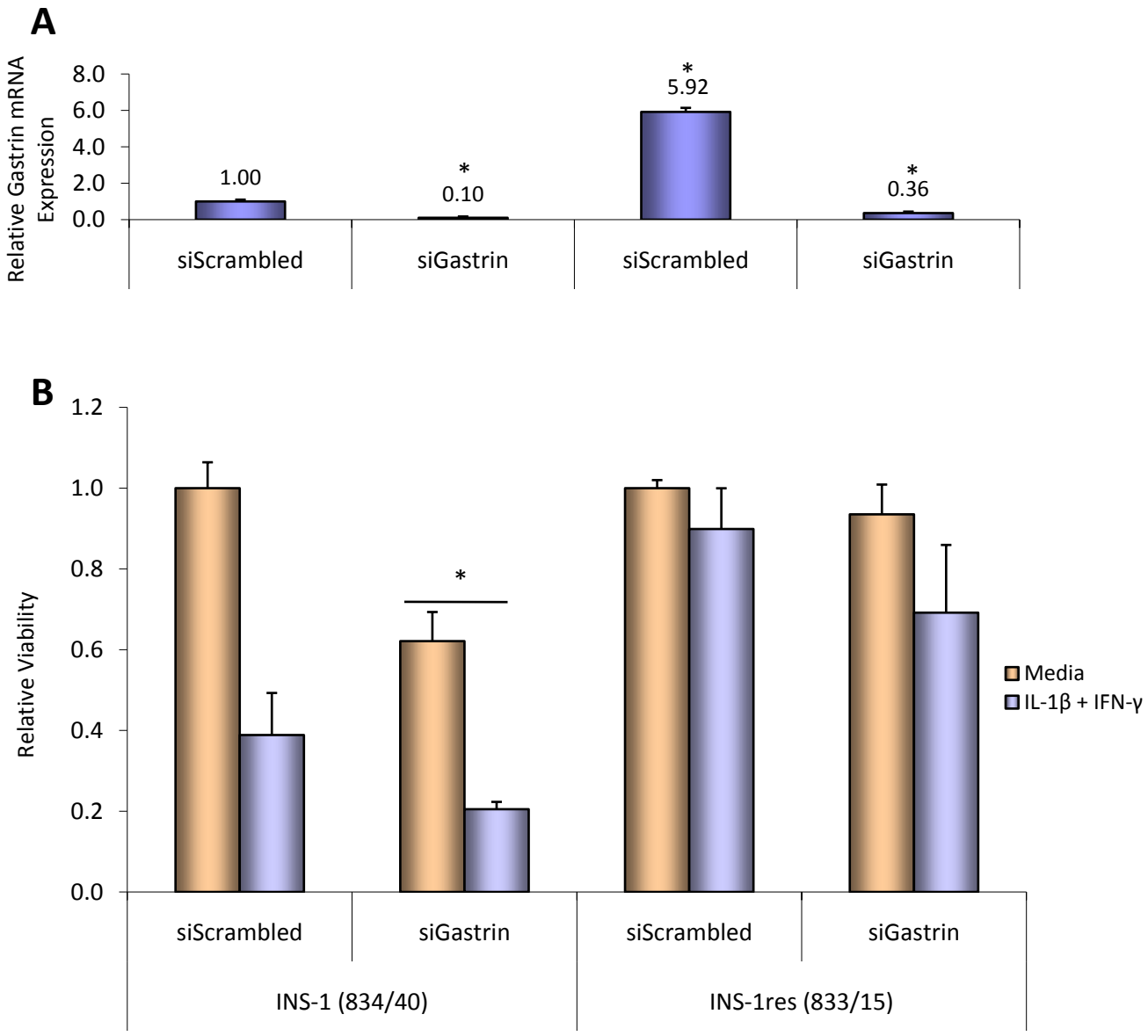


Figure 3-7. Suppression of gastrin expression in INS-1 cell lines results in diminished viability. Cells were grown to 80% confluence, trypsinized, harvested, and treated according to the AMAXA^{TM} Biosystems nucleofection protocol (Materials and Methods) with either control oligos (siScrambled) or oligonucleotides specific for rat gastrin (siGastrin). 24 hours after nucleofection, cytokine-containing media (100 U/mL IL-1β and 10 ng/mL IFN-γ) was added, and the cells were grown for an additional 48 hours. Total RNA was collected and an MTT viability assay was performed on each of the treatment groups. **A. Gastrin mRNA expression.** Expression was reduced by 90% in the INS-1 834/40 cell line and by 95% in the INS-1_{res} 833/15 cell line treated with siGastrin oligonucleotides. Despite a 95% reduction, overall gastrin expression was still 3-4 fold higher in siGastrin-treated 833/15 cells than 834/40 cells (0.36 vs. 0.1). **B. MTT viability assay.** siGastrin-treated 834/40 cells show a 38±7% reduction in viability in the absence of cytokines. Viability is further diminished upon cytokine treatment to 20±2% of baseline, compared to 39±10% for siScrambled-treated 834/40 cells. siGastrin treatment of 833/15 cells did not cause a statistically significant reduction in viability in either culture condition. * indicates $p \leq 0.05$ vs. siScrambled.

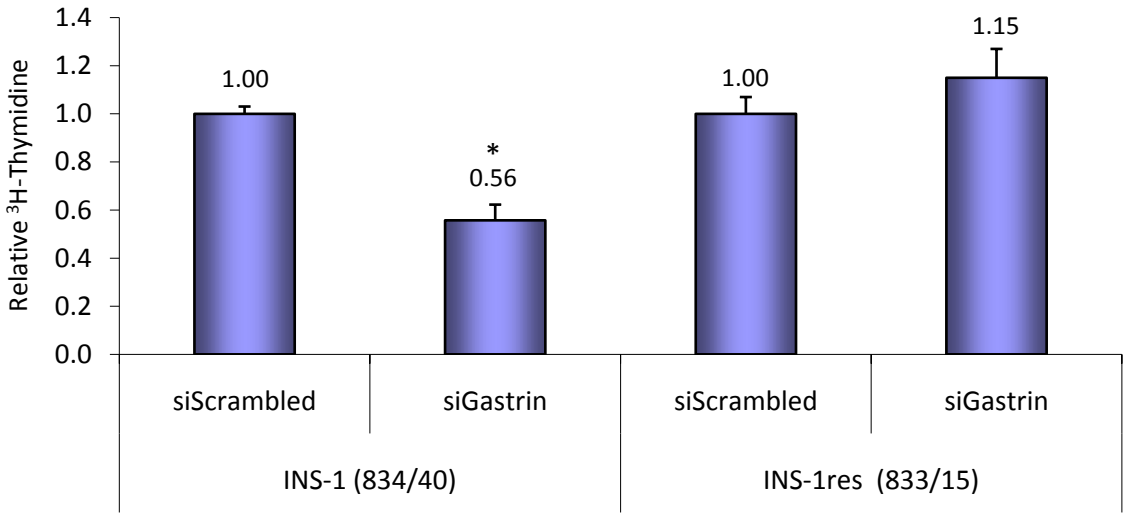


Figure 3-8. Suppression of gastrin expression in INS-1 but not INS-1_{res} cell lines results in diminished proliferation. INS-1 834/40 and INS-1_{res} 833/15 cells were grown and treated with the AMAXA^{TM} nucleofection system per Figure 3-5. 48 hours post-nucleofection, media was exchanged to include ^{3}H-thymidine (1 μCi/mL) and the cells grown for an additional 4 hours. Cells were harvested and subjected to a trichloroacetic acid precipitation per the protocol described in Materials and Methods. ^{3}H-thymidine incorporation was measured by dissolving the precipitate in 0.3 N NaOH and counting radioactivity in a scintillation counter for 1 minute per sample. Counts per minute were normalized to the protein content of each sample (Bio-Rad Protein reagent). INS-1 834/40 cells treated with siGastrin demonstrated a 44±7% reduction in growth as measured by ^{3}H-thymidine incorporation compared to siScrambled controls. There was no statistically significant change in proliferation measured in INS-1_{res} 833/15 cells. * indicates $p \leq 0.05$ vs. siScrambled.

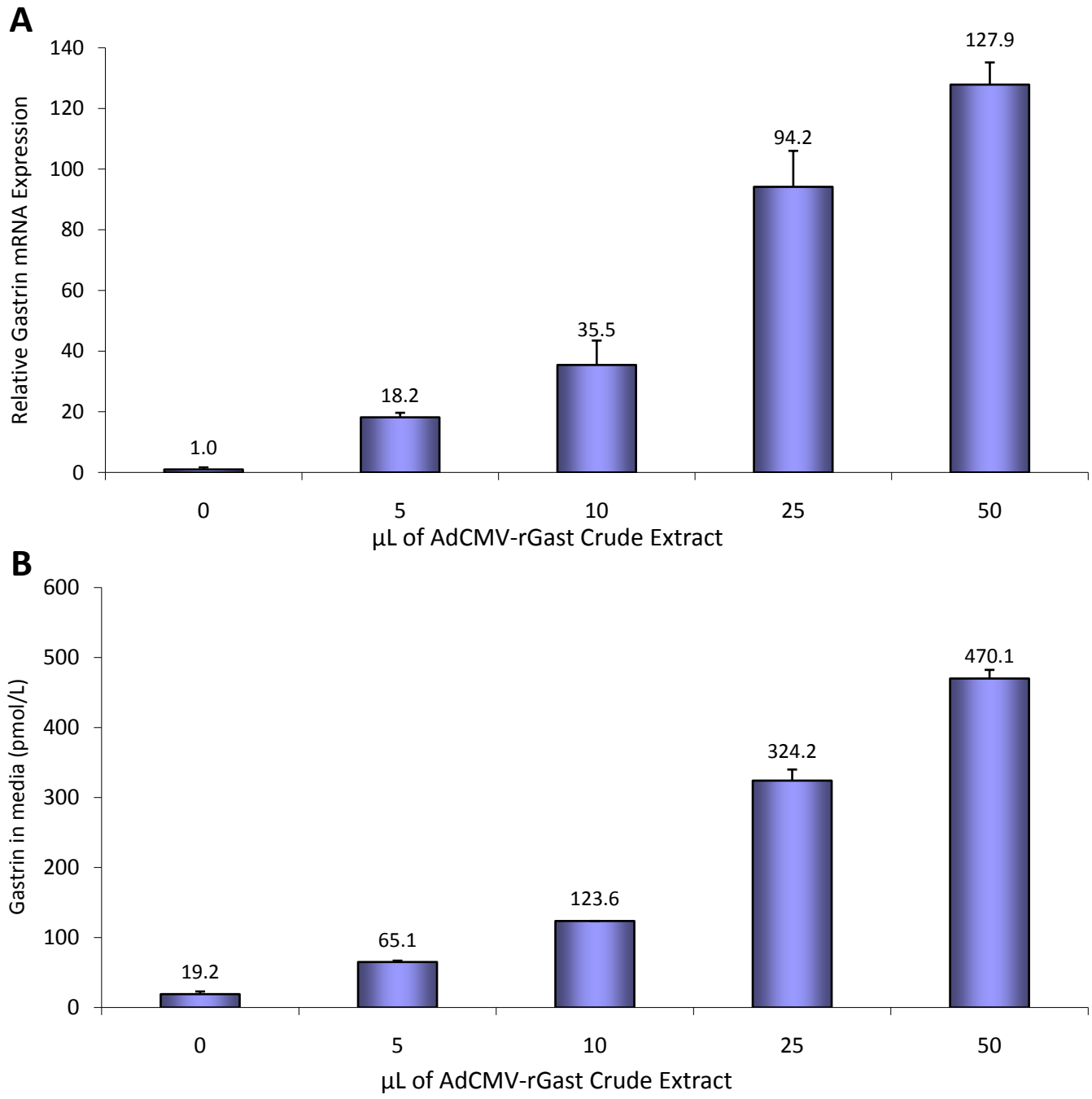


Figure 3-9. AdCMV-rGast induces robust overexpression of gastrin in INS-1 834/40 cells. A recombinant adenovirus containing the rat preprogastrin cDNA (Genbank NM_012849) was constructed per the protocol described in Materials and Methods. INS-1 834/40 cells were split and at 24 hours post-seeding treated with AdCMV-rGast for 4 hours, followed by a media change. 48 hours after treatment, total RNA and media were harvested. **A.** RNA was purified, reverse transcribed to cDNA, and gastrin expression measured using a Taqman® primer + probe set as described in Materials and Methods. **B.** 834/40 INS-1 cells treated with AdCMV-rGast secrete processed gastrin into the media 48 hours after viral treatment. In both measurements, a dose-dependent upregulation of gastrin expression is clearly evident.

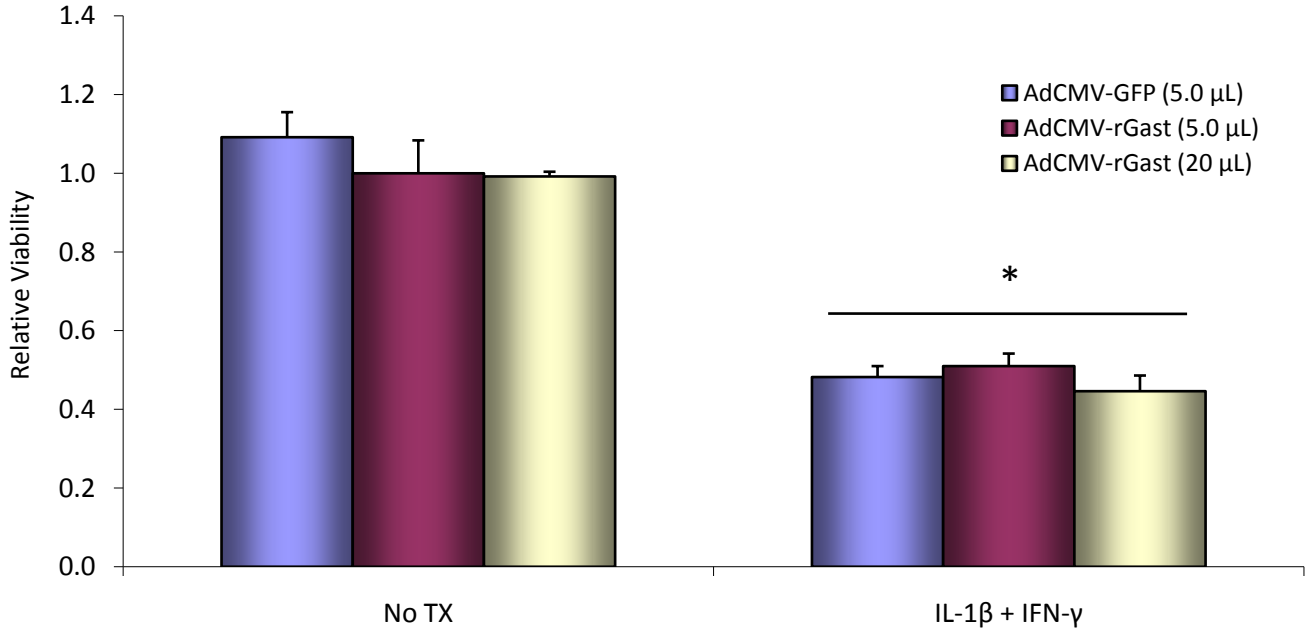


Figure 3-10. Gastrin overexpression in INS-1 834/40 cells has no effect on cytokine resistance. INS-1 834/40 cells were split and 24 hours post-seeding treated with either AdCMV-GFP or AdCMV-rGast for 4 hours, followed by a media change. 48 hours after treatment an MTT viability assay was performed as described in Materials and Methods. AdCMV-rGast treatment has no measurable effect on cytokine resistance in 834/40 INS-1 cell lines. * indicates $p \leq 0.05$ vs. no treatment (No TX).

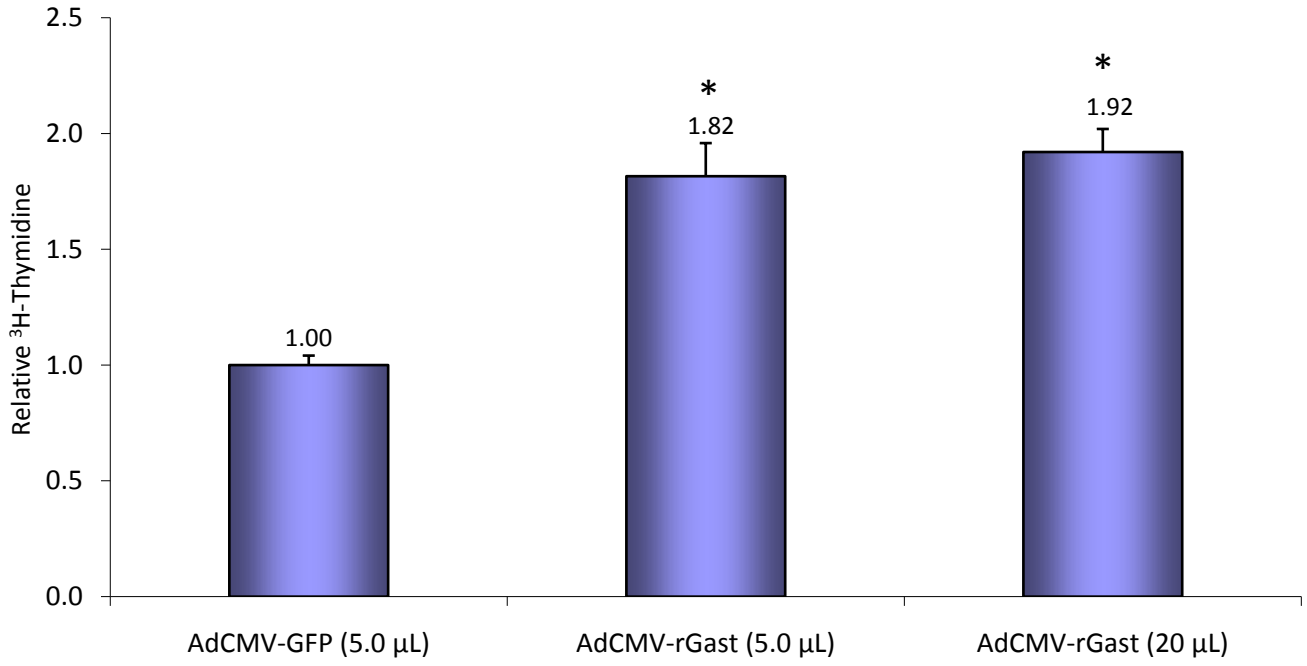


Figure 3-11. Gastrin overexpression induces ^{3}H-thymidine incorporation in the INS-1 834/40 cell line. A recombinant adenovirus was used to drive preprogastrin expression using the CMV promoter (AdCMV-rGast). 834/40 INS-1 cells were exposed to either AdCMV-GFP (5 µL crude lysate) or AdCMV-rGast (5 µL or 20 µL crude lysate) for 4 hours, followed by a media change. ^{3}H-thymidine incorporation was measured 48 hours later for a 4 hour time-frame. Both treatment groups showed significant increases in ^{3}H-thymidine incorporation following AdCMV-gastrin treatment. * indicates $p \leq 0.05$ vs. AdCMV-GFP.

CHAPTER FOUR

INTRACELLULAR GASTRIN AND CHOLECYSTOKININ STIMULATE ISLET BETA CELL PROLIFERATION

Introduction

The expression of gastrin during pancreatic development was recognized over thirty years ago (Greider and McGuigan 1971). However, despite the significant time that has elapsed since that discovery, our knowledge of the function of gastrin in the pancreas is still quite limited. Disruption or overexpression of gastrin in transgenic mice results in no overt pancreatic phenotype (Wang, Bonner-Weir *et al.* 1993; Friis-Hansen, Sundler *et al.* 1998), yet the combination of gastrin with growth factors such as epidermal growth factor (EGF) or transforming growth factor α (TGFα) can induce significant increases in β cell mass (Wang, Bonner-Weir *et al.* 1993; Rooman, Lardon *et al.* 2002).

In the course of this dissertation project, gastrin emerged as a gene potentially involved in conferring cytokine resistance, based on microarray analyses of the INS-1 and INS-1_{res} cell lines. While studies summarized in Chapter 2 demonstrate that gastrin overexpression does not confer cytokine resistance in cytokine sensitive cell lines, intracellular expression of the preprogastrin gene, but not exogenous administration of the processed peptides gastrin-17, sulfated gastrin-17, or gastrin-34, caused significant increases in cell growth.

In this chapter we expand our analysis of gastrin to primary rat islets. We have discovered that similar to the INS-1 cell lines, preprogastrin overexpression in primary rat islets via a recombinant adenovirus can promote potent β cell proliferation.

Additionally, we have expanded our analysis to include preprocholecystokinin (preproCCK) since both hormones bind to the cholecystokinin 2 receptor (Dufresne *et al.* 2006). In addition to demonstrating that both preprogastrin and preproCCK overexpression can induce β cell growth, we demonstrate that a key β cell function, glucose-stimulated insulin secretion (GSIS), is not impaired by our manipulations. In fact, with preproCCK we see a significant potentiation of GSIS. In studies involving administration of exogenous gastrin and CCK peptides, islets lacking CCK receptors, and various signaling assays, we also begin to investigate the mechanisms by which preprogastrin and preproCCK expression can stimulate β cell replication.

Materials and Methods

Islet Procurement and Culture

Rat islets were isolated from 200-250 g Wistar rats (Charles River Breeding Laboratories) as previously described (Johnson *et al.* 1990; Bain, Schisler *et al.* 2004) and cultured in RPMI-1640 (Sigma R8758) media containing 10% fetal calf serum (Atlanta Scientific S11150 Lot M0054), 8 mM D-glucose, 100 units/mL penicillin, and 100 μg/mL streptomycin (Sigma) at 37 °C and 5% CO_2. All animal protocols were approved by the Duke University Institutional Animal Care and Use Committee.

CCK1R, CCK2R, and CCK1/2R knockout mice on the 129SvEv background were obtained from Alan Kopin at Tufts University and bred in the laboratory of our collaborator, Dr. Alan Attie, at the University of Wisconsin-Madison. Mice were housed on a 12 hour light-dark cycle, lights on at 6:00 AM. Mice had free access to a chow diet of roughly 6% fat (by mass, Purina 5008) and water, except during fasts. Islets were harvested from 10-12 week old mice (20-25 g weight). Research protocols were approved by the University of Wisconsin Institutional Animal Care and Use Committee.

Recombinant rat gastrin-17 (BaChem) was reconstituted in 0.1% NaOH and used at 100 ng/mL unless otherwise noted. Recombinant rat cholecystokinin-8 (Pierce Endogen) was reconstituted in diH_2O and used at 100 ng/mL in all experiments.

^{3}H-Thymidine Incorporation Assay

^{3}H-thymidine incorporation measurements in isolated rat islets were performed in a similar manner to that described in Chapter 2 for the INS-1 cell lines. Isolated rat islets were cultured in RPMI-1640 media containing ^{3}H-thymidine (1 μCi/mL) for 24 hours.

At the end of the incubation, islets were placed on ice for 10 minutes, washed three times with ice-cold serum-free RPMI-1640 media, and DNA was precipitated by three washes with 10% trichloroacetic acid. The precipitate was solublized in 0.3 N NaOH and the radioactivity measured using liquid scintillation counting in a Beckman Coulter LS 6500 scintillation counter. A fraction of the solubilized product was kept to measure total protein (Bio-Rad Protein reagent). Sample counts were individually normalized to protein, and an average for each treatment group was determined. All treatment groups were normalized to a AdCMV-βGal or AdCMV-GFP control virus group within each experiment.

Glucose-Stimulated Insulin Secretion Assay – Isolated Primary Rat Islets

Isolated islets were treated with various recombinant adenoviruses (see later sections for description of construction and utilization of specific adenoviruses) and incubated in ^{3}H-thymidine to assay for cell proliferation as previously described. Just prior to precipitation of total protein by trichloroacetic acid in the ^{3}H-thymidine assay, β cell function was assessed by measuring glucose-stimulated insulin secretion (GSIS). Groups of 25-30 islets were washed in secretion assay buffer (SAB) containing 2.5 mM glucose and 0.5% bovine albumin (Sigma) for 1 hour at 37 °C. The SAB wash was discarded and islets were incubated for an additional hour at 37 °C in SAB containing 2.5 mM glucose and 0.5% bovine albumin. SAB was collected and the islets were incubated for a final hour at 37 °C in SAB containing 16.7 mM glucose and 0.5% bovine albumin. A radioimmunoassay (RIA) for insulin was performed on the supernatants (DPC Coat-A-Count) collected from islets exposed to either the low (2.5 mM) or high (16.7 mM)

glucose washes. Counts were normalized to protein levels, as measured by Bio-Rad protein reagent.

Gastrin and CCK Radioimmunoassays

Secreted total gastrin-17/34 and CCK-8/22/33 levels were measured using RIA (ALPCO) according to the manufacturer's directions, and radioactivity was measured on Perkin Elmer 1470 automatic gamma radiation counter. The sensitivity of the gastrin assay is 5 pmol/L, while that for CCK is 0.3 pmol/L. These values are both well below the 100-1000 pmol/L of gastrin and the 20-50 pmol/L of CCK that is secreted from INS-1 cells and isolated islets treated with recombinant adenoviruses expressing the respective peptides. Coefficients of variation for the two kits are 2.2-3.0% (gastrin-17) and 2.0-5.5% (CCK-8) with detection ranges of 5-500 pmol/L and 0.3-20 pmol/L, respectively.

Isolation and Purification of Total RNA for qPCR

Total RNA was purified from islets by a chloroform + ethanol extraction. Extracts were passed through a Qiagen Rneasy mini-column, treated with DNase, and eluted from the column with RNase-free H_2O. The purified RNA samples were then quantified spectrophotometrically at 260 nm and 280 nm. 1 μg RNA from each sample was then reverse-transcribed using the Bio-Rad iScript^{TM} cDNA synthesis kit.

Construction of Recombinant Mutant Gastrin Adenoviruses

Mutant gastrin adenoviruses were created using a PCR "stitching" mechanism. Using this method, the preprogastrin gene was subdivided into 7 oligonucleotide pairs

approximately 45-60 base pairs in length, which were annealed and ligated to reconstitute the entire rat preprogastrin cDNA, including 5' EcoRI and 3' BamHI restriction enzyme sites. This mechanism allowed for rapid mutant production by substituting wild-type primer pairs for new primer pairs containing the desired mutant(s).

PCR Stitching Oligos:
FIRST SET
5'- aatt cgctctgcag acaag***ATG***cc tcgactgtgt gtgtgcatgc tagtc - 3'
gcgagacgtc tgttctacgg agctgacaca cacacgtacg atcagaatca cgacc - 5'P

SECOND SET
P5'- ttagt gctggctcta gctaccttct cggaagcttc ttggaaacct cgctc - 3'
gagat cgatggaaga gccttcgaag aacctttgga gcgagggtcg atgtc - 5'P

THIRD SET
P5'- ccagc tacaggatgc atcctctgga ccaaggacca atggggccct ggaac - 3'
ctacg taggagacct ggttcctggt taccccggga ccttgtcgtg gtcga - 5'P

FOURTH SET
P5'- agcac cagctagaaa agctgggccc agcctctcac catcggagac agctg - 3'
tcttt tcgacccggg tcggagagtg gtagcctctg tcgaccccgg ggttc - 5'P

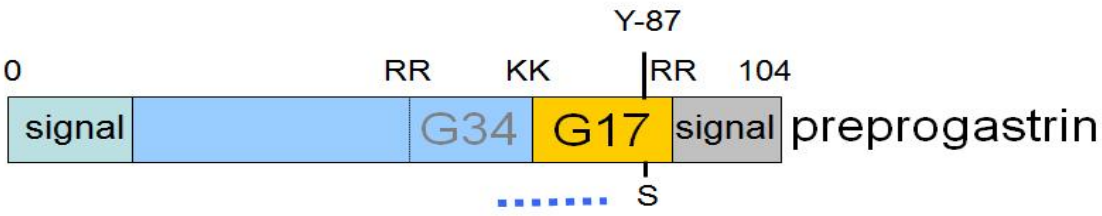
FIFTH SET
P5'- gggcc ccaaggtccg caacacttca tagcagatct gtccaagaaa cagag - 3'
caggc gttgtgaagt atcgtctaga caggttcttt gtctccggtg gttac - 5'P

SIXTH SET TTT - mutation to Phenylalanine
P5'- gccac caatggagga agaagaggaa gcatacggat ggatggactt - 3'
ctcct tcttctcctt cgtatgccta cctacctgaa accagcggca - 5'P
AAA

SEVENTH SET
P5'- tggtcgccgt agcgctgagg aagaagacca gtataactag caatgctctt ccagagcccg - 3'
tcgcgactcc ttcttctggt catattgatc gttacgagaa ggtctcgggcctag - 5'

RED - sulfation site mutation
BLUE - dibasic cleavage site mutation
GREEN - ER signal sequence: no mutations to date

Translation:
MPRLCVCMLV LVLALATFSE ASWKPRSQLQ DASSGPRTNG ALEQHQLEKL GPASHHRRQL GPQGPQHFIA
DLSKKQRPPM EEEEEAYGWM DFGRRSAEEE DQYN



Using this method, the following mutants were constructed: preprogastrin lacking the sulfation site (AdCMV-GastΔS), preprogastrin lacking a single dibasic cleavage site for fully processed gastrin-17 (AdCMV-GastΔ34), and preprogastrin lacking 3 dibasic cleavage sites (AdCMV-progastrin). These mutations force the expression of non-sulfated gastrin-17, gastrin-34, and progastrin, respectively. Co-transfections and viral purification were completed as described for AdCMV-rGast in Chapter 3.

Construction of Recombinant CCK Adenovirus

Mouse CCK cDNA (GenBank accession number NM_031161) was obtained from Dr. Alan Attie (University of Wisconsin) in a pIRES-EGFP vector. The 631 base pair insert for mouse CCK was amplified from the pIRES-EGFP vector with the PCR primers 5'-AACTTAGCTGGACTGCAGCCTTCTC (sense) and 5'-GCATAGCAATAGGTCTGGGAGTC (antisense) with the addition of the desired PmeI and HindIII restriction enzyme sites. The CCK fragment was cloned into the FF001 plasmid, a derivative of pACCMV.pLpA with an engineered PmeI restriction site. Co-transfections and viral purification were completed as described for AdCMV-rGast in Chapter 3.

Quantitative PCR Measurements of RNA Levels

Quantitative PCR reactions were performed using the ABI PRISM® 7000 sequence detection system and Applied Biosystems Taqman® primers and probes (An, Muoio *et al.* 2004). Pre-validated Taqman® primer and probe sets used are listed in Appendix C. Triplicate reactions from independent RNA samples were carried out in a

final volume of 25 μL containing 20 ng of cDNA template. Samples were normalized to 18S rRNA expression levels and compared to values obtained for cells treated with appropriate control adenoviruses (AdCMV-βGal, AdCMV-GFP, or Ad-siScrambled).

BrdU Labeling, Immunohistochemistry, and Immunofluorescence in Primary Rat Islets

Three days post-viral treatment, primary rat islets were incubated in a 1/100 dilution of 5-bromo-2'-deoxyuridine (BrdU) labeling reagent (Invitrogen) for 24 hours. Islet immunohistochemistry was performed as previously described (Cozar-Castellano, Takane *et al.* 2004). Pools of 200 islets were collected and washed three times with 1 ml of PBS with centrifugation at 300 x G for 3 minutes between washes. Islets were fixed in Bouin's solution for 2 hours in 1.7 mL microcentrifuge tubes and maintained in 10% neutral-buffered formalin. Prior to embedding in paraffin, islets were centrifuged at 300 x G for 3 minutes and the formalin aspirated. 50 μL of Affi-Gel blue bead slurry (Bio-Rad) was added to the islets to aid in visualization during sectioning. Agar was gently added to the mixture in the microcentrifuge tube and allowed to solidify. The agar was removed from the tube and embedded in paraffin. 5 μm serial sections on glass slides were deparaffinized with xylene and rehydrated in a graded series of ethanol. Antigen retrieval was performed by microwaving the slides for 13.5 minutes in 10 mM sodium citrate buffer with 0.05% Tween-20, pH 6.0. Endogenous peroxidase activity was quenched with 3% H_2O_2 in methanol for 10 minutes. BrdU immunodetection was carried out using the BrdU detection kit (Invitrogen) which uses a biotinylated mouse anti-BrdU antibody (1 hour incubation at room temperature). 3,3'-diaminobenzidine-tetra-

hydrochloride (DAB) was used as the HRP substrate for colorimetric detection. For insulin immunostaining, the HistoMouse-MAX kit (Invitrogen) was used with pre-diluted guinea pig anti-insulin (Invitrogen) primary antibody incubated for 1 hour at room temperature. DAB (brown) or 3-amino-9-ethylcarbazole (AEC, red) was used as HRP substrate for secondary antibody colorimetric detection. In the case of BrdU and insulin co-staining, detection of anti-insulin antibody was used with an alkaline phosphatase-conjugated (AP) secondary antibody using Fast Red (Invitrogen) as the AP substrate. Sections were lightly counterstained with hematoxylin.

Islet sections for immunofluorescence were prepared in an identical manner to that described for immunohistochemistry. The following primary antibodies and dilutions were used: mouse anti-BrdU conjugated to Alexa fluor546 (1:25, Invitrogen), guinea pig anti-insulin (1:50, Invitrogen), secondary FITC-conjugated goat anti-guinea pig (1:1000, Jackson Labs). Images were acquired on an Eclipse E400 microscope (Nikon) with a Photometrics Coolsnap® color CCD camera (Roper Scientific).

Immunoblot analysis

Total protein extracts were made by lysing harvested primary rat islets in protein extract buffer (MPER, Pierce) containing protease and phosphatase inhibitors and by briefly sonicating. 20 μg aliquots of protein were resolved on 4-12% Bis-Tris-HCl buffered (pH = 6.4) polyacrylamide gels (Invitrogen) and transferred to polyvinylidene difluoride (PVDF) membranes. Primary antibodies and dilutions were as follows: anti-cyclin E (1:500, Santa Cruz Biotechnologies), anti-phospho Akt-Ser^{473} (1:1000, Cell Signal), anti-phospho STAT3-Tyr^{705} (1:1000, Cell Signal), and anti-Akt (1:1000, Cell

Signal). All dilutions were in tris buffered saline with 5% bovine serum albumin (Sigma) and 0.1% Tween-20 (Sigma). PVDF membranes were incubated overnight at 4^{o} C and primary antibodies were detected using appropriate HRP-linked secondary antibodies (1 hour incubation) and visualized using ECL Advance (Amersham) on a Versadoc 5000 (Bio-Rad). Membranes were stripped (ReBlot, Chemicon) and re-probed with additional antibodies as described above.

Statistical Methods

The two-tailed Student's t-test was used in statistical analysis of the data to calculate *p* values. Unequal variances were assumed in all calculations. *P* values ≤ 0.05 were considered significant.

Results

Gastrin overexpression induces robust ^{3}H-thymidine incorporation in primary rat islets

The ability of gastrin to stimulate islet proliferation was investigated in isolated rat islets using similar techniques to those employed in our analysis of gastrin function in the INS-1 cell lines. For these experiments, purified stocks of all the adenoviruses used were made via the CsCl method outlined in Materials and Methods.

Islets were isolated from adult (6 to 8 weeks old) Wistar rats weighing 200-250 g and cultured in RPMI-1640 media with 10% fetal calf serum (FCS) for 2 hours prior to treatment. Groups of 200 islets were treated with either 100 ng/mL recombinant rat gastrin-17 peptide (Sigma), 4 μL AdCMV-βGal, or 20 μL AdCMV-rGast for 18 hours. At the end of treatment, media was changed in all groups, with gastrin-17 peptide maintained at 100 ng/mL in the peptide treated group. Culture was maintained for 48 hours before the islets were transferred to media containing 1 μCi/mL ^{3}H-thymidine and allowed to incubate for an additional 24 hours. After ^{3}H-thymidine incubation, 3 groups of 25 islets were isolated from each treatment group and subjected to a glucose-stimulated insulin secretion (GSIS) assay followed by precipitation of total protein and DNA using the tricholoroacetic acid method described in Materials and Methods. Radioactivity was measured for each sample and the counts normalized to protein.

In primary rat islets, preprogastrin is virtually undetectable. AdCMV-rGast stimulated preprogastrin mRNA expression to Ct values of 18-20, a level approximately 20-fold higher than the maximal levels stimulated in the INS-1 cell experiments described in Chapter 3. The level of gastrin accumulated in the media from AdCMV-

rGast treatment of the islets was comparable to that seen in the INS-1res cell lines (>500 pmol/L, the sensitivity range of the gastrin RIA). This level of preprogastrin expression caused a greater than 4-fold induction of ^{3}H-thymidine incorporation relative to AdCMV-βGal control islets (Figure 4-1). In contrast, gastrin-17 peptide-treated islets showed no measurable increase in proliferation as measured by ^{3}H-thymidine incorporation. An additional experiment in isolated primary rat islets was conducted to test if sulfated gastrin-17, gastrin-34, or the combination of TGFα with any of these peptides could induce β cell proliferation. None of these manipulations altered ^{3}H-thymidine incorporation in isolated primary rat islets (data not shown).

The experiments in Figure 4-1 were carried out in media that contained 10% fetal calf serum (FCS). To account for potential growth stimulating properties of FCS, we conducted additional ^{3}H-thymidine incorporation assays on AdCMV-rGast treated islets in the presence and absence of FCS (Figure 4-2). Importantly, the replacement of 10% FCS with 0.1% bovine serum albumin did not abolish the ability of AdCMV-rGast to increase ^{3}H-thymidine incorporation. Furthermore, as seen with the INS-1 cell lines, the addition of TGFα to the culture media at 10 or 100 ng/mL had no effect on islet proliferation as measured by ^{3}H-thymidine incorporation (data not shown).

Overexpression of gastrin increases BrdU immunoreactivity in β cells

Although islets are primarily composed of the insulin-secreting β cells, there are a significant number of other cell types present, such as glucagon producing α cells, somatostatin producing δ cells, endothelial cells, and fibroblasts. The robust increase in ^{3}H-thymidine incorporation seen in Figures 4-1 and 4-2 could result from proliferation of

one of these cell types, and not necessarily the β cells. To validate or refute the hypothesis that islet β cells are replicating upon preprogastrin overexpression, we cultured islets in the nucleotide analog 5-bromo-2'-deoxyuridine (BrdU) and measured its incorporation via immunohistochemistry. As shown in Figure 4-3A, islets treated with AdCMV-rGast exhibited a significant increase in the number of BrdU positive cells. Sections from AdCMV-rGast treated islets showed significant numbers of BrdU positive cells (10.3±1.8 cells per section) relative to AdCMV-GFP treated controls (1.9±1.4 positive cells per section) with a $p < 0.005$. The ratio of BrdU positive cells in AdCMV-rGast to AdCMV-GFP is 4.7±1.8 to 1, which approximates the fold change in ^{3}H-thymidine incorporation seen with AdCMV-rGast treatment.

To establish the identity of the replicating cells, sections stained for BrdU were co-stained with antibodies specific for insulin or glucagon and the number of co-stained cells counted. 82±5% of the BrdU positive cells co-stained with insulin, while 17±6% co-stained with glucagon. While the majority of cells proliferating in AdCMV-rGast treated islets were β cells, the ratio of α to β cell replication is approximately equal to the ratio of these cells within an islet (Cerf *et al.* 2005). A representative slide showing BrdU co-staining with insulin (red), but not glucagon (blue), is presented in Figure 4-3B. Immunofluorescence was also performed on sections from AdCMV-rGast treated islets with similar results (Figure 4-4). BrdU positive cells (red) co-staining with insulin positive cells (green) are noted with white arrows.

Cholecystokinin overexpression, like gastrin, induces robust ^{3}H-thymidine incorporation in primary rat islets

A recombinant adenovirus expressing mouse preprocholecystokinin (preproCCK) under control of the CMV promoter (AdCMV-mCCK) was constructed as described in Materials and Methods. Islets were isolated as previously described in the AdCMV-rGast experiments and treated with the following: 100 ng/mL recombinant rat gastrin-17 or CCK-8 peptide, 4 μL AdCMV-βGal, 20 μL AdCMV-rGast, or 3 μL AdCMV-mCCK. PreproCCK mRNA levels were robustly stimulated over basal (Ct 17 versus Ct 32 in controls) as measured by qPCR. This stimulation resulted in a 9-fold increase in proliferation as measured by ^{3}H-thymidine incorporation (Figure 4-5). Peptide treatments with gastrin-17 and CCK-8 had no measurable effect on islet proliferation.

AdCMV-mCCK overexpression stimulates ^{3}H-thymidine incorporation in primary islets of mice lacking both CCK1R and CCK2R

In human colon cancers and intestinal cell lines, proliferation mediated by exogenous gastrin requires signaling through the cholecystokinin receptor 2 (CCK2R) (Upp *et al.* 1989). Our experiments with exogenously administered gastrin-17 in islets and INS-1 cells, as well as the conditioned media exchange between the INS-1 and INS-1_{res} cell lines, suggests that the classical gastrin receptor is not involved in β cell proliferation in response to preprogastrin or preproCCK overexpression. To definitively establish the necessity of CCK1R or CCK2R in β cell proliferation due to preprogastrin or preproCCK overexpression, islets from CCK1/2R double knockout mice were treated with AdCMV-rGast and AdCMV-mCCK.

Islets were isolated from 10 to 12 week old wild type (WT) and CCK1/2R double knockout 129SvEv mice as described in Materials and Methods. Groups of 150 islets from both WT and CCK1/2R double knockout mice were treated with 4 μL AdCMV-βGal, 20 μL AdCMV-rGast, or 10 μL AdCMV-mCCK for 18 hours. Islets were cultured per previous experiments for an additional 48 hours and then incubated in media containing ^{3}H-thymidine. Unexpectedly, AdCMV-rGast-treated wild type (WT) mouse islets showed no changed in ^{3}H-thymidine incorporation compared to AdCMV-βGal-treated controls. AdCMV-rGast treatment also did not stimulate proliferation in CCK1/2R double knockout mouse islets. In contrast, AdCMV-mCCK treatment caused a robust 4.5-5 fold induction in growth as measured by ^{3}H-thymidine incorporation in both WT and CCK1/2R double knockout mouse islets, with no statistical difference in the responses measured in the WT and CCK1/2R knockout groups (Figure 4-6).

Overexpression of gastrin and CCK do not negatively affect normal β cell glucose-stimulated insulin secretion

To date, some progress has been made in developing methods for stimulating β cell proliferation (Welsh, Welsh *et al.* 1988; Beattie *et al.* 1991; Beattie *et al.* 1996; Otonkoski *et al.* 1996; Yuan *et al.* 1996; Beattie *et al.* 1997; Laybutt *et al.* 2002; Cozar-Castellano, Takane *et al.* 2004; Roccisana *et al.* 2005; Suarez-Pinzon, Lakey *et al.* 2005; Wente *et al.* 2006). However, most of the reported methods that induce β cell proliferation are accompanied by significant loss of function, as measured by glucose-stimulated insulin secretion (GSIS) (Hohmeier and Newgard 2005). We measured GSIS to determine if preprogastrin or preproCCK overexpression altered β cell function.

Overexpression of either preprogastrin or preproCCK caused a 77±23% and 75±28% increase, respectively ($p < 0.05$ for both), in basal insulin secretion at 2.5 mM glucose (Figure 4-7). For AdCMV-rGast treated islets, this resulted in a small, but statistically significant, decrease in the fold responsiveness of the treated islets (2.7±0.6 versus 4.0±0.7, $p < 0.05$). At stimulatory glucose concentrations (16.7 mM), there was no statistically significant potentiation of insulin secretion by preprogastrin overexpression, but there was a 51.4±14% increase in GSIS with AdCMV-mCCK treatment. There was also no significant difference in fold responsiveness of AdCMV-mCCK treated islets versus AdCMV-βGal treated control islets.

Gastrin and CCK potentially work through different pathways to induce primary islet β cell proliferation

To investigate the mechanisms by which overexpression, but not extracellular administration, of gastrin and CCK can induce proliferation in islets, we first evaluated the mRNA expression levels of the trefoil factor 3 (TFF3) gene. This gene has come to the attention of our laboratory as another gene that is upregulated in the INS-1_{res} cell lines (Appendix A) and is currently under investigation by Patrick Fueger, Ph.D. in our laboratory for its growth-promoting effects in primary rat islets. TFF3 overexpression via a recombinant adenovirus (AdCMV-TFF3) is able to increase β cell proliferation in isolated rat islets approximately 4-fold, as measured via ^{3}H-thymidine incorporation (Figure 4-8, used with permission). Dr. Fueger is able to reduce AdCMV-TFF3 induced proliferation by co-overexpressing a dominant negative form of Protein Kinase B (Akt). The impairment of AdCMV-TFF3-induced β cell proliferation by AdCMV-dnAkt

indicates involvement of the PI_3/Akt signaling pathway in AdCMV-TFF3 induced proliferation. TFF3 expression was measured via qPCR in AdCMV-rGast and AdCMV-mCCK treated islets and found to be significantly (22-fold) upregulated in response to preprogastrin, but not preproCCK, overexpression (Figure 4-9).

Modeled after the TFF3/dnAkt experiments, preprogastrin and preproCCK were each co-overexpressed with dnAkt in primary rat islets and growth measured using the ^{3}H-thymidine assay (Figure 4-10A). Despite significant expression of dnAkt (Figure 4-10B), there was no statistically significant reduction in thymidine incorporation caused by dnAkt expression in the AdCMV-rGast and AdCMV-mCCK treated islets ($p = 0.75$ and $p = 0.47$, respectively). Furthermore, there was no significant increase in Akt phosphorylation (Ser^{473}) in any of the treatment groups, including stimulation with exogenous insulin despite strong expression of the dnAkt construct as measured by immunoblot (Figure 4-10B). Additional immunoblots were performed on p70S6K, β-catenin, phosphor-GSK3α/β with either negative or inconclusive results (data not shown).

We also evaluated various genes involved in the cell cycle. Previous work in our lab on the homeobox transcription factor Nkx6.1 by Jonathan Schisler, Ph.D. has demonstrated that overexpression of Nkx6.1 via a recombinant adenovirus has similar growth-promoting effects as that seen with AdCMV-rGast and AdCMV-mCCK treatments (Schisler *et al.* In Review). AdCMV-Nkx6.1 overexpression induces a significant upregulation of a number of the cell cycle regulatory genes, including cyclins A, B, E, and Cdk-2. To investigate the effects of preprogastrin and preproCCK expression on cell cycle regulatory genes, rat islets were treated with AdCMV-rGast, AdCMV-mCCK, or AdCMV-βGal and expression of retinoblastoma protein (pRb),

cyclins A2, B1, B2, D1, D2, E1, CDC25A, Cdk1, and Cdk4 were measured using qPCR. Of these genes, there was a significant increase in mRNA expression of cyclins A2, B1, B2, E1, and Cdk1 (Figure 4-11A) in islets treated with AdCMV-mCCK, but not AdCMV-rGast. Within this group, cyclin E1 demonstrated the most significant induction (8.3 fold). Moreover, immunoblot analysis of cyclin E protein levels demonstrated a clear upregulation of expression in response to AdCMV-mCCK treatment, but not AdCMV-rGast treatment, relative to AdCMV-βGal controls (Figure 4-11B). The significance of this upregulation is not yet known, but may be important in the transition of differentiated cells, like the β cell, out of the quiescent G_0 phase and into active replication (Honda *et al.* 2005). Interestingly, as shown in Figure 4-11B, co-overexpression of dnAkt with preproCCK resulted in a substantial reduction of cyclin E protein levels compared to islets with preproCCK overexpression alone, despite equal rates of ^{3}H-thymidine incorporation in the two groups (Figure 4-10).

Overexpression of a constitutively active form of the transcription factor STAT3 has recently been shown to induce islet β cell proliferation (Tsukiyama *et al.* 2006). This result along with the known involvement of STAT3 in CCK2R signaling (Figure 1-2) prompted us to examine AdCMV-rGast and AdCMV-mCCK overexpressing islets for phosphorylation or upregulation of STAT3 (Figure 4-12). AdCMV-mCCK + AdCMV-βGal treated islets show significant phosphorylation of STAT3 at tyrosine 705. Interestingly, treatment with AdCMV-dnAkt + AdCMV-mCCK partially reverses this phosphorylation event. Like the dnAkt reversal of AdCMV-mCCK induced cyclin E upregulation, the significance of dnAkt reversal of STAT3-Tyr^{705} phosphorylation is not yet known. AdCMV-rGast islets showed no phosphorylation of STAT3.

Adenoviruses expressing mutant forms of the preprogastrin gene were constructed using the PCR stitching method described in Materials and Methods. The first of these adenoviruses, AdCMV-GastΔS, was constructed with the Tyr^{87} sulfation site mutated to a phenylalanine. The gastrin radioimmunoassay was used to verify that this virus properly expresses the rGastΔS construct (data not shown). AdCMV-GastΔS treatment of isolated rat islets was able to induce a dose-dependent increase in ^{3}H-thymidine incorporation similar to that seen with AdCMV-rGast treatment (Figure 4-13).

Discussion

The two types of diabetes share a common mechanism of an insufficient β cell mass necessary to maintain glucose homeostasis. In Type 1 diabetes, this deficiency results from autoimmune β cell destruction. In Type 2 diabetes, the existing β cell mass is insufficient to compensate for the increased metabolic demands placed upon it by the body, either because of peripheral insulin resistance or the eventual failure of the β cells caused by glucose and lipid toxicity. The studies presented in this dissertation began with the goal of developing novel and robust genetic engineering strategies that could be used to protect insulin-secreting cells from the immune system. To this end, we used the INS-1_{res} model system to screen for candidate genes involved in cytokine resistance and, later, β cell replication. Here we provide evidence that one of the genes from that screen, preprogastrin, stimulates islet β cell proliferation without impairing β cell function. Furthermore, our work on gastrin led to the discovery that preprocholecystokinin (preproCCK), a gene closely related to preprogastrin, might also be able to stimulate islet β cell proliferation.

Previous work has shown that gastrin is upregulated in islets in the fetal pancreas during islet expansion (Greider and McGuigan 1971; Larsson, Rehfeld *et al.* 1976; Brand, Andersen *et al.* 1984). Similarly, CCK is upregulated in the exocrine pancreas during bursts of proliferation (Brand and Morgan 1981; Gasslander *et al.* 1990; Rivard, Guan *et al.* 1991; Povoski *et al.* 1994; Yoshinaga, Ishizuka *et al.* 1996), but only two results to date have implicated CCK in islet β cell proliferation (Kuntz, Pinget *et al.* 2004; Raess *et al.* In Review). Numerous studies have demonstrated that extracellular gastrin can promote cell cycle progression by binding to CCK2R (Greider and McGuigan 1971;

Larsson, Rehfeld *et al.* 1976; Smith and Watson 2000; Rooman and Bouwens 2004; Suarez-Pinzon, Yan *et al.* 2005), but an intracellular mode of action for the gastrin gene has not been demonstrated.

In the studies presented in this chapter we demonstrate that the proliferation previously reported in INS-1 cells in response to overexpression of preprogastrin is reproducible in isolated primary rat islets, with a robust 4-fold increase of ^{3}H-thymidine incorporation. As observed in the INS-1 cell lines, exogenous administration of recombinant rat gastrin-17, sulfated gastrin-17, and gastrin-34, alone or in combination with the EGFR ligand TGFα, had no effect on proliferation. This result appears to contradict the published literature, until we take into account the model systems that were used previously to show gastrin-induced β cell mass expansion. Of the studies reviewed in Chapter One, all required some change in the homeostatic balance of the pancreas in order to promote β cell growth. These included chemical damage to β cells with alloxan or streptozotocin (Brand, Tagerud *et al.* 2002; Rooman and Bouwens 2004), surgical injury such as ductal ligation or partial pancreatectomy (Hultquist, Karlsson *et al.* 1979; Rooman, Lardon *et al.* 2002), or a significant genetic change such as transgenic overexpression or abnormal immune function (NOD mice) (Wang, Bonner-Weir *et al.* 1993; Suarez-Pinzon, Yan *et al.* 2005). In our experiments, islets were not pretreated chemically or harvested from transgenic animals in order to make them permissive to growth. Furthermore, while we cannot exclude the possibility that CCK2R mediates growth in response to preprogastrin overexpression in β cells, this seems unlikely as we were unable to detect CCK2R mRNA in isolated rat islets using qPCR. This finding is consistent with previous immunofluorescence studies that have limited the scope of

CCK2R expression to the somatostatin-producing δ cells within the islet (Morisset, Wong *et al.* 2000; Morisset, Julien *et al.* 2003).

In some growth systems the presence of fetal calf serum (FCS) promotes the induction or potentiation of growth, irrespective of the design of the experimental manipulation. To test for this variable in our experiments, isolated rat islets were treated with AdCMV-GFP or AdCMV-rGast and grown in either normal culture media with 10% FCS or 0.1% bovine serum albumin. As shown in Figure 4-2, the presence of FCS in the culture media did not have a significant impact on the growth-promoting effect of AdCMV-rGast treatment.

To identify the cell type(s) undergoing proliferation in AdCMV-rGast treated islets, 5-bromo-2'-deoxyuridine (BrdU) was incorporated into treated islets and immunohistochemistry performed on islet sections. BrdU-treated islets showed a significant increase in anti-BrdU immunoreactivity in β cells (Figure 4-3A). Incorporated BrdU was co-stained for insulin (β cells) or glucagon (α cells) with anti-insulin or anti-glucagon antibodies to identify and quantify the proliferating cells (Figure 4-3B). The majority of proliferating cells were β cells (82±5%), closely matching the percentage of cells in islets that are β cells. However, some glucagon-producing α cells did co-stain with BrdU, indicating that the insulin-producing β cells were not the only cells to undergo replication. Repeated attempts to co-stain BrdU with somatostatin, which is produced from a very small minority of cells (δ cells) in the islet, were unsuccessful due to weak or non-existent anti-somatostatin staining of the sectioned islets. Immunofluorescence experiments confirmed the co-localization of the majority of BrdU positive cells with insulin producing cells (Figure 4-4).

Through a collaboration with Dr. Alan Attie (University of Wisconsin) the gene encoding mouse preproCCK was cloned into an adenovirus (AdCMV-mCCK) and tested alongside rat preprogastrin for growth-promoting properties. PreproCCK overexpression, but not mature CCK-8 peptide, caused a 9-fold increase in proliferation as measured by ^{3}H-thymidine incorporation (Figure 4-5).

In an attempt to establish if preprogastrin and preproCCK overexpression require the cholecystokinin receptors to stimulate proliferation, we conducted ^{3}H-thymidine measurements of growth in islets isolated from 129SvEv mice in which both CCK1R and CCK2R had been knocked out (Chen *et al.* 2002; Wang *et al.* 2004), as part of our collaboration with the Attie lab. In three independent experiments, both wild type and CCK1/2R double knockout mice demonstrated a 4.5-5 fold induction of ^{3}H-thymidine incorporation after treatment with AdCMV-mCCK (Figure 4-6). These experiments confirm that neither CCK1R nor CCK2R are required for islet proliferation upon preproCCK overexpression.

Unfortunately, in all of these experiments, AdCMV-rGast failed to induce any proliferation in wild type mouse islets relative to AdCMV-βGal-treated control islets. In the absence of this important positive control, we are presently unable to make any conclusions about the requirement of CCK1R and CCK2R for promoting growth with preprogastrin overexpression. Radioimmunoassay of the culture media of the WT mouse islets indicated significant accumulation of processed gastrin (~500 pmol/L) which is consistent with our experiments in rat islets. These data indicate that, unlike our findings in rat islets, rat preprogastrin expression in mouse islets is not sufficient to induce β cell replication. It is possible that this negative result stems from a species difference, as

there are substantial N-terminal differences in rat and mouse preprogastrin. It is also possible that due to the sequence differences in rat preprogastrin, the processing enzymes in mice (prohormone convertases and carboxypeptidase E) are unable to properly process progastrin to the peptide form necessary to induce β cell proliferation. Additionally, we have only attempted this experiment in one mouse subtype, the 129SvEv mice. It is possible that the negative result from these experiments is limited to this particular mouse strain.

Inducing β cell proliferation is of little value to our end goal of therapeutic intervention if the resulting β cell mass is not functional. Therefore, we measured GSIS in all of the experiments in which ^{3}H-thymidine incorporation was measured. Both AdCMV-rGast and AdCMV-mCCK induced small, but significant, increases in basal GSIS at 2.5 mM glucose concentrations (Figure 4-7). The reasons for these increases in basal GSIS are currently unknown. In AdCMV-rGast treated rat islets the increase in basal insulin secretion was large enough to significantly impair the fold responsiveness of the islets when switched from low to high glucose. In AdCMV-mCCK treated islets the fold responsiveness was equivalent to that seen in AdCMV-βGal treated controls. AdCMV-mCCK treatment caused a significant increase in GSIS at high glucose (16.7 mM). The change in GSIS at high glucose upon AdCMV-rGast treatment trends upward as well, but is not statistically significant in these studies. Our group has recently demonstrated that overexpression of the β cell specific transcription factor Nkx6.1 induces β cell proliferation that is accompanied by a significant *increase* in function (Schisler, Fueger *et al.* In Review). PreproCCK now joins Nkx6.1 as a rare molecule

capable of eliciting both an increase in replication and insulin secretion from islet β cells at stimulatory glucose concentrations.

Elucidating the molecular mechanisms that drive β cell proliferation in response to preprogastrin and preproCCK overexpression is a significant task, especially considering that at least for preproCCK this mechanism is independent of CCK1R and CCK2R. Our lab has been studying another gene, trefoil factor 3 (TFF3), which, like gastrin and CCK, can promote β cell proliferation when overexpressed via a recombinant adenovirus. We have discovered that TFF3 induction of β cell proliferation is mediated at least in part by Akt activation (Figure 4-8, used with permission by Patrick Fueger). In these studies, Dr. Fueger was able to partially block TFF3-mediated β cell proliferation by co-overexpressing a dominant negative form of Akt (Suhara *et al.* 2001). AdCMV-dnAkt overexpresses an Akt protein that has had threonine 308 and serine 478 substituted with alanines, preventing phosphorylation of the protein and subsequent activation of the Akt signaling cascade. This construct still needs further validation in the INS-1 cell system and primary rat islets to verify the dominant negative phenotype of the overexpressed Akt. To accomplish this, downstream targets of Akt such as mTOR, GSK3β, and BAD will be evaluated for phosphorylation using immunoblots after stimulation of INS-1 cell lines with TGFα in serum-free media.

TFF3 expression was measured in rat islets treated with AdCMV-rGast and AdCMV-mCCK (Figure 4-9). Preprogastrin overexpressing islets demonstrated a 22-fold increase in TFF3 expression, but preproCCK overexpressing islets showed no significant change in TFF3 expression. This could indicate that preprogastrin and preproCCK stimulate islet β cell proliferation through different mechanisms. Similar to

the experiments conducted by Dr. Fueger, the AdCMV-dnAkt was used to investigate the involvement of Akt signaling in preprogastrin and preproCCK induced β cell proliferation (Figure 4-10). Despite strong expression of dnAkt (lower band, panel B), there was no significant change in β cell proliferation as measured by ^{3}H-thymidine incorporation. However, because the vast majority of cells in the islet are non-replicating, even in our experiments inducing proliferation with preprogastrin and preproCCK, it is extremely difficult to measure for Akt phosphorylation in the few cells that are actively dividing. Presumably, because of this difference, the signaling through the Akt pathway will be significantly less from TTF3 upregulation secondary to AdCMV-rGast treatment and may not be detectable in these islets using immunoblot analysis. Until further validation of the dnAkt adenovirus is completed, it is not possible to ascertain if Akt signaling is necessary for AdCMV-rGast and AdCMV-mCCK induced β cell proliferation. Currently, AdCMV-rGast and AdCMV-mCCK islet total protein extracts have been evaluated for phosphorylation of GSK3β, β-catenin, and phospho-Akt with either negative or inconclusive results. Phosphorylation of p70S6K and ribosomal protein S6 appears to be stimulated in AdCMV-mCCK, but not AdCMV-rGast, -treated islets, indicating possible involvement of the Akt pathway in AdCMV-mCCK treated islets. Further studies will be required to investigate this point.

Current understanding of the genes involved in cell cycle progression in the β cell is poor compared to other cell types. Our group has recently made significant headway in this field through studies on the Nkx6.1 homeobox transcription factor. Jonathan Schisler discovered that overexpression of Nkx6.1 causes a significant induction of several cell cycle genes (Schisler, Fueger *et al.* In Review). Following this line of investigation, we

investigated cell cycle gene changes in response to overexpression of preprogastrin and preproCCK (Figure 4-11A). AdCMV-mCCK induced clear upregulation of mRNAs encoding several cell cycle regulatory proteins, including cyclins A2, B1, B2, E1, and Cdk1. Preprogastrin overexpression caused an upward trend in expression of several of these genes (cyclins A2, B1, E1), but none were significant at a *p* value cutoff of 0.05 (*p* = 0.08 and higher for cyclins A2, B1, E1). With preproCCK overexpression, cyclin E upregulation is detectable at the protein level (Figure 4-11B). Interestingly, co-overexpression of dnAkt with preproCCK blunts the upregulation of cyclin E, but as previously discussed has no negative impact on β cell proliferation (Figure 4-10). The lack of a significant increase in the mRNA expression of cell cycle regulatory genes in response to preprogastrin overexpression indicates that the proliferative response seen with this manipulation may not be occurring at a genetic level, or may not be discernable based on the sensitivity of qPCR. It is very possible that regulation of cell cycle progression occurs instead through phosphorylation events or protein stabilization (*e.g.* changes in ubiquitinization). These possibilities have not yet been tested for in the AdCMV-rGast treated islets.

Recent work by Tsukiyama *et al.* (2006) has demonstrated that STAT3 overexpression can promote β cell proliferation. We investigated whether STAT3 was phosphorylated in response to preprogastrin and preproCCK overexpression (Figure 4-12). PreproCCK, but not preprogastrin, induced measureable phosphorylation of STAT3 at tyrosine 705. This phosphorylation event has been shown to be necessary for STAT3 translocation into the nucleus (Bild *et al.* 2002). STAT3-Tyr^{705} phosphorylation is

somewhat blunted in islets overexpressing both preproCCK and dnAkt. The significance of this finding is not yet known.

To gain further insight into the mechanism by which preprogastrin overexpression can stimulate β cell proliferation, a group of adenoviruses expressing various mutants of preprogastrin have been constructed. The first of these viruses, AdCMV-GastΔS, contains the full rat preprogastrin gene with a single mutation made so that tyrosine 87 is now a phenylalanine. This mutation results in the removal of a single –OH group from the side chain of the amino acid, thereby preventing sulfation by the sulfotransferase during progastrin peptide processing. The gastrin RIA was used to verify proper expression of the mutant. Treatment of isolated rat islets with AdCMV-GastΔS resulted in a dose-dependent increase in ^{3}H-tymidine incorporation (Figure 4-13), indicating that the sulfation site in preprogastrin is not necessary for the gene to drive β cell proliferation**.**

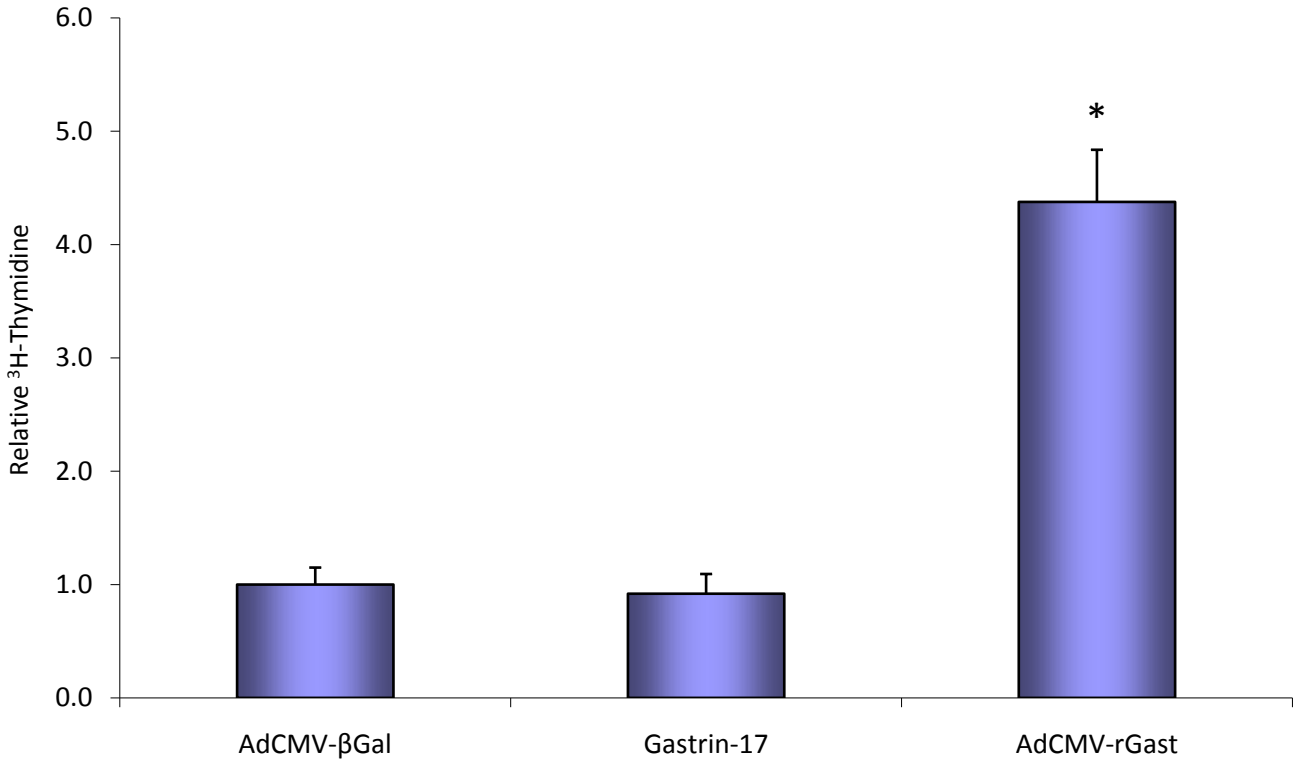


Figure 4-1. AdCMV-rGast induces robust proliferation in isolated rat islets. Islets were isolated from adult Wistar rats per Methods and Materials. 200 individual islets were grouped and exposed to either 4 μL purified AdCMV-βGal adenovirus, 100 ng/mL recombinant gastrin-17 peptide, or 20 μL purified AdCMV-rGast adenovirus for 18 hours, followed by a media change. After 48 hours and one additional media change, the islets were transferred to media containing 1 μCi/mL ^{3}H-thymidine and allowed to incubate for 24 hours before harvesting. Gastrin-17 peptide was maintained throughout all media changes but adenoviral treatments were not. For harvesting, 3 groups of 25 islets were isolated from each treatment group. Counts were determined and then normalized to protein content. Treatment exogenously with recombinant gastrin-17 resulted in media gastrin levels approximately twice that of the AdCMV-rGast treatment group, as measured by radioimmunoassay (data not shown). Data represent the mean ± SEM for eight independent experiments. * indicates $p \leq 0.05$ versus the AdCMV-βGal control group.

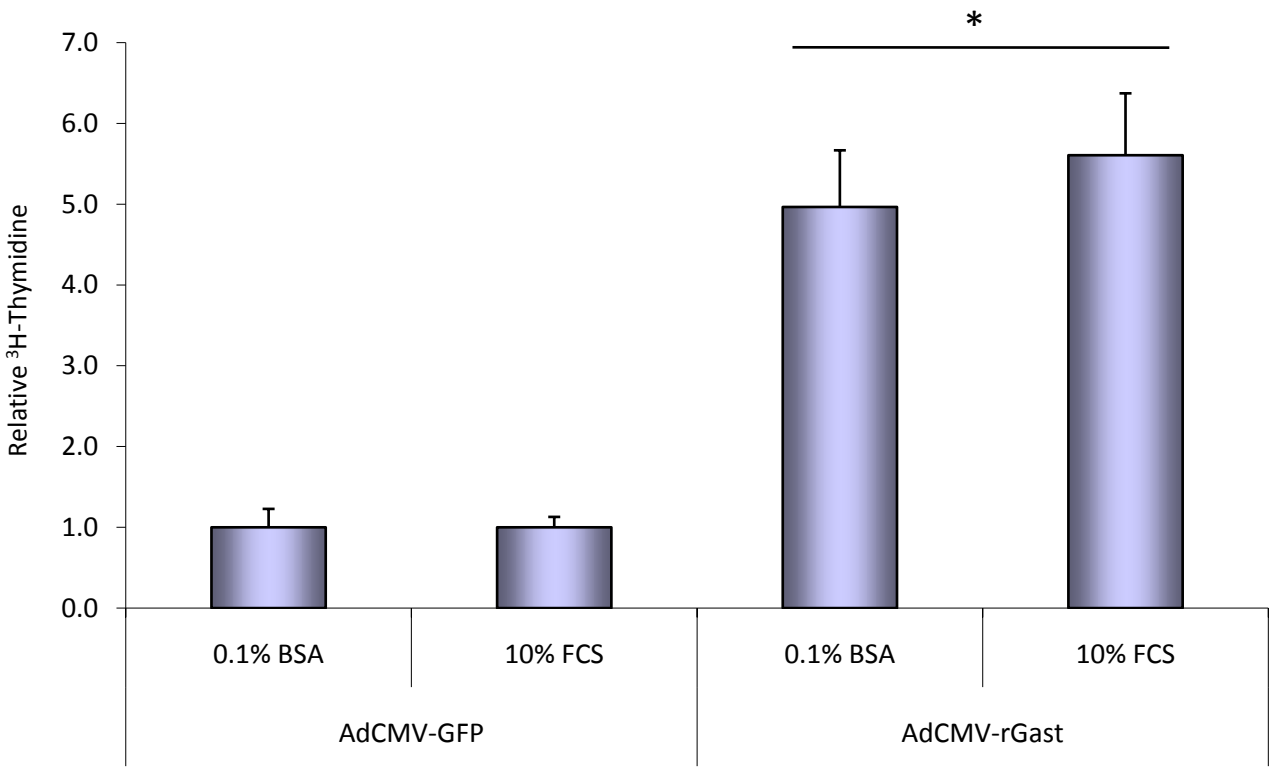


Figure 4-2. Islet proliferation induced by AdCMV-rGast is independent of culture media serum conditions. Islets were isolated from adult Wistar rats per Methods and Materials and Figure 4-1. 200 individual islets were grouped and exposed to either 20 μL purified AdCMV-GFP or AdCMV-rGast adenoviruses for 18 hours, followed by a media change to RPMI-1640 media containing 0.1% bovine serum albumin (BSA) or 10% fetal calf serum (FCS). After 48 hours and one additional media change, the islets were transferred to media containing 1 μCi/mL ^{3}H-thymidine and allowed to incubate for 24 hours before harvesting. 3 groups of 25 islets were isolated from each treatment group. Counts were determined and then normalized to protein content. * indicates $p \leq 0.05$ vs. the AdCMV-GFP control group.

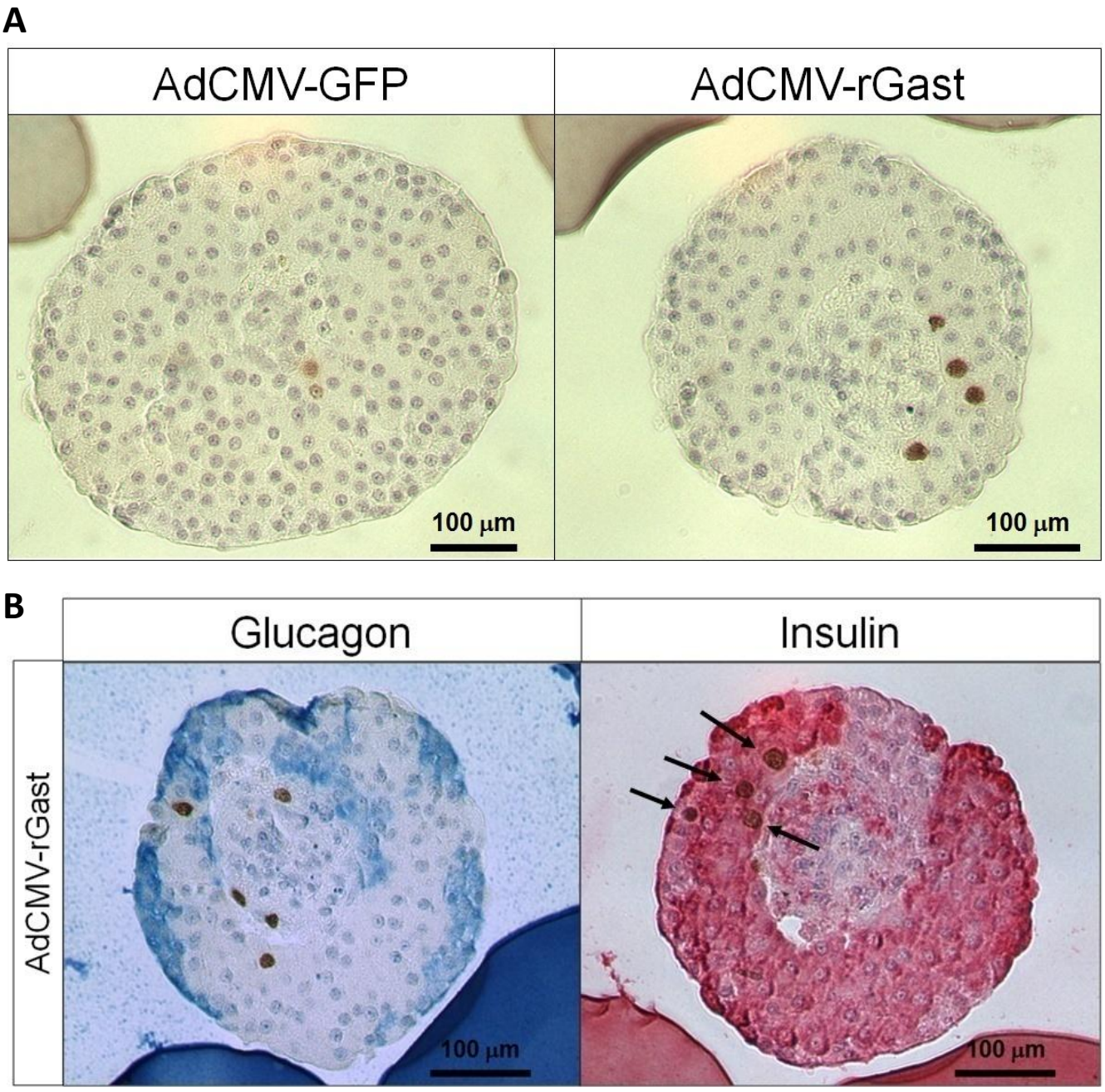


Figure 4-3. Immunohistochemistry of BrdU incorporation in primary rat islets treated with AdCMV-rGast. Islets were isolated from adult Wistar rats and treated with adenoviruses per Figure 4-1. 24 hours prior to harvest, culture media was treated with a 1:100 dilution of the nucleotide analog 5-bromo-2'-deoxyuridine (BrdU). Islets were harvested, fixed, and embedded in agar for paraffin sectioning. Sectioned slides were stained with anti-BrdU antibodies. **A.** Representative slide of AdCMV-GFP control and AdCMV-rGast islet sections. BrdU positive nuclei (brown) are clearly present in the AdCMV-rGast-treated islets. **B.** Co-staining with BrdU and glucagon or insulin antibodies. Cells co-positive for glucagon and BrdU were rarely detected. Cells co-positive for insulin and BrdU are highlighted by arrows.

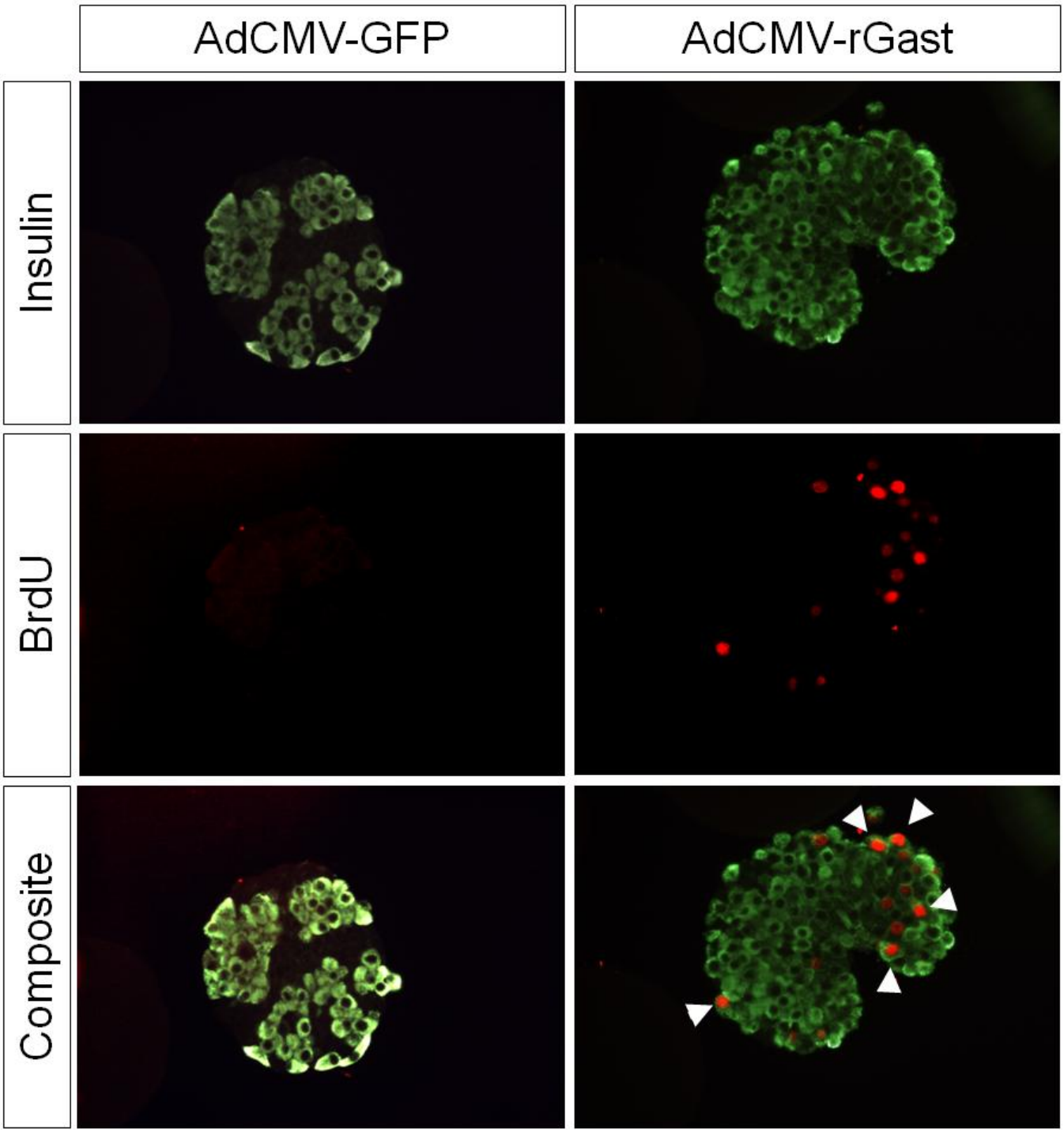


Figure 4-4. Immunofluorescence of BrdU incorporation in primary rat islets treated with AdCMV-rGast. Islets were isolated, treated with AdCMV-rGast, and sectioned per Figure 4-5. Representative slide of AdCMV-GFP control and AdCMV-rGast islet sections. BrdU positive nuclei (red) are clearly present in greater numbers in the AdCMV-rGast-treated islets and co-stain with insulin positive cells.

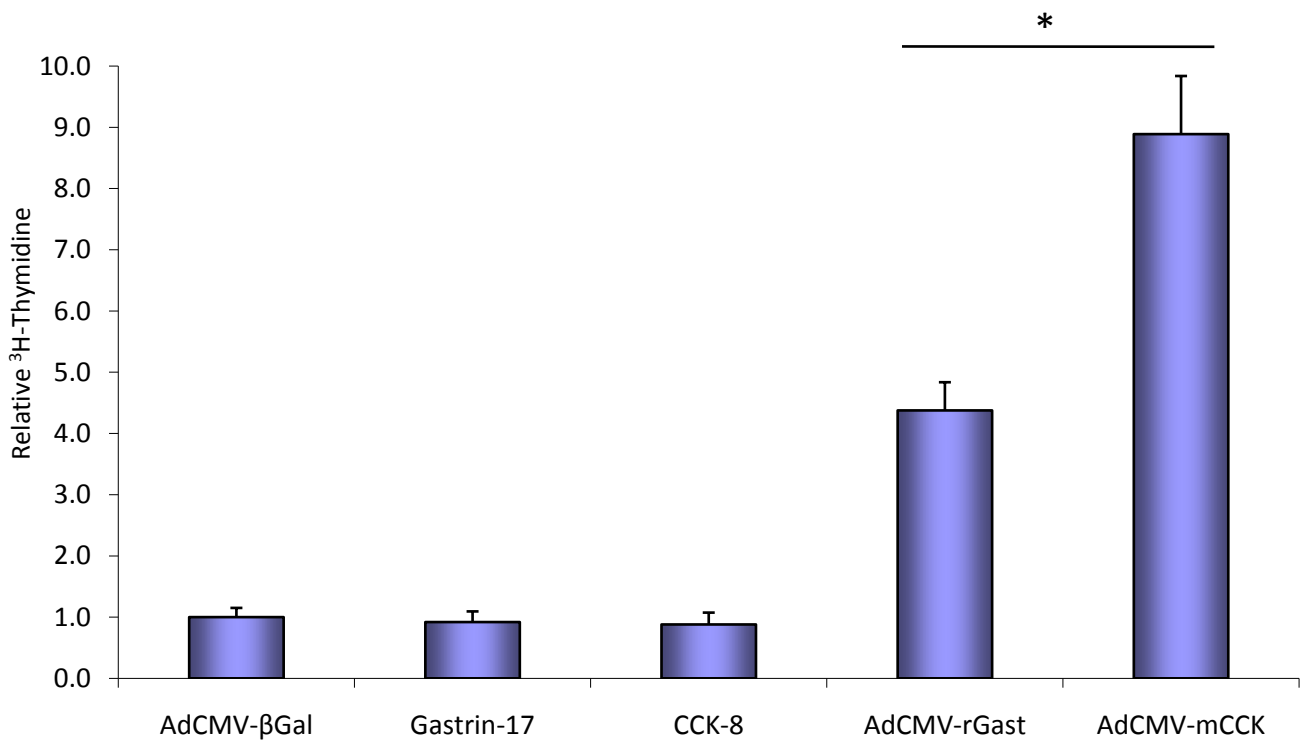


Figure 4-5. AdCMV-mCCK induces robust proliferation in isolated rat islets. Islets were isolated from adult Wistar rats and treated per Figure 4-1. Islets were exposed to AdCMV-βGal (4 μL), AdCMV-rGast (20 μL), AdCMV-mCCK (3 μL) or 100 ng/mL recombinant gastrin-17 or CCK-8 peptide for 18 hours, followed by a media change. After 48 hours and one additional media change, the islets were transferred to media containing 1 μCi/mL ^{3}H-thymidine and allowed to incubate for 24 hours before harvesting. Gastrin-17 and CCK-8 peptides were maintained throughout all media changes but adenoviral treatments were not. For harvesting, 3 groups of 25 islets were isolated from each treatment group. Counts were determined and then normalized to protein content. Data represent the mean ± SEM for eight independent experiments for the AdCMV-rGast groups and ten independent experiments for the AdCMV-mCCK. * indicates $p \leq 0.05$ vs. the AdCMV-βGal control group.

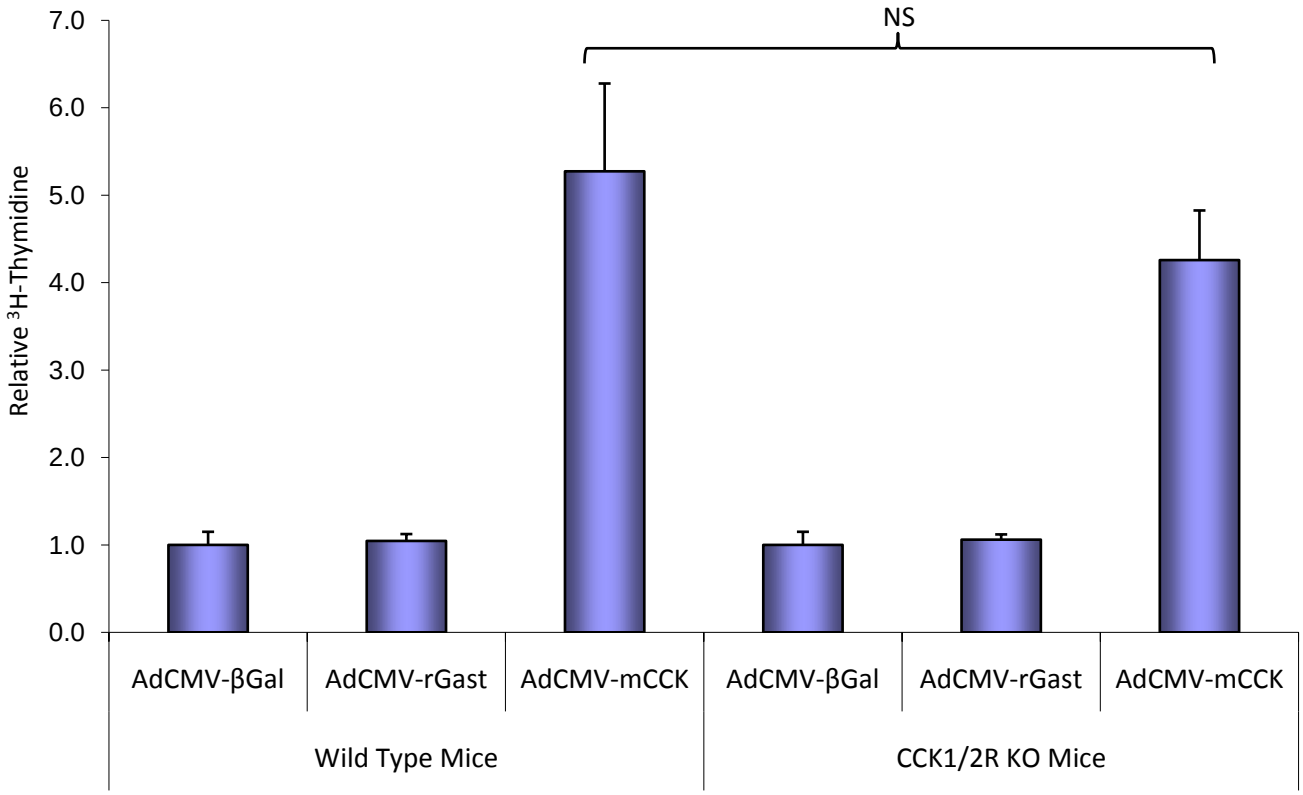


Figure 4-6. AdCMV-mCCK but not AdCMV-rGast induces robust proliferation in CCK1/2R knockout isolated mouse islets. Islets were isolated from adult wild type and CCK1/2R knockout mice bred on the 129SvEv background and treated per Figure 4-1. Data represent the mean ± SEM for three independent experiments. There was no statistically significant (NS) difference between proliferation rates in the AdCMV-mCCK-treated wild type and double receptor knockout islets ($p = 0.52$).

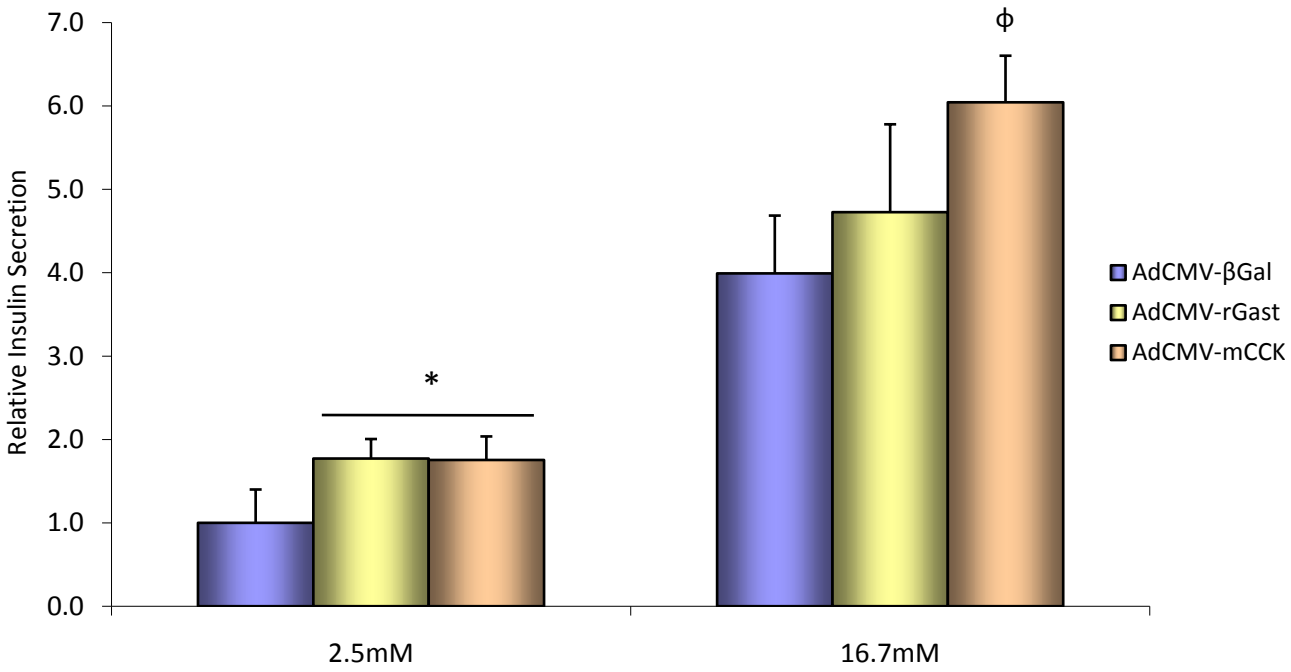


Figure 4-7. Glucose-stimulated insulin secretion is enhanced in primary rat islets treated with AdCMV-mCCK. Isolated rat islets were treated per conditions outlined in Figure 4-3. Prior to ^{3}H-thymidine precipitation, glucose-stimulated insulin secretion (GSIS) was measured by culturing islets in 2.5 mM and 16.7 mM secretion assay buffer. Data are normalized to the AdCMV-βGal-treated 2.5 mM glucose group and represent the mean ± SEM for five independent experiments, each performed in triplicate. AdCMV-mCCK treated islets demonstrated a 51.4±14% increase in GSIS at high glucose (16.7 mM) compared to AdCMV-βGal. All samples normalized to protein content. Both treatment groups demonstrated higher basal levels of insulin secretion at 2.5 mM glucose. * denotes $p \leq 0.05$ compared to AdCMV-βGal 2.5 mM glucose treatment group and ϕ denotes $p \leq 0.05$ AdCMV-βGal 16.7 mM glucose treatment group.

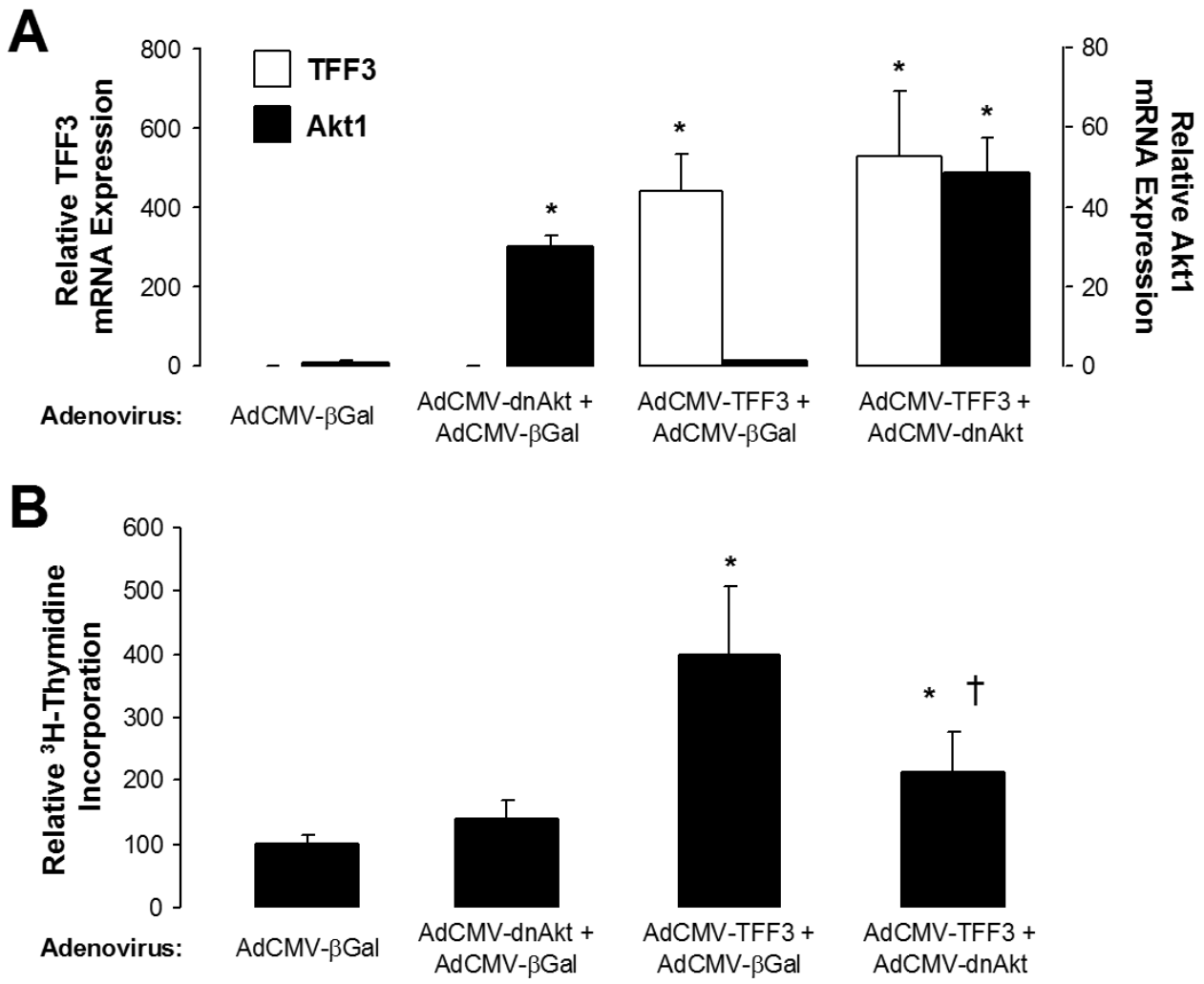


Figure 4-8. TFF3-stimulated islet proliferation is mediated, in part, through Akt. **A.** AdCMV-TFF3 and AdCMV-dnAkt treatment of rat islets induces overexpression of trefoil factor 3 (TFF3) and Akt, respectively. **B.** TFF3 overexpression induces a similar proliferative response to that seen with gastrin overexpression, and this effect is partially blocked by overexpression of dominant negative Akt (dnAkt). Data are mean ± SEM for five experiments. * denotes $p < 0.05$ versus AdCMV-βGal. † denotes < 0.05 versus AdCMV-TFF3 + AdCMV-βGal. Figure used with permission of Patrick Fueger, Ph.D.

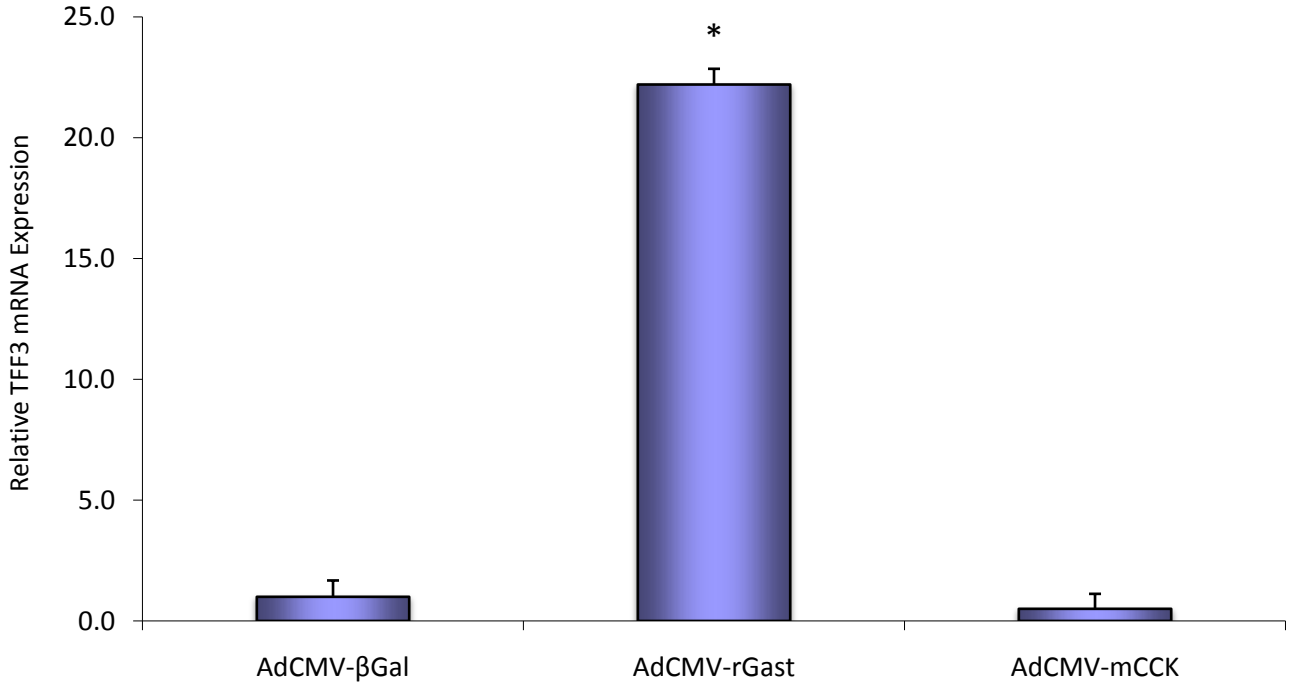


Figure 4-9. Trefoil factor 3 expression is strongly upregulated by AdCMV-rGast, but not AdCMV-mCCK, in isolated rat islets. Isolated rat islets were treated per conditions outlined in Figure 4-3. 25 islets were isolated from each treatment group and harvested for total RNA. RNA was reverse transcribed to cDNA and Trefoil Factor 3 (TFF3) expression was measured using a Taqman® primers and probe set specific for rat TFF3. AdCMV-rGast-treated islets demonstrated a robust (> 22-fold) increase in TFF3 expression relative to AdCMV-βGal control and AdCMV-mCCK-treated islets. Preprogastrin and preproCCK were verified to be robustly upregulated via qPCR (ddCt of 17 and 16, respectively). * denotes $p \leq 0.05$ versus AdCMV-βGal.

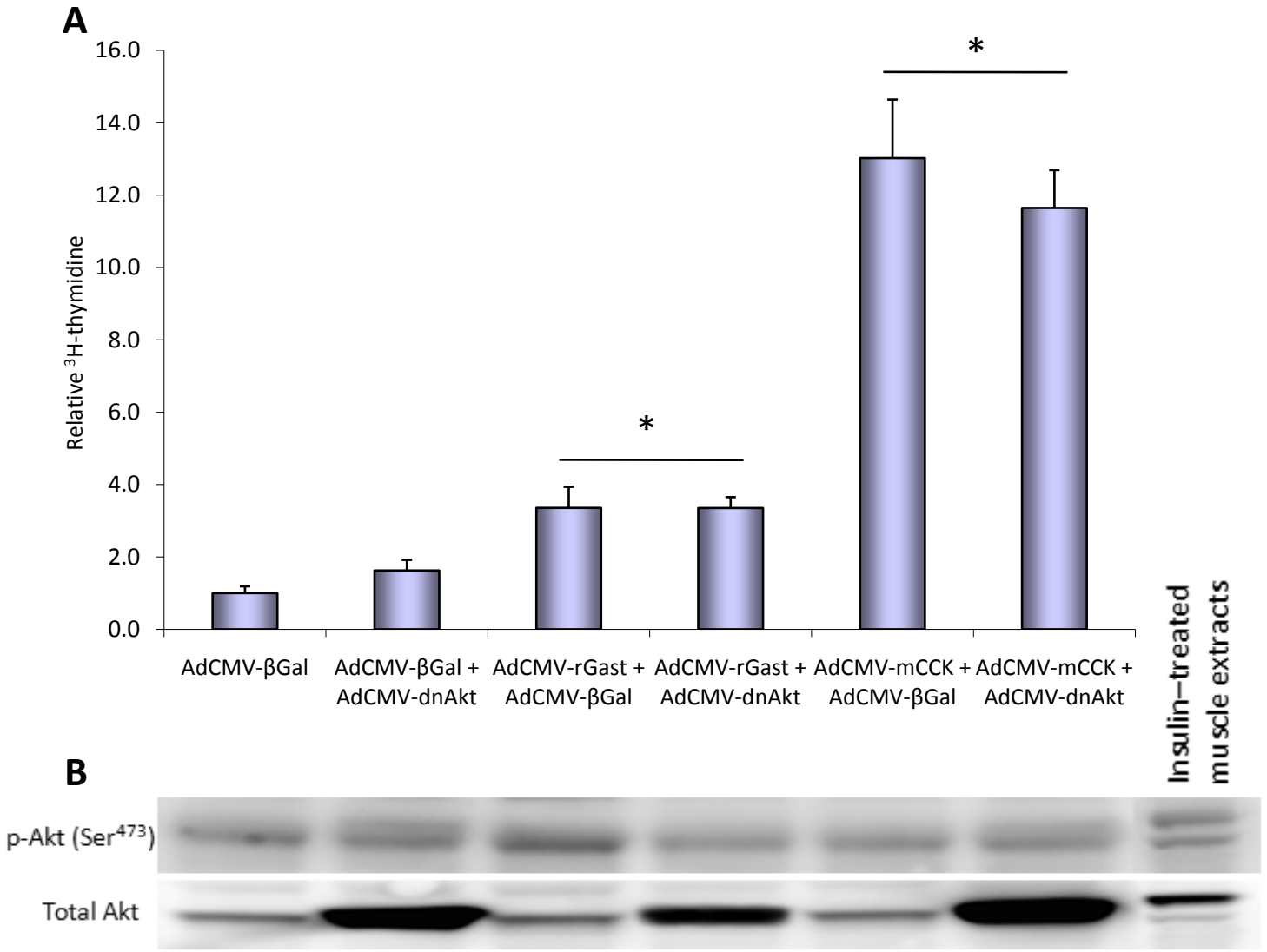


Figure 4-10. Dominant negative Akt co-expression with gastrin and CCK in isolated rat islets. Groups of 300 isolated rat islets were harvested and treated per conditions outlined in Figure 4-5 with the inclusion of either AdCMV-βGal (as a titer control) or AdCMV-dnAkt. **A.** ^{3}H-thymidine incorporation in adenovirus treated isolated rat islets. Both AdCMV-rGast and AdCMV-mCCK induce robust ^{3}H-thymidine incorporation regardless of dominant negative Akt expression. Data represent mean ± SEM for three independent experiments. * denotes $p \leq 0.05$ relative to AdCMV-βGal control. **B.** Immunoblot of Ser^{473} Akt phosphorylation (top) and total Akt expression (bottom). Akt phosphorylation is not detectable in rat islets, although AdCMV-dnAkt treatment causes significant increases in total Akt expression (bottom).

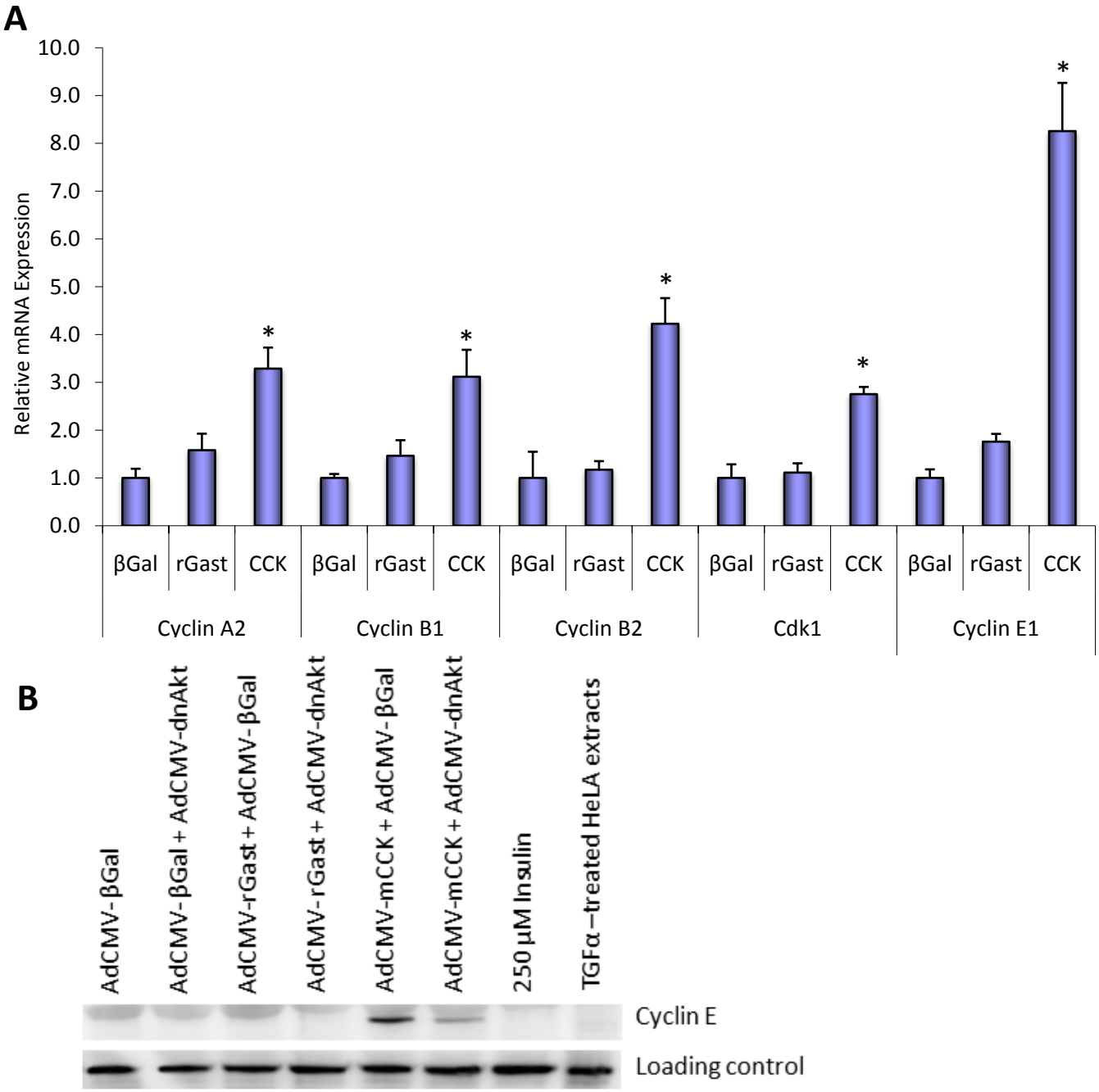


Figure 4-11. Expression of cyclin genes in adenovirus-treated isolated rat islets. Isolated rat islets were treated per conditions outlined in Figure 4-3. 25 islets were isolated from each treatment group and harvested for total protein and total RNA. **A.** RNA was reverse transcribed to cDNA and mRNA expression measured with Taqman® primers and probe sets for the respective cyclins. AdCMV-mCCK-treated islets demonstrated significant upregulation of Cyclin A2, B1, B2, E1, and Cdk1 expression relative to AdCMV-βGal control. * denotes $p \leq 0.05$ vs. AdCMV- βGal control. **B.** Immunoblot of total Cyclin E. Cyclin E expression is increased in AdCMV-mCCK treated islets. AdCMV-dnAkt blunts the upregulation of Cyclin E induced by AdCMV-mCCK, but does not affect proliferation.

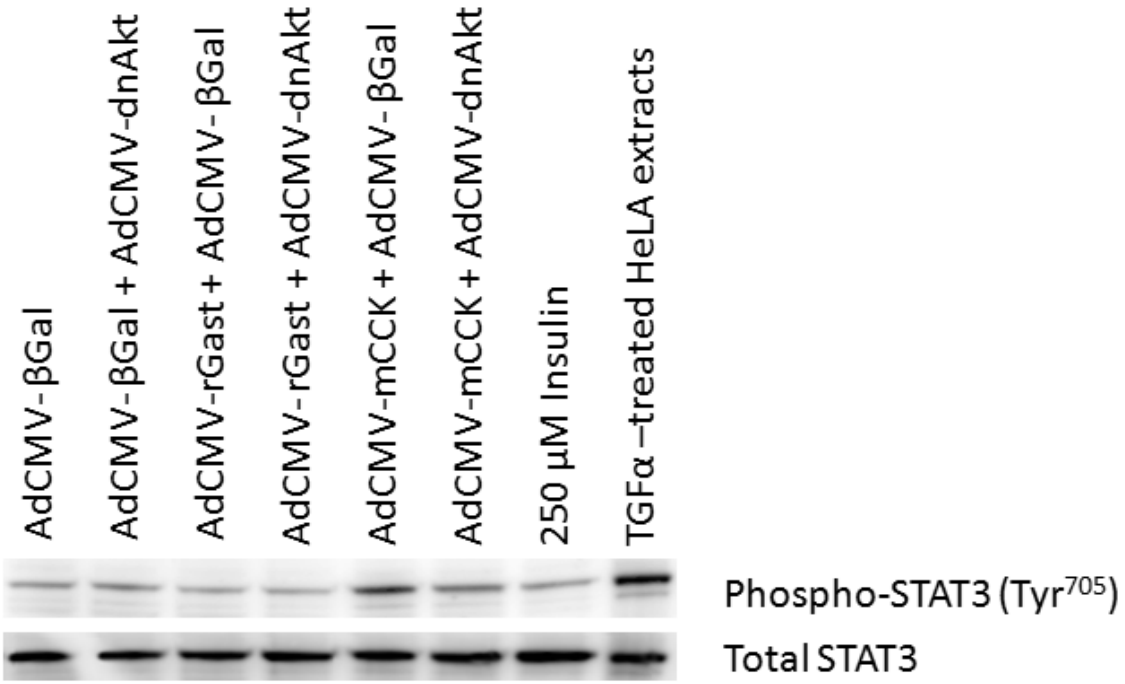


Figure 4-12. Phosphorylation of STAT3 in adenovirus-treated isolated rat islets. Isolated rat islets were treated per conditions outlined in Figure 4-11 and total protein isolated from groups of 300 islets. STAT3 is phosphorylated in response to AdCMV-mCCK treatment, but not AdCMV- βGal control or AdCMV-rGast treatments.

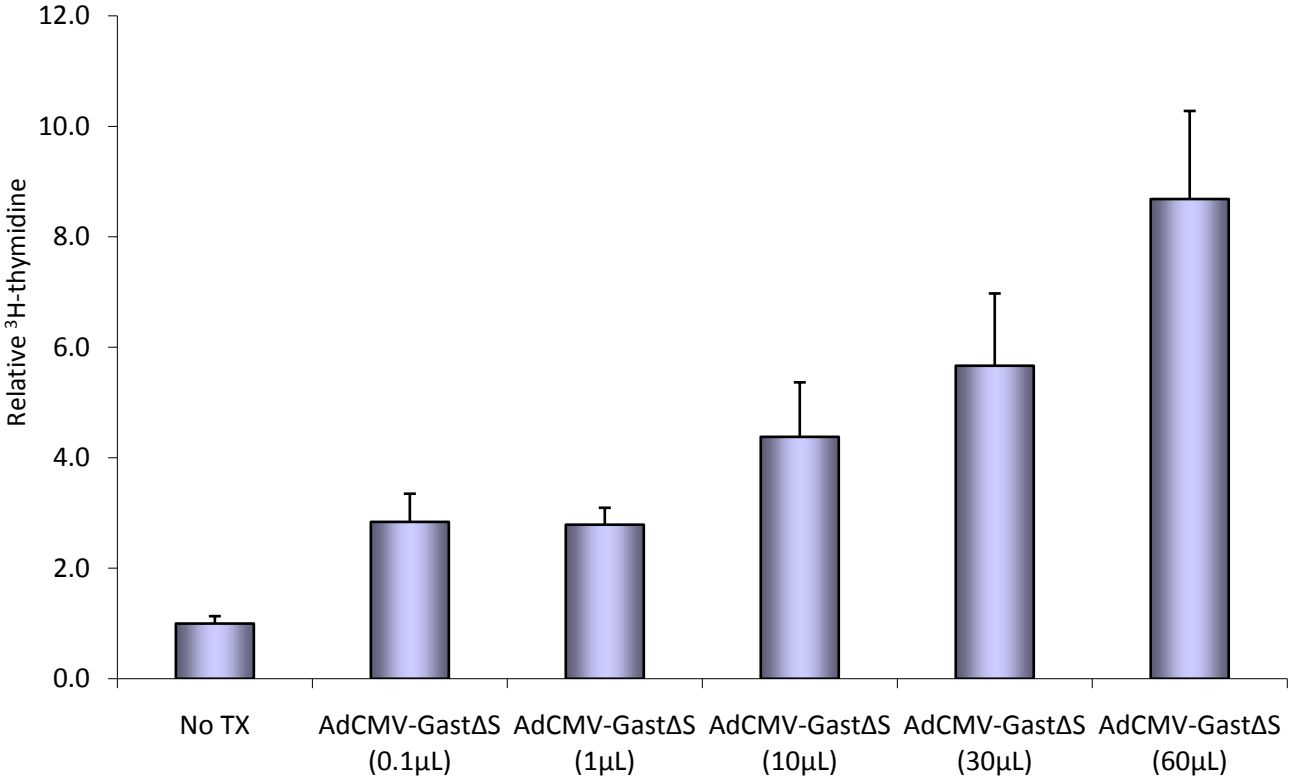


Figure 4-13. AdCMV-GastΔS induces robust proliferation in isolated rat islets. Islets were isolated from adult Wistar rats and treated per Figure 4-1. Islets were exposed to increasing doses of AdCMV- GastΔS. Media was changed in the treated islets after 18 hours. After 48 hours and one additional media change, the islets were transferred to media containing 1 μCi/mL ^{3}H-thymidine and allowed to incubate for 24 hours before harvesting. For harvesting, 3 groups of 25 islets were isolated from each treatment group. Counts were determined and then normalized to protein content.

CHAPTER FIVE

CONCLUSIONS AND FUTURE DIRECTIONS

In this dissertation I have described novel roles of the peptide hormones gastrin and cholecystokinin (CCK) in islet β cell proliferation. Overexpression of either preprogastrin or preproCCK, but not exogenous administration of various peptides encoded by their prohormone precursors, promotes robust β cell proliferation without impairment in β cell function. In the case of preproCCK overexpression, β cell proliferation is also accompanied by a significant enhancement of glucose-stimulated insulin secretion, placing it in a rare class of genes capable of these dual functions.

These discoveries were greatly aided by the utilization of a broad-based, unbiased gene screening approach as that described in Chapter 2. By analyzing the differential expression of genes in the cytokine resistant (INS-1_{res}) and cytokine sensitive (INS-1) cell lines, gastrin emerged as a potential mediator of cytokine resistance. Although further experimentation determined that gastrin does not promote cytokine resistance, the significant mitogenic capacity of preprogastrin, and subsequently preproCCK, that emerged from these studies is potentially just as valuable. It is highly unlikely that we would have investigated these genes, and subsequently discovered their potential for β cell regeneration, if the microarray experiments on the INS-1_{res} cell lines had not been undertaken. Furthermore, three years later, the data harvested from the INS-1_{res} microarray analyses is still providing us with valuable insights into β cell biology. Trefoil factor 3 (TFF3), another gene with significant mitogenic capacity when

overexpressed in pancreatic islets, is an additional candidate from the INS-1_{res} microarray experiment that is being investigated for potential use in expanding β cell mass.

Despite investigation of a number of different hypotheses in these studies, the mechanism by which preprogastrin expression stimulates β cell proliferation remains unknown. Our data suggests that preprogastrin may be working via an intracellular signaling mechanism. Evidence for this hypothesis includes the fact that the classical gastrin-17 receptor, CCK2R, is marginally detectable in INS-1 cell lines and undetectable in isolated rat islets using quantitative PCR (qPCR). Moreover, extracellular treatment of rat islets with high concentrations of the G17, G17S, and G34 peptides had no measurable effect on islet proliferation. In an attempt to test the hypothesis that preprogastrin overexpression stimulates β cell proliferation in a CCK1R/CCK2R independent fashion, wild type and CCK1R/CCK2R deficient mouse islets were transduced with AdCMV-rGast. Unfortunately, preprogastrin overexpression did not stimulate proliferation of wild-type mouse islets in these experiments, making it impossible to make any clear conclusion about the role of CCK1R and CCK2R in the proliferative response we observed in isolated rat islets.

The potential reasons for the failure of preprogastrin overexpression to stimulate proliferation in mouse islets are many, but do not include a failure to overexpress preprogastrin, based on a clear increase in secreted gastrin levels comparable to that seen in our rat islet experiments (500-1000 pmol/L in the media of mouse islets, as measured by RIA for a 24 hour specimen collection). Other factors that could prevent preprogastrin induction of mouse β cell proliferation include: 1) The rat preprogastrin cDNA contains 11% sequence variation with mouse preprogastrin at the level of protein

sequence. Mouse preprogastrin is also shorter than rat preprogastrin due to the absence of the Gln-Asn-Tyr C-terminal residues. While these differences do not seem large, it is unknown if they are sufficient to prevent rat preprogastrin from inducing the same β cell proliferation in mouse islets as we observe in rat islets. 2) The AdCMV-rGast virus failed to penetrate to the β cell core of mouse islets, causing the majority of the preprogastrin overexpression to occur in peripheral α cells. In fact, a number of laboratories have reported poor penetration of adenoviral constructs into mouse islets, whereas the experience in rat islets in our laboratory has always been a high level of transduction, including in the β cell core of the islet (Becker, Noel *et al.* 1994; Becker *et al.* 1996). We have noted some significant differences between the islet harvest protocols used in our lab and the Attie lab, which potentially could contribute to a higher efficiency of β cell transduction in our studies. One of these differences involves the collagenase digestion of the pancreas after harvesting, which aids in removing exocrine cells and loosening the islet structure. We perform a 30 minute digestion while the Attie lab uses a 16 minute digestion. We hope that we can improve the delivery of the adenoviral constructs into the β cell core of mouse islets by increasing the collagenase digestion in the mouse islets. Studies are currently ongoing in the Attie laboratory to determine if the islet isolation protocol used in our laboratory enhances adenovirus-mediated gene transfer to mouse β cells.

In a separate approach for investigating potential species-specific problems with preprogastrin overexpression, I have recently cloned the mouse preprogastrin gene into an adenoviral expression vector in an identical manner to that described for AdCMV-rGast. This construct will be tested in mouse islets that have been harvested and cultured

in our lab with the same techniques used to harvest rat islets, to eliminate the variability introduced by differences in islet isolation protocols. The ability or failure of AdCMV-mGast to induce significant proliferation in mouse islets isolated in our lab will help us to determine if the lack of islet proliferation seen with AdCMV-rGast in mouse islets is species specific or procedural in nature. Ideally, our collaborators in the Attie lab will test this construct on islets isolated from mice using their harvesting protocol as well.

It is possible that preprogastrin and preproCCK promote β cell proliferation by a mechanism that does not involve classical cell surface receptors, and that the signal for proliferation is intracellular in nature. In this context, a new and exciting field of intracrine signaling through nuclear G-protein coupled receptors (GPCRs) has emerged in the last five to seven years. Growing evidence indicates that GPCRs, which are localized to the nuclear envelope instead of the plasma membrane, mediate distinct functions from their traditionally ascribed roles (Bhattacharya *et al.* 1998; Gobeil *et al.* 2002; Marrache *et al.* 2002). While we do not yet have direct evidence for an intracrine mode of signaling by preprogastrin and preproCCK, nuclear localization of CCK2R has been demonstrated in multiple hepatoma and pancreatic cell lines (Caplin *et al.* 1999; Stubbs *et al.* 2002; Savage *et al.* 2006). These studies demonstrated that CCK2R can undergo nuclear localization from the plasma membrane and/or is expressed directly to the nuclear envelope, although no direct signaling modality has been ascribed to nuclear CCK2R. Furthermore, as mentioned in Chapter 1, the epidermal growth factor receptor (EGFR) can potentiate the proliferative effects of gastrin and CCK-mediated CCK2R activation (Suarez-Pinzon, Lakey *et al.* 2005). While not a GPCR, EGFR has also been shown to

localize to the nucleus, and this localization appears to be directly responsible for the high proliferative rate of tissues in which it is expressed (Lin *et al.* 2001).

Our finding that overexpression of preproCCK in CCK1R/CCK2R double knockout mice induces islet proliferation argues against an important intracrine signaling role of these receptors in CCK-mediated islet growth, but their potential role in preprogastrin signaling remains to be evaluated. Moreover, recently published radioligand binding studies have demonstrated significant evidence for an additional gastrin receptor, one specifically involved in the promotion of cell growth (Copps *et al.* 2006). It is possible that this receptor is the hypothesized gastrin-gly receptor, which would preferentially bind the non-amidated forms of gastrin and CCK. Several groups have hypothesized the existence of this receptor based on radioligand binding studies (Negre *et al.* 1996; Iwase *et al.* 1997; Monstein *et al.* 1997; Ahmed *et al.* 2004), but it has yet to be definitively identified and characterized and it is unclear if it is the same receptor that Copps et al. (2006) have identified. Furthermore, this receptor is presumed to be in the plasma membrane, however subcellular localization studies have not been conducted to confirm this assumption.

Even if unidentified gastrin/CCK receptors are expressed in the nucleus, how is it possible for preprogastrin and preproCCK, both of which are the precursors for secreted prohormone peptides, to signal in an intracrine fashion? Mounting evidence suggests that many proteins lacking "classical" nuclear localization sequences (NLS) or variations of it can be actively transported into the nucleus (Christophe *et al.* 2000). Furthermore, the characterization of non-classical NLSs is a rapidly evolving field. Both preprogastrin and preproCCK contain a significant number of basic residues that could be part of non-

conventional NLS motifs, particularly when one considers the extremely high intracellular concentrations of preprogastrin and preproCCK protein produced by adenoviral overexpression. These extremely high intracellular concentrations could aid in binding to the importin nuclear translocation machinery if the NLS of preprogastrin and preproCCK was marginal. Furthermore, preproCCK contains more of the basic lysine and arginine residues that constitute an NLS than preprogastrin, and if these are important in nuclear import it could explain why preproCCK overexpression is a more potent inducer of islet β cell replication.

At this point we have significant evidence that CCK1R and CCK2R do not contribute to preprogastrin and preproCCK-mediated β cell proliferation (animal knockouts, qPCR), providing impetus to evaluate the possibility of an intracrine mode of signaling. To test this hypothesis, we will perform co-localization experiments using confocal immunofluorescence microscopy of anti-gastrin/anti-CCK stained islet sections with the nuclear fluorescence stain 4',6-diamidino-2-phenylindole (DAPI). We expect that if gastrin or CCK is actively transported into the nucleus, then it should be readily visible in sectioned islets using these methods. Additionally, BrdU/preprogastrin co-localization will be performed to verify that positive preprogastrin expressing cells are actively dividing. The islet sections for these experiments have already been collected, banked, and are ready for immunofluorescence analysis. Additional co-localization studies will be performed on these samples to determine if CCK1R, CCK2R, or EGFR are expressed and localized to the nucleus of actively dividing β cells. With this experiment, we aim to determine if preprogastrin and preproCCK are actively transported

to the nucleus in replicating islet β cells that have been treated with AdCMV-rGast and AdCMV-mCCK.

If preprogastrin and preproCCK are determined to co-localize to the nucleus of actively-dividing β cells, then we will undertake additional experiments to determine if this nuclear transport is necessary to stimulate β cell proliferation. First pass analysis of the necessity for gastrin nuclear import will consist of a series of mutations of the putative NLS. Mutant preprogastrin overexpression viruses have already been constructed and are available to test this hypothesis. These viruses have one or more pairs of the dibasic prohormone convertase cleavage sites mutated to double alanines and thereby preventing expression of smaller, processed forms of gastrin. The mutant constructs include a $Lys^{74}Lys^{75} \rightarrow Ala^{74}Ala^{75}$ double mutant designed to prevent cleavage of gastrin-34 to gastrin-17 thereby forcing gastrin-34 overexpression (AdCMV-GastΔ34), and a variant with $Arg^{57}Arg^{58} \rightarrow Ala^{57}Ala^{58}$, $Lys^{74}Lys^{75} \rightarrow Ala^{74}Ala^{75}$, and $Arg^{94}Arg^{95} \rightarrow Ala^{94}Ala^{95}$ triple dibasic sites mutated to force progastrin expression (AdCMV-GastΔ80).

While these mutants were originally designed to test for the necessity of specific processed forms of progastrin in β cell proliferation, they will be equally useful in testing our hypothesis of the presence of a non-classical NLS in the preprogastrin gene. This is because the dibasic residues that constitute the cleavage sites for prohormone convertases 2 and 1/3 double as part of the NLS. If the dibasic residues removed in AdCMV-GastΔ80 are important to nuclear localization, we would expect: 1) β cell proliferation as measured by ^{3}H-thymidine incorporation would drop to basal; 2) nuclear import of preprogastrin will be impaired or fully blocked. If these mutants do not co-localize to the

nucleus and yet are still able to stimulate β cell proliferation, then we would consider that significant evidence that a non-processed form of gastrin, possibly progastrin or G34, is sufficient to induce β cell proliferation through a mechanism that does not involve nuclear import of one of these gastrin precursor molecules. If we see this result, we will consider knocking down the prohormone convertases 2 and 1/3 and carboxypeptidase E using the AMAXA^{TM} nucleofection system in INS-1 cells, as a quick pass analysis of the requirement of processed gastrin in β cell proliferation. A positive result from this experiment would be followed up by the construction of siRNA adenoviruses that target these proteins for evaluation in isolated rat islets.

In addition to these preprogastrin mutants, we are also considering variants lacking the C-terminal flanking section or containing a mutation in the N-terminal signal peptide responsible for directing preprogastrin into the endoplasmic reticulum. This will allow us to evaluate these peptide sequences for involvement in preprogastrin-mediated β cell proliferation. The necessity of the C-terminal flanking peptide in preprogastrin-mediated β cell proliferation would support recently published data demonstrating this fragment of the preprogastrin gene in promoting cellular growth (Smith *et al.* 2006). Abolishment of β cell proliferation upon removal of the N-terminal signal peptide would slightly more difficult to interpret and could indicate that it is necessary to shuttle preprogastrin to the endoplasmic reticulum for further processing or that part or all of the signal peptide itself is involved in β cell proliferation, directly.

Despite our inability to block preprogastrin-mediated β cell proliferation by co-overexpression of a dominant negative form of Akt, we feel that the potential involvement of trefoil factor 3 (TFF3) deserves further investigation. To pursue this line

of investigation we will first verify that expression of TFF3 at the mRNA level correlates with an increase in TFF3 protein. This will be accomplished by immunoblot analysis for TFF3 in both total protein extracts and media collected from AdCMV-rGast treated islets. If these results confirm TFF3 expression, like we believe that they will, then the potential link between TFF3 expression and preprogastrin-mediated β cell proliferation will be evaluated in detail.

TFF3 expression will be suppressed in AdCMV-rGast treated INS-1 cells by the use of AdCMV-siTFF3, a recombinant adenovirus expressing a siRNA hairpin construct that we have validated for effectively suppressing TFF3 expression. Construction of this virus is currently in process by Dr. Fueger. Once viral titers and effective TFF3 suppression have been determined in the INS-1 cell lines, work will proceed in isolated rat islets where we will attempt to block AdCMV-rGast induced β cell proliferation by co-treatment with AdCMV-siTFF3. If suppression of TFF3 expression upon AdCMV-rGast treatment results in a significant impairment of β cell proliferation, then we will be able to conclude that TFF3 expression is at least partially responsible for preprogastrin-mediated β cell proliferation.

Through work on TFF3 in the 823/13 INS-1 cell line, Dr. Fueger has discovered that TFF3-mediated proliferation requires the presence of fetal calf serum (FCS) in the culture media. Furthermore, as previously mentioned, the proliferative effect of TFF3 overexpression appears to be mediated, at least in part, through the Akt signaling pathway, possibly through the epidermal growth factor receptor (EGFR). Based on the evidence to date, we believe that TFF3 acts synergistically with EGF to stimulate islet proliferation, possibly through a mechanism that by which TFF3 increases the immediate

local concentrations of EGF at the plasma membrane receptor. It is possible that if preprogastrin is stimulating TFF3 expression, it is in order to increase EGFR signaling. Dr. Fueger is preparing a series of experiments in which he will attempt to block TFF3-induced β cell proliferation by 1) inactivation of the EGFR with monocolonal antibodies, 2) suppression of intracellular EGFR tyrosine kinase activity by small molecule inhibitors, for which multiple drugs are now are available (Baker 2004), and 3) siRNA-mediated suppression of the EGFR gene. These experiments will be duplicated by me in AdCMV-rGast treated islets to evaluate for TFF3 signaling through EGFR secondary to preprogastrin overexpression. A thorough evaluation will include AdCMV-rGast stimulated islets grown in the presence of FCS or 0.1% bovine serum albumin (BSA) and in the presence or absence of recombinant rat EGF. TFF3 levels will be measured using both qPCR and immunoblot of total islet and culture media.

Work is also nearing completion on another viral construct, AdCMV-CCK2$_{i4sv}$R. AdCMV-CCK2$_{i4sv}$R is being constructed in our lab through a collaboration with Dr. Mark Hellmich at the University of Texas-Galveston to overexpress a constitutively active splice variant of the gastrin receptor/CCK2R. CCK2$_{i4sv}$R was discovered as a natural splice variant expressed in colonic adenocarcinomas (Hellmich *et al.* 2000). In this splice variant, part of intron 4 is included in the third intracellular loop of CCK2R, resulting in constitutive activation of the receptor through coupling with heterotrimeric G-proteins. This construct should serve as an excellent tool to evaluate the traditional gastrin signaling pathway in islet β cells and tell us what effect constitutive activation of this pathway has in islet β cells.

In addition to the aforementioned co-localization experiments and viral mutants, we intend to continue examining different proliferative pathways in the involvement of preprogastrin-mediated β cell growth. Principal candidates include not only the proteins of the known signaling modalities of the CCK receptors (Figure 1-2), but also cell cycle proteins such as the cyclins, cyclin dependent kinases, and the retinoblastoma family (pRb, p107, p130). These analyses were begun in Chapter 4 where we presented data indicating the potential involvement of both Cyclin E and STAT3 in preproCCK, but not preprogastrin, mediated β cell proliferation. In these experiments is it critical to re-evaluate, at the protein level, all of the cyclin molecules for which we have qPCR data. This is necessary because changes in protein expression or phosphorylation that are independent of genetic expression could mediate the proliferative response. Our analysis of these candidate genes using the previously described immunoblot techniques has already begun.

Not only is it important to enhance our understanding of the molecular mechanisms by which preprogastrin and preproCCK promote islet β cell proliferation *ex vivo*, it is critical to demonstrate that our manipulations using these genes have translational value *in vivo*. We propose testing for *in vivo* islet function by re-introducing AdCMV-rGast and AdCMV-mCCK treated rat islets into immunocompromised mice. This technique has been used by many researchers (Grey *et al.* 2003; Lopez-Talavera *et al.* 2004; Yin *et al.* 2006) to test for islet function after *in vitro* manipulations of β cells and is currently being used in our lab with AdCMV-Nkx6.1 treated islets. Groups of 100 rat islets treated with AdCMV-rGast, AdCMV-mCCK, or AdCMV-βGal will be transplanted under the kidney capsule of severe combined immunodeficient (SCID) mice

that have had their β cell mass ablated with high-dose streptozotocin treatment (160 mg/kg). The transplanted islet mass in the AdCMV-βGal treated control mice should be sufficient to partially restore euglycemia for several days to weeks (Grey, Longo *et al.* 2003). Transplantation of a subcritical mass of islets allows us to measure the length of time that the transplanted β cell mass is able to partially restore euglycemia, with the assumption that the active β cell replication we see in AdCMV-rGast and AdCMV-mCCK treated islets will allow for a longer period of partial glycemic control or even complete glycemic control for long periods compared to mice receiving AdCMV-βGal treated islets. Mice will also be administered BrdU at 100 mg/kg once daily in this experiment. This will allow us to assay for growth of the transplanted β cells by recovering them from under the kidney capsule at the conclusion of the experiment.

It is our hope that by uncovering the molecular mechanisms responsible for preprogastrin and preproCCK-mediated stimulation of β cell growth we can come one step closer to developing a robust, safe, and clinically-relevant method for expanding the available β cell mass that can be used for islet transplantation.

APPENDIX A

PancChip 5.0 Microarray analysis of INS-1_{res} 833/15 vs. INS-1 834/40 cell lines

POSITIVE SIGNIFICANT GENES (833/15 v 834/40)

Genebank ID (rat)	Gene Name	Score (d)	Fold Change	Gene Notes
NM_012671	TGF-alpha (or precursor)	19.27	4.27	marker of angiogenesis; may be important for transplanted islets
NM_012901 (2)	alpha 1 microglobulin/bikunin (Ambp)	11.30	4.65	pancreas development
X78949	P4ha1 (P4ha) – prolyl 4-hydroxylase alpha subunit	9.03	2.68	catalyzes the formation of 4-hydroxprolines in collagen
NM_013124	Pparg (Nr1c3; PPAR-gamma)	7.76	3.04	regulator of lipid homeostasis and cellular differentiation
NM_013042 (U48825)	Trefoil Factor 3 (OR P-domain neuropeptide P1.B)	7.22	2.43	intestinal peptide; important for wound healing
BC061717 (2)	Rabggtb – immediate early response 2 (cDNA clone MCG:72578 IMAGE:5598375 in rat)	6.44	2.17	immediate early response (IER) 2 and 5 proteins
NM_133591	rabphilin 3A-like	6.26	2.06	involved in exocytosis by interacting with the cytoskeleton
X14044 (2)	Glutamate dehydrogenase	6.03	1.97	catalyzes the formation of glutatmate from NH3 and a-KG; may be important for insulin secretion
XM_232937	AI314180 (similar to KIAA0368 (LOC313196))	5.79	1.56	upregulated upon chemical exposure in skin cells
	1500012F01Rik	5.67	1.54	????
BC061847	Vimentin	5.59	1.71	possible marker of angiogenesis
XM_222906	similar to Transgelin 2, sm22-alpha homolog (Sm22B; Sm22a; 2700094C18Rik)	5.43	1.68	*links actin filaments together*
BC061541	Pyruvate kinase, muscle	5.38	2.08	terminal step of glycolysis
XM_238806	similar to harmonin isoform b3 (LOC308596)	5.32	1.80	*Scaffolding protein*
_	AI429612	5.31	1.68	????
NM_022852	Ipf1; Mmp14 (Ipf1; Pdx1; IDX-1; IPF-1; Mody4; STF-1; MT1-MMP; Membrane type 1-MMP)	5.19	1.75	islet T.F., upregulated to maintain beta cell phenotype?
_	IMAGE=5682307 (no BLAST match)	5.17	1.52	????
BC063171 (3)	Pbp (HCNP; Pbpr) – phosphatidylethanolamine binding protein	5.13	1.86	inhibits the ERK pathway, which may inhibit proliferation
BC061740	low BLAST match w/ ecto-ATPase precursor	5.12	1.47	*ATP hydrolysis*
XM_222275	Coro1c; 2010001K21Rik (coronin 3) – similar to slingshot 1 (LOC304580)	5.09	1.62	protein phosphatase; may be associated with cell cycle changes
NM_053420	Bnip3 (Nip3) – BCL2/adenovirus E1B 19 kDa-interacting protein 3, nuclear gene for mitochondrial product	5.00	1.78	member of the Bcl-2 family; involved in hypoxia and apoptosis pathways
NM_053462	Nfs1 (m-Nfsl; nifS-like (sic)) – cysteine desulfurase	4.98	1.67	produces elemental sulfur for biogenesis of iron-sulfur proteins
V01242	mid BLAST match w/ rat insulin 1	4.92	2.15	*insulin*
_	IMAGE=6431991 (no BLAST match)	4.90	1.54	????
X52984 (2)	Mug1 – alpha(1)-inhibitor 3, variant I	4.81	1.84	regulated pretranslationally following inflammation

XM_215513 (3)	Krt1-18 (K18; Endo B.; keratin 18) – cytokeratin	4.78	1.69	released during apoptosis, pancreatic ductal stem cell marker, intermediate filament protein
XM_215073	IMAGE=5680449 (similar to RIKEN cDNA 4833422P03 (LOC293489))	4.78	1.59	????
NM_022512	Acads (Bcd1; SCAD; Bcd-1) – short chain acyl-coenzyme A dehydrogenase	4.71	1.62	fatty acid oxidation
BX883045	IMAGE=6430278 (low BLAST match w/ chromosome 20, major histocompatibility complex)	4.66	1.73	????
	Pea15 (Mat1; Pkcs15) – not in database	4.54	1.52	PKC substrate; T2DM association
XM_342025 (2)	1110006I15Rik (similar to mitsugumin 23 (LOC361732))	4.53	1.69	transmembrane protein on ER and nuclear membranes
XM_227402	Sema4a (SemB; SemB; SEMAB; Semab) – similar to sema domain, immunoglobulin domain (Ig), transmembrane domain I and short cytoplasmic domain, (138ignaling138138) 4A	4.49	1.70	important for neuronal survival; anti-apoptotic
X78847	Glutathione s-transferase	4.49	1.77	138ignaling138138g138e of reactive oxygen species; CREB target gene
	IMAGE=6434886 – not in database	4.48	1.49	????
XM_223837	similar to kappa B-ras 1 (2400004O09Rik)	4.41	1.50	RAS small GTPases
U36585	Pcx (Pc) – pyruvate carboxylase	4.38	1.94	pyruvate cycling; anaplerotic
NW_047563	2310043N10Rik	4.37	1.76	????
NM_012526	Chromogranin B – Chgb (Scg-1; secretogranin I)	4.36	1.80	secretory granule formation
BC062086	Keratin 5	4.36	2.02	skin protein; keratin5 promoter used for skin-targeted transgenic mice
AF286722	IMAGE=6434132 (Low BLAST match w/ rat peripheral cannabinoid receptor)	4.27	1.82	pertussis toxin sensitive; activates MAPK pathways
BC061781 (2)	Tpi (Tpi-1) – triosephosphate isomerase 1	4.24	2.20	glycolytic enzyme
8321	IMAGE=6434858 – not in database	4.23	1.63	upregulated in islet tumors
6840	Slc40a1 (MTP; Ol5; Dusg; FPN1; MTP1; IREG1; Slc11a3; ferroportin1; metal transporting protein 1) – not in database	4.22	1.38	Iron transporter
BC051951	138ignaling138138-linked glycosylation 2 homolog (yeast, alpha-1,3-mannosyltransferase)	4.20	1.61	formation of glycoproteins
	Coro1c; 2010001K21Rik (coronin 3) (no BLAST match)	4.19	1.69	*binds and crosslinks F actin*
	IMAGE=656839 (no BLAST match)	4.18	1.69	????
	IMAGE=5944763 (no BLAST match	4.13	1.56	????
AY500369	50% match w/ N-myc downstream regulated 1 (Ndr1)	4.13	1.77	serine-threonine kinase; functionally analogous to the cyclins
XM_213463	Rab5c	4.12	1.44	phosphorylatable protein involved in membrane trafficking
AY500369	N-myc downstream regulated (Ndr1)	4.12	1.93	serine-threonine kinase; functionally analogous to the cyclins
XM_214953	similar to p53 apoptosis-associated target – Perp-pending (1110017A08Rik)	4.10	1.66	expressed in p53 dependent manner; expression is highest in apoptotic cells
J03583	Clathrin heavy chain	4.09	1.99	coated vesicle biogenesis
	IMAGE=6430934 (no BLAST match)	4.07	1.38	????
XM_341950	Protein tyrosine phosphatase, receptor type, epsilon polypeptide – Ptpre (PTPe; PTPepsilon)	4.07	1.32	signal transduction

BC061737 (2)	Scd2 (Scd-2) – stearoyl-Coenzyme A desaturase 2	4.04	1.79	monounsaturated fatty acid (oleate, palmitoleate) synthesis – catalyzes formation of PUFAs
XM_217038 (2)	Dazap2 (Brbp; Prtb; gt6-12) – DAZ associated protein 2	3.96	1.56	139ignalin role in mRNA transport
BC061791	IMAGE 1481411 (low BLAST match w/ phosphofructokinase type B, liver)	3.95	1.50	*glycolytic enzyme*
	IMAGE=5945359 (no BLAST match)	3.92	1.68	????
NM_031508	glutamate receptor, ionotropic, 139ignali 5 (Grik5)	3.91	1.59	139ignali glutamate receptor
NM_080477	low BLAST match w/ rat fructose-2,6-bisphosphatase 2 – not in database	3.87	1.51	regulator of glycolysis and gluconeogenesis
XM_226213	VE-Cadherin	3.87	1.54	expression is increased in hypoxia islets; may function in angiogenesis
NM_022193	Acetyl-CoA carboxylase	3.84	1.43	Fatty acid synthesis
NM_013079 (2)	Asns – 139ignaling139139 synthetase	3.83	1.70	139ignaling139139 synthesis; role in nucleotide biosynthesis?
XM_341700	coactosin-like 1	3.82	1.62	F-acting binding protein
XM_222151	similar to Histone-lysine N-methyltransferase	3.80	1.63	heterochromatic silencing
7992	IMAGE=613673_15 (no BLAST match)	3.79	1.74	corresponds to drosophila metabolic gene transcript
XM_214908 (2)	Glucose phosphate isomerase	3.79	1.77	glycolytic enzyme
NM_013144	Insulin-like growth factor binding protein 1	3.79	2.32	growth promoting function in islets
XM_218336	IMAGE=5944682 (no BLAST match)	3.79	1.53	zinc-finger motif transcription factor
NM_022005	FXYD domain-containing ion transport regulator 6	3.79	1.37	control of ion transport; FXYD domain is invariant throughtout the isoforms
AY325243	Cc1-27	3.77	1.38	*liver regeneration-related protein LRRG163*
	IMAGE=6432204 (no BLAST match)	3.77	1.52	????
XM_213783 (2)	Anapc5 (2510006G12Rik) – anaphase-promoting complex subunit 5	3.75	1.59	Component of the APC or cyclosome
NM_054009	Ctrl – Chymotrypsin-like	3.73	1.96	chymopasin: a novel serine protease
NM_024146	Fgfr1 (FLG; Flt-2; Fgfr-1) – Fibroblast growth factor receptor 1	3.71	1.45	mediates vulnerability and protection against oxidative damage depending on the isoform
NM_012849 (2)	Gast (GAS) – gastrin	3.71	1.94	hormone that stimulates COX-2 expression
XM_236444	Myo6 (sv; Snells waltzer) – similar to myosin VI	3.70	2.06	involved in endocytosis, secretion and cell migration
6561	Ucp2 – not found in database	3.68	1.65	Uncoupling protein 2
NM_053291	Pgk1 (Pgk-1) – phosphogylcerate kinase 1	3.67	1.85	glycolytic enzyme
BC065579	Gnb2 (Gnb-2) – guanine nucleotide binding protein 2	3.65	1.40	G protein coupled receptor
AY462245	mid-low match w/ CREB-binding protein	3.63	1.59	*transcriptional coactivator*
XM_234552	similar to RIKEN cDNA 6720458F09 (LOC314462)	3.61	1.44	*O-methyltransferase*
XM_225599	similar to hypothetical protein (LOC307138)	3.60	1.52	????
NM_138873	Nbn (Nbs1) – nibrin	3.60	1.55	DNA double-strand break repair; upregulated by c-Myc
AJ430859	procollagen-lysine, 2-oxoglutarate 5-dioxygenase 3 (plod3 gene)	3.59	1.64	*Post-translational modification; hydroxylation of lysine residues*
2841	IMAGE=6431218 (no BLAST match)	3.59	1.44	????
12850	blank1176	3.57	1.63	????
NM_175764 (2)	Jmjd1 – jumonji domain containing 1	3.57	1.39	zinc-finger containing protein, transcriptional repressor

BC058478 (2)	Tegt – testis enhanced gene transcript (2 days neonate thymus thymic cells), rat match w/ ab1-011 and cc1-27	3.57	1.60	Suppressor of apoptosis; aka BI-1 (Bax inhibitor-1)
AF085693	(GIT1) GPCR kinase-associated ADP ribosylation factor GTPase-activating protein	3.57	1.33	Activates p21-activated kinases
BC070881	Gas6 (GAS 6; Gas-6; growth arrest-specific)	3.57	1.90	ligand for receptor tyrosine kinase Axl; prevents apoptosis
NM_053965	Slc25a20 (Cact; mCAC; 1110007P09Rik) – solute carrier family 25 (carnitine/acylcarnitine translocase), member 20	3.53	1.44	FA transporter
NM_172035	AL033370; Fzd2 – frizzled 2 homolog	3.51	1.40	*WNT receptor*
XM_236909	similar to 140ignaling140-associated activator of morphogenesis 1	3.49	1.41	*wimgless signal transduction pathway??*
BC064440 (3)	Aldo1 (Aldo-1) – aldolase A	3.49	1.72	glycolytic enzyme
BC062064	Cd164 (A24; A115; MGC-24) – CD164 antigen	3.47	1.85	cell adhesion; negative regulator of cell proliferation
NM_080888	Bnip3l (Nix; D14Ertd719e) – BCL2/adenovirus E1B 19kDa-interacting protein 3-like	3.47	1.36	member of the Bcl-2 family; involved in hypoxia and apoptosis pathways
5640	IMAGE=613673_16 (no BLAST match)	3.46	1.63	????
132	IMAGE=6433499 (no BLAST match)	3.45	1.38	????
XM_214557 (3)	Nedd4l (Nedd4b; Nedd4-2; 1300012C07Rik) – ubiquitin-protein ligase Nedd4-2	3.45	1.41	possibly part of the protein degradation pathway
NM_012533	Carboxypeptidase B1 (Cpb1)	3.43	1.48	proteolysis
7991	IMAGE=613673_7 (no BLAST match)	3.43	1.76	????
XM_216411	Atp6v1g1 (VAG1; ATP6J; Vma10; Atp6g1; 1810024D14Rik; lysosomal 13kDa) – similar to ATPase, H+ transporting, V1 subunit G isoform 1	3.43	1.42	Vacuolar proton-translocating ATPase
BX883046	Cdkap1 (Doc1; ST19; Apc10; DORC1) – chromosome 20 MHC, assembled from 40 BACs	3.42	1.30	mediates the activity of the anaphase-promoting complex by contributing to substrate recognition
XM_217058 (2)	Eukaryotic translation initiation factor 4B (eIF-4B)	3.38	1.47	enhances ribosomal scanning along an mRNA template, involved in translation
XM_214228 (4)	Itm2b (E25BMM; D14Sel6) – E25B protein	3.38	1.42	pro-apoptotic by inducing a loss of mitochondrial membrane potential
BC061842 (2)	H1 histone family member 0	3.38	1.62	accumulates in terminally differentiated cells
	IMAGE=6431752 (no BLAST match)	3.37	1.49	????
10199	IMAGE=6430123 (no BLAST match)	3.36	1.29	????
XM_239258	Supt6h (SPT6)	3.34	2.03	chromatin structure regulator
NM_053297 (2)	pyruvate kinase (pituitary, muscle)	3.33	1.64	glycolytic enzyme
AY017337 (3)	Nucleoside diphosphate kinase DR-nm23	3.33	1.48	interconverts 2 ADP -> ATP + AMP, promotes apoptosis
XM_234441	Ddx41 (ABS; 2900024F02Rik) – similar to DEAD-box protein 140ignalin homolog	3.33	1.45	RNA helicase
8316	2310081H14Rik (not in database)	3.32	1.37	????
	IMAGE=6430568 (no BLAST match)	3.31	1.52	????
BC060578 (4)	Lamr1 (MLR; P40; Lamr; 67kDa; P40-3; P40-8; Lamrl1) – laminin receptor 1	3.31	1.44	ribosomal protein involved in apoptosis; prion disease
XM_234841	similar to wizL (LOC314598)	3.30	2.04	*zinc finger protein??*

10344	IMAGE=613673_14 (no BLAST match)	3.29	1.67	????
XM_217597 (2)	Plp2 (mIMA4) – proteolipid protein 2 (intestinal membrane A4 protein)	3.28	1.35	ion channel
BC061784 (3)	ribosomal protein L6	3.28	1.61	translation accuracy; aka Taxreb107; potential autoantigen
622	IMAGE=6436007 (no BLAST match)	3.27	1.42	????
5639	IMAGE=613673_8 (no BLAST match)	3.24	1.63	????
BC061993	AI848715 (low BLAST match w/ myosin binding protein H)	3.22	1.35	*myosin binding protein*
AF318150	Dhfr – dihydrofolate reductase	3.21	1.65	folate metabolism
XM_341032 (2)	Rpo1-3 (16kDa; mRPA16) – partial blast match w/ similar to hypothetical protein MGC9850	3.18	1.43	Component of DNA-dependent RNA polymerase
5052	IMAGE=613673_12 (no BLAST match)	3.16	1.60	????
3872	IMAGE=5681039 (no BLAST match)	3.15	1.36	????
AY005464	Msra (MSR-A; 2310045J23Rik) – methionine sulfoxide reductase	3.15	1.60	Reduces ROS; oxidative stress protection
13021	Sepp1 (Se-P; selp; D15Ucla1) – not in database	3.13	1.49	mettaloprotease
AY239016	Psat1 (PSA; D8Ertd814e) – phosphoserine aminotransferase	3.13	1.39	Serine metabolism
6524	IMAGE=6435903 (no BLAST match)	3.13	1.28	????
U05013	Hmgn2 (Hmg17; HMG-17) – heme oxygenase-2 non-reducing isoform	3.13	1.38	nucleosomal protein; autoantigen
NM_053596	endothelin converting enzyme 1 (Ece1)	3.13	1.44	degrades beta amyloid
5115	IMAGE=6433611 – not in database	3.12	1.37	????
AF016049	Pafah1b1 (Lis1; Mdsh; LIS-1; Pafaha; lissencephaly-1 protein) – platelet-activating factor acetylhydrolase beta subunit	3.10	1.68	regulatory subunit of platelet-activating factor acetylhydrolase
NM_199370 (3)	Krt2-8 (K8; EndoA; Krt-2.8; cytokeratin8; cytokeratin 8; cytokeratin-8) – keratin complex 2, basic, gene 8	3.10	1.44	Intermediate filament protein
7404	IMAGE=613673_11 (no BLAST match)	3.10	1.61	????
NM_139333	D19Wsu55e – neuronal differentiation-related gene (LOC246216)	3.09	1.54	mRNA splicing factor
NM_012536 (3)	Chymotrypsinogen B (Ctrb)	3.09	1.61	protease gene
NM_181626	HesB Protein	3.08	1.39	binds iron; iron-sulfur containing protein synthesis
XM_231042	Noxa1 (NY-CO-31; SDCCAG31) – NADPH oxidase activator 1	3.08	1.37	activator of NADPH oxidase
XM_341497	Auh (W91705)	3.08	1.36	enoyl-CoA hydratase activity??
XM_215486	2310016C16Rik – similar to RIKEN cDNA 2310016C16 (LOC294774)	3.06	1.32	????
BC058454 (2)	thioredoxin	3.05	1.47	redox-regulating protein
XM_213699 (3)	heat shock protein 105 kDa alpha	3.04	1.43	molecular chaperone; ER stress pathway?
XM_342890 (2)	Yars – similar to prion protein interacting 1 like	3.04	1.50	exonuclease or DNA polymerase??
XM_214014	Igfbp7 (Fstl2; mac25) – similar to mac25	3.03	1.35	autocrine/paracrine factor that specifies the proliferative response to IGFs
XM_242848	Cntfr (Cntfralpha) – ciliary neurotrophic factor receptor alpha precursor	3.03	1.57	receptor for CNTF (cytokine); JAK/Stat pathway
AF218576	Rad50	3.02	1.93	DNA repair
XM_340775	Arhgdig (Gdi5; RIP2; Rho-GDI2; Rho-GDI-3) – similar to Rho-GDI1	3.01	1.44	GDP-dissociation inhibitor for Rho proteins

XM_342872 (3)	AA960307; Jak1 – janus kinase 1	3.01	1.44	cytokine 142ignaling; transautophosphorylate and phos cyto receptors
12691	IMAGE=613673_5 (no BLAST match)	3.00	1.72	????
NM_013111	cationic amino acid transporter-1 (CAT-1); solute carrier family 7, member 1	3.00	1.58	Arginine uptake
NM_019132	Gnas – guanine nucleotide-binding protein G-s, alpha subunit	3.00	1.38	GPCR (see Chen et al Endocrinology. 2004 May 27)
Y12635	vacuolar adenosine triphosphate 142ignali B	3.00	1.43	ATPase
XM_215931	similar to hypothetical protein (MGC28864 (LOC296372)	2.99	1.28	????
XM_215165	similar to 2610301D06Rik protein	2.99	1.53	????
NM_031051 (3)	Mif (GIF; Glif) – macrophage migration inhibitory factor	2.98	1.66	cell proliferation and angiogenesis; increases Myc
BC062028 (9)	Arbp (36B4) – acidic ribosomal protein P0	2.98	1.55	essential for translation elongation; interacts with elongation factors
AF249673	amino acid transporter system A (ATA2)	2.98	1.38	short-chain neutral amino acid transporter
XM_225548	Net1 (Net1a; mNET1; Net1 homolog; 0610025H04Rik; 9530071N24Rik)	2.95	1.41	RhoA-specific GEF
M11188	18S rRNA gene	2.95	2.05	Component of 18S ribosome
X90486	mid BLAST match w/ promoter and enhancer of inhibitor of metallopoteinases-1	2.93	1.34	????
BC062000	Rdh6 (CRAD) – retinol dehydrogenase type II	2.92	1.39	Conversion of retinol to retinoic acid (ligand for RXR)
NM_053961	Erp29 (Erp28; Erp31; PDI-Db; 1200015M03Rik; 2810446M09Rik) – endoplasm reticulum protein 29	2.92	1.41	ER Chaperone; involved in secretory protein production
X62908	Cfl1 (Cof; cofilin)	2.92	1.30	Actin binding protein
	4632413K17Rik – no BLAST match	2.92	1.80	????
AY089719	Nell2 (mel91; A330108N19Rik) – neuron-specific epidermal growth factor-like repeat domain-containing protein	2.91	1.38	thrombospondin-like protein; promotes survival; promotes JNK phosphorylation and ERK dephosphorylation
XM_215270 (2)	Nt5c2 (GMP; NT5B; PNT5; cN-II) – cytosolic purine 5-nucleotidase	2.90	1.39	hydrolyzes 5'-AMP to adenosine
NM_012743	Foxa2 (Hnf3b; Tcf3b; Hnf-3b; Tcf-3b) – forkhead box A2, hepatocyte nuclear factor 3a	2.87	1.40	Transcription factor
12692	IMAGE=613673_13 (no BLAST match)	2.85	1.66	????
NM_032615	Mir16-pending (1200003M13Rik) – membrane interacting protein of RGS16	2.85	1.56	glycerophosphoinositol phosphodiesterase regulated by stimulation of G protein-coupled receptors
58	GENBANK=BM878001 (no BLAST match)	2.85	1.37	????
XM_224235	similar to zinc finger protein 198 (fused in myeloproliferative disorders protein)	2.85	1.46	????
	IMAGE=6436701 (no BLAST match)	2.82	1.37	????
XM_343803 (2)	Rps4x (Rps4; Rps4-1) – ribosomal protein S4, X-linked	2.82	1.38	component of 40S ribosome
BC061543	Hsd17b12 (KIK-I; 2610510O05Rik) – smooth muscle-specific 17 beta-hydroxysteroid dehydrogenase type 3	2.81	1.36	Steroid hormone synthesis
AJ278147 (2)	partial EF-2 gene and partial dlk gene for DAP-like kinase	2.81	1.54	translation, apoptotic when cytoplasmic
8044	IMAGE=6434084 (no BLAST match)	2.80	1.26	????
XM_213765	Cct6a (Cct6; Cctz-1; chaperonin containing TCP-1)	2.79	1.24	Component of type II chaperonin complex

1801	IMAGE=5682147 (no BLAST match)	2.78	1.35	????
NM_133556 (2)	Atp5g2 (1810041M08Rik) – ATP synthease, mitochondrial F0 complex, subunit c (9), isoform 2	2.78	1.31	ATP synthase; correlated with mitochondrial damage and metabolic stress
AY569013	Cn2-pending (0610010E05Rik) – cytosolic non-specific dipeptidase	2.78	1.59	protein processing
XM_340897	Insulin-like growth factor binding protein 4 precursor	2.76	1.55	inhibits IGF signalling
XM_238028 (3)	0610010O12Rik – hypothetical LOC291665	2.76	1.34	????
XM_222002	Cops6 (Sgn3; VIP/MOV34; COP9 complex S6)	2.75	1.38	nucleocytoplasmic shuttling protein; component of COP9 signalsome
NM_017138 (3)	Lamr1 (MLR; P40; Lamr; 67kDa; P40-3; P40-8; Lamrl1) – laminin receptor 1	2.75	1.62	ribosomal protein involved in apoptosis
668	IMAGE=6435569 (no BLAST match)	2.74	1.37	????
NM_017208	Lbp (Ly88) – lipopolysaccharide binding protein	2.73	1.47	upregulated by IL-1beta and TNFalpha
XM_344695	2810405I11Rik – similar to CHMP1.5 protein	2.73	1.21	143ignali trafficking
NM_022607 (2)	MIPP65 protein	2.72	1.44	mitochondrial phosphoprotein
XM_343821	Bex2 – brain expressed X-linked protein 2	2.72	1.34	signal transduction; suppressed by retinoic acid
10697	IMAGE=6437071 (no BLAST match)	2.72	1.38	????
	Arfgap3 (1810004P07Rik) – no BLAST match	2.71	1.31	intracellular traffic of proteins and vesicular transport; cytosolic protein and concentrated in the perinuclear region
X62322 (2)	Grn (epithelin; progranulin; acrogranulin; PC cell-derived growth factor)	2.70	1.46	secreted mitogen
XM_216661	5730503J23Rik; Pum2 – similar to Pum1 in rat BLAST	2.70	1.77	RNA-binding proteins; translational repressors
NM_022519	Serpina1a; Serpina1b (PI1; Spi1-1; PI2; Spi1-2; D12Ucla2) – serine (or cysteine) proteinase inhibitor, clade A, member 1	2.70	1.59	alpha-1-protease inhibitor
	AW212591; Nkd2 – no BLAST match	2.69	1.38	Dishevelled binding protein; negative regulator of Wnt & Beta-catenin 143ignaling; myristoylation-dependent escort of TGF alpha to the basolateral plasma membrane of polarized epithelial cells
NM_022542	Arhb; Gnb2-rs1 (Arh6; RhoB; p205; Rack1; GB-like)	2.69	1.50	adaptor/scaffold protein; binds activated PKC, Gbetagamma
NM_021578	Tgfb1 (Tgfb; Tgfb-1; TGF-beta 1) – transforming growth factor beta 1	2.69	1.40	growth modulatory and differentiation factor
XM_233268	Thea; BC026682 (BFIT; BFIT1; Them1; MGC:25974; 2010309H15Rik) – BLAST match w/ similar to CDNA sequence BC026682	2.69	1.26	acyl-CoA thioesterase
7403	IMAGE=613673_3 (no BLAST match)	2.68	1.64	????
XM_233720	1110035L05Rik	2.67	1.32	????
10306	IMAGE=5669984 (no BLAST match)	2.67	1.27	????
XM_227492	Mab21l2 – similar to mab-21-like protein 2	2.66	1.39	Developmental gene; perhaps transcription factor
NM_053886	lectin, mannose-binding, 1 (Lman1)	2.66	1.32	Chaperone for secreted proteins
XM_213782	Arpc3 (21kDa; p21-Ar; 1110006A04Rik; Arp2/3 complex subunit p21-Arc) – Actin-related protein 2/3 complex subunit 3	2.66	1.56	Initiates actin polymerization
NM_022180	hepatocyte nuclear factor 4, alpha (Hnf4a)	2.66	1.34	transcription factor for glucose metabolic genes
XM_233287	Tes (TESS; Tes1; Tes2; testin; testin2; D6Ertd352e)	2.66	1.31	structurally related to the mouse cysteine proteinase precursor but devoid of any protease/anti-protease activity

NM_024377	Rpf1-pending (2310066N05Rik) – low BLAST match w/ G protein gamma-5 subunit (Gng5)	2.65	1.43	GPCR
XM_343065	Nfkbia (Nfkbi; I(Kappa)B(alpha); nuclear factor of kappa light polyp gene enhancer in B-cell 1)	2.65	1.26	inhibits/heterodimerizes with NF-kappaB, preventing its translocation to the nucleus
XM_224406	Lcp1 (Pls2; L-fimbrin; D14Ertd310e) – lymphocyte cytosolic protein 1	2.65	1.32	component of actin cytoskeleton
XM_214981	Isg20 (20kDa; DnaQl; HEM45; 1600023I01Rik; 2010107M23Rik) – interferon-stimulated protein	2.65	1.46	3' to 5' exonuclease
12103	IMAGE=613673_1 (no BLAST match)	2.63	1.63	????
10055	IMAGE=6435979 (no BLAST match)	2.61	1.24	????
NM_172039 (3)	Hdlbp (D1Ertd101e; 1110005P14Rik) – high density lipoprotein-binding protein	2.61	1.39	transcriptional coactivator; contributes fasting-induced transcriptional activation of mitochondrial acetyl-CoA synthetase; cholesterol removal?
V01270 (3)	C87963; 1110003P22Rik – BLAST match w/ genes for 18S, 5.8S, and 28S ribosomal RNAs	2.61	1.44	ribosomal RNAs, GTP-activator activity on Rab-like GTPases
X82396	IMAGE=6432163 BLAST match w/ cathepsin B	2.60	1.36	????
NM_012551 (2)	early growth response 1 (Egr1)	2.60	1.50	Transcription factor
13461	IMAGE=763540 (no BLAST match)	2.59	1.29	????
M23572	1300002F13Rik – gene 33 DNA, exon 4 and 3' end	2.59	1.25	transcription factor??; transcriptionally enhanced by glucocorticoids, insulin, or cyclic AMP
X96721	Cyp3a11 (Pcn; Cyp3a; IIIAm1; steroid inducible) – cytochrome P450IIIA23	2.59	1.39	drug metabolism
NM_013055	Pcbp2; Map3k12 (Hnrpx; alphaCP-2; DLK; MUK; Zpk)	2.58	1.37	RNA-binding protein; posttranscriptional events
XM_217039	Eplin-pending (D15Ertd366e; 1110021C24Rik) – similar to Epithelial protein lost in neoplasm	2.58	1.30	regulates actin dynamics by cross-linking and stabilizing filaments
	IMAGE=6436700 (no BLAST match)	2.58	1.32	????
5051	IMAGE=613673_4 (no BLAST match)	2.57	1.59	????
9906	IMAGE=6430231 (no BLAST match)	2.57	1.51	????
5892	IMAGE=5668160 (no BLAST match)	2.57	1.33	????
NM_199099	Impdh2 (IMPD; IMP dehydrogenase) – inosine monophosphate dehydrogenase 2	2.56	1.47	de novo pathway of guanine nucleotide biosynthesis
9470	IMAGE=6435031 – not in database	2.55	1.45	????
M86708	Idb1 (Id1; D2Wsu140e) – inhibitor of DNA binding	2.55	1.33	Negative regulator of gene transcription
XM_213263	1810041F13Rik – similar to protein disulfide isomerase (pancreatic)	2.54	1.32	involved in unfolded protein response
XM_215655	Acp6 (mPACPL1; 5730559A09Rik) – similar to acid phosphatase 6, lysophosphatidic	2.54	1.26	Histidine acid phosphatase
7447	Rpl31; Tbc1d8 (no BLAST match)	2.53	1.23	ribosomal protein
5198	IMAGE=5645931 (no BLAST match)	2.52	1.21	????
13931	IMAGE=6433572 (no BLAST match)	2.52	1.38	????

XM_233581	Ddost – oligosaccharyltransferase	2.52	1.42	Protein processing; catalyzes the transfer of a high-mannose oligosaccharide (GlcNac2Man9Glc3) from a dolichol-linked oligosaccharide donor (dolichol-P-GlcNac2Man9Glc3) onto the 145ignaling145145 acceptor site within an Asn-X-Ser/Thr consensus motif in nascent polypeptide chains across the membrane of the endoplasmic reticulum
XM_238534	Prkcsh – alpha glucosidase II, beta subunit	2.51	1.26	PKC substrate OR hydrolyzes glycogen
8292	1110003E12Rik – not in database	2.51	1.24	????
5385	IMAGE=6434973 – not in database	2.51	1.27	????
BC058494 (3)	Rpl3 (F2; J1) – ribosomal protein L3	2.50	1.46	ribosomal protein L3 participates in the formation of the peptidyltransferase center
XM_342140	Vps26 (HB58; H beta 58) – similar to vacuolar protein sorting 26	2.50	1.24	Endosome-to-Golgi retrieval
NM_031510	Idh1 (Id-1; Idh-1; E030024J03Rik) – isocitrate dehydrogenase 1	2.49	1.31	TCA enzyme
BC059126 (2)	Taldo1 – transaldolase 1	2.49	1.46	pentose phosphate pathway, which is responsible for generation of reducing equivalents to protect cellular integrity from reactive oxygen intermediates
11259	2810451E09Rik – not in database	2.48	1.32	????
BC062072	voltage-gated Ca channel	2.48	1.30	voltage-gated Ca channel
AF153013 (2)	Tgfb2 (Tgfb-2) – short form precursor	2.48	1.48	apoptotic
Bc070958	BCL2/adenovirus E1B 19 kDa-interacting protein 3	2.48	1.36	member of the Bcl-2 family; involved in hypoxia and apoptosis pathways
BC061991	Suox (MGC:28458) – sulfite oxidase	2.48	1.32	catalyzes the terminal reaction in the sulfur amino acid degradation
XM_342687 (7)	D6Ertd772e (D6Ertd404e; 1200003J11Rik) – Tax1 binding protein 1	2.47	1.35	regulates transcription by protein-protein interaction, but not DNA binding
XM_342755	Bcap37 (Bap37) – B-cell receptor-associated protein 37	2.46	1.53	membrane-bound chaperone for the stabilization of mitochondrial proteins
XM_343413	Pdcd7 (ES18)	2.46	1.37	apoptotic cell death of T-cells; induced by ceramide
XM_216612	EXCretory canal abnormal EXC-7, ELAV type RNA binding protein	2.45	1.24	pre-mRNA splicing factor
XM_218427	Pvrl2 (MPH; Pvr; Pvs; Cd112; nectin-2) – similar to poliovirus receptor homolog (LOC308417)	2.45	1.29	ligand for DNAM-1 (leukocyte adhesion molecule)
7845	IMAGE=6430076 (no BLAST match)	2.45	1.32	????
BX883049	Snx3 (SDP3) – BLAST match w/ chromosome 20, major histocompatibility complex	2.43	1.60	regulates endosomal function/trafficking
M57719	cytochrome P-450 IVA2 (CYP4A2) gene	2.43	1.46	hydroxylate epoxyeicosatrienoic acids to form high affinity peroxisome proliferator-activated receptor ligands
AJ298278 (3)	Pabpc1 (PABP; Pabp1; PabpI; Pabpl1) – poly(A) binding protein	2.43	1.44	Arginine methyltransferase; in translation initiation complex and regulates mRNA decay; polyadenylates mRNA
XM_236970	Pgk2 (Pgk-2; Tcp-2; Tcp-2) – phosphoglycerate kinase	2.43	1.28	glycolytic enzyme
AY392036	Map1lc3 (1010001C15Rik) – microtubule-associated protein 1 light chain 3	2.42	1.27	component of autophagic vacuoles/autophagosomes
XM_216558	Sdhb (0710008N11Rik) – succinate dehydrogenase Ip subunit	2.42	1.41	complex 2 of the respiratory chain,succinate-ubiquinone oxidoreductase
AY190520	Rnf130 (GP; G1RP; G1RZFP; GOLIATH; 2510042A13Rik) – liver r-goliath	2.42	1.65	transcription factor found in mitochondria
5203	1810015M01Rik (no BLAST match)	2.42	1.24	????

XM_234377	Ches1 (5430426H20Rik) – checkpoint suppressor 1	2.42	1.51	forkhead/winged helix transcription factor
XM_343483	Dag1 (DG; D9Wsu13e; dystrophin associated glycoprotein 1) – dystroglycan 1	2.42	1.40	adhesion molecule which connects the extracellular matrix to the cytoskeleton
XM_228689	Cul4b (2700050M05Rik) – cullin 4B	2.41	1.30	ubiquitin ligase
XM_340941 (2)	Ptdsr (PtdSerR; 5730436I23Rik) – similar to mKIAA0585 protein	2.41	1.36	phosphatidylserine receptor; apoptotic cell recognition
NM_024155 (2)	Anxa4 (Anx4; Xanx-4) – ZAP 36/annexin IV (Anxa4)	2.40	1.25	exocytosis; contributes to granule formation, presumably by interaction with the glycosylphosphatidylinositol-anchored glycoprotein GP-2
BC062230	Ucp2 – Uncoupling protein 2	2.40	1.53	regulator of reactive oxygen species production in mitochondria
NM_012620	Serpine1 (PAI1; PAI-1; Planh1) – serine (or cysteine) proteinase inhibitor, member 1	2.39	1.45	proteolysis
XM_340954	0610008N23Rik (D16Bwg0193e)	2.39	1.26	????
BC061561	Rps26 – ribosomal protein S26	2.39	1.37	ribosomal protein; storage of pre-mRNAs inactive in splicing
NM_017283	Psma6 (IOTA) – proteasome (prosome, macropain) subunit, alpha type 6	2.38	1.33	component of the 20S proteasome
BC063809 (8)	Gnb2-rs1 (p205; Rack1; GB-like) – guanine nucleotide binding protein, beta polypeptide 2-like 1	2.38	1.43	adaptor/scaffold protein; binds activated PKC, Gbetagamma
XM_215970	2700038C09Rik	2.38	1.24	????
12104	IMAGE=613673_9 (no BLAST match)	2.38	1.61	????
XM_215255	Aldh2; Cyp2c50; Cyp2c37 (Ahd5; Ahd-5) – Cytochrome P450 2C6 (CYPIIC6) (P450 PB1)	2.37	1.30	Mitochondrial aldehyde dehydrogenase 2 (ALDH2) plays a major role in ethanol metabolism
8909	IMAGE=6434870 (no BLAST match)	2.37	1.24	????
X54626	Cebpb (LAP; CRP2; NF-M; Nfil6; NF-IL6; IL-6DBP; C/EBP BETA) – liver-enriched transcriptional activator protein	2.37	1.58	basic region-leucine zipper transcription factors
NM_019195	Cd47; B430305P08Rik (IAP; CD47; Itgp) – integrin-associated protein (Cd47)	2.37	1.41	Thrombospondin receptor; novel form of apoptosis
XM_216891 (2)	Cct2 (Cctb) – similar to chaperone containing TCP-1 beta subunit	2.37	1.32	Cytolsolic chaperone
NM_138530	MAWD Binding Protein	2.37	1.35	MAWD is phosphorylated by TGFbeta II receptor
XM_217042	low BLAST match to hypothetical protein FLJ20436	2.36	1.18	????
X94242 (2)	ribosomal protein L14	2.36	1.47	one of the most conserved ribosomal proteins and appears to have a central role in the ribonucleoprotein complex
AB017696 (3)	Cdh1 (Um; UVO; Ecad; E-cadherin; uvomorulin)	2.36	1.35	Activates anaphase promoting complex (APC) ubiquitin ligase
XM_342544	2810442O16Rik (1110028I04Rik) – similar to chromosome 20 open reading frame 140	2.35	1.20	GTPase activating protein
XM_216024 (2)	Rpl7a (Surf-3; surfeit 3) – similar to 60S ribosomal protein L7a, Surfeit locus protein 3, PLA-X polypeptide	2.34	1.32	ribosomal large subunit protein
AB105193	Xpo1 (Crm1) – nuclear export factor CRM1	2.34	1.33	mediates the nuclear export of a variety of protein and RNA substrates
NM_017245 (3)	Eef2 (Ef-2) – eukaryotic translation elongation factor 2	2.34	1.45	translation
XM_347275	similar to RIKEN cDNA 9030221M09	2.33	1.44	Phosphatidylserine decarboxylase
XM_217243	Srprb – similar to Ab2-417	2.33	1.22	Sar1p-like members of the Ras-family of small GTPases

XM_342989	LOC362671	2.32	1.39	????
XM_236058	Esam-pending (W117m; 2310008D05Rik) – endothelial cell-selective adhesion molecule	2.32	1.21	immunoglobulin receptor
NM_022602	Pim3 (KID-1/kinase induced by depolarization) – serine threonine kinase pim3	2.31	1.30	serine threonine kinase; transcriptional repressor
XM_235618	AI043245; Asb8 – ankyrin repeat and SOCS box-containing protein 8	2.31	1.27	Inhibit cytokine signalling
NM_012941 (2)	Cyp51 – cytochrome P450, subfamily 51	2.30	1.23	Sterol 14 alpha-demethylase
XM_216781	D12Ertd771e (2610305M23Rik)	2.30	1.50	????
XM_220128	similar to mKIAA0350 protein (LOC302902)	2.30	1.18	????
Z29649	Prx – periaxin	2.30	1.42	Structural protein?
AB088765	Trfr (CD71; Mtvr1; Mtvr-1; 2610028K12Rik; E430033M20Rik) – pseudogene for basic transcription element binding protein 2 (btebp2)	2.29	1.35	Transferrin receptor; forms “immunological synapse”; in plasma membrane and 147ignaling endosomes
9020	8430417A20Rik (no BLAST match)	2.28	1.29	????
BC061978 (2)	AL024364; Oprs1 – opioid receptor, sigma 1	2.27	1.45	basic region-leucine zipper transcription factors
2679	IMAGE=5678544 (no BLAST match)	2.27	1.34	????
NM_012889	Vcam1 (CD106; Vcam-1) – vascular cell adhesion molecule 1	2.27	1.52	cell surface sialoglycoprotein highly expressed on endothelial cells following cytokine stimulation
7495	IMAGE=6432314 (no BLAST match)	2.27	1.25	????
BC062036 (2)	Rnp24-pending (Sid394; 1110032D12Rik; 1810020N21Rik) – BLAST match w/ cDNA clone MGC:72390 IMAGE:5623841	2.27	1.27	RNA binding protein? OR 147ignali trafficking and protein transport
BC058132	low BLAST match w/ epididymal secretory protein 1	2.27	1.24	lipid recognition; receptor?
U54632	ubiquitin-conjugating enzyme UbcE2A	2.26	1.25	ubiquitin-conjugating enzyme
XM_217310	low BLAST match w/ similar to putative calcium binding transporter	2.26	1.28	Ca+ Transporter
XM_222279 (2)	Sart3 – tumor-rejection antigen SART3	2.26	1.30	U4/U6 snRNP recycling
13088	IMAGE=6432432 (no BLAST match)	2.26	1.23	????
NM_013170	Gucy2c (GC-C) – guanylate cyclase 2C	2.26	1.25	receptor for guanylin and uroguanylin
XM_213540 (2)	Actg – gamma actin-like protein	2.25	1.30	smooth muscle gamma actin
XM_215758	Fkbp7 (23kDa; FKBP23) – FK506-binding protein	2.25	1.24	Ca+ dependent binding to BiP (Hsp70) in ER; peptidyl-prolyl cis-trans isomerase
9493	D2Bwg1356e (4930500L08Rik) – no BLAST match	2.25	1.53	????
NM_057132 (3)	plysia ras-related homolog A2 (Arha2)	2.24	1.40	Ras-related small G-protein
NM_053892	Pla2g6 – phospholipase A2, group VI	2.24	1.22	calcium-independent PLA
658	IMAGE=6435047 – not in database	2.24	1.42	????
XM_343249	IMAGE=779681 – similar to Hypothetical protein KIAA0196	2.24	1.30	????
BC061727	Ak2 (Ak-2; D4Ertd220e) – adenylate kinase 2	2.24	1.24	cytosolic insulin binding protein; ATP:AMP phosphotransferase (makes 2 ADPs)
NM_017356 (3)	Hpcal1 (Nvp3; Vnsl3; VILIP3; visinin like 3; neural visinin-like 3) – neural visinin-like Ca2+-binding protein type 3	2.23	1.26	Ca+ sensor protein

XM_223494	similar to mKIAA1916 protein (LOC305417)	2.23	1.25	????
X95088	2010003O02Rik – low BLAST match w/ parathyroid hormone regulated sequence	2.23	1.35	????
XM_223834 (2)	similar to 40S ribosomal protein S7 (S8)	2.23	1.22	40S ribosomal protein
AJ301677	Mlp (F52; Mrp; D4Bc1; Macs2; Macs3; MacMARCKS) – Mac-MARCKS protein (F52 gene)	2.21	1.22	prevents the export of intron-containing transcripts
BC062036	Rps6; Tcfcp2 – ribosomal protein S6 (Rps6)	2.21	1.21	Ribosomal protein S6 located in the mRNA binding site of the 40S subunit of cytosolic ribosomes
V01233	preproelastase (pancreatic elastase II)	2.21	1.30	angiotensin II generation
XM_343278	AL024098; Rpl8 – ribosomal protein L8	2.21	1.36	ribosomal protein
11565	Slc31a1 (Ctr1; 4930445G01Rik) – not in database	2.21	1.29	copper transporter
NM_053609	HLA-B-associated transcript 3 (Bat3)	2.21	1.23	apoptosis?
XM_341131 (2)	Arpc5 (165kDa; p16-Arc; 5830443F10Rik) – ARP2/3 complex 16 kDa subunit	2.21	1.25	actin polymerization
XM_230589	Mcpr (Apc1; tsg24; 2610021O03Rik) – similar to tsg24 (LOC311412)	2.20	1.21	largest protein of the anaphase-promoting complex or cyclosome
BC065577	Car2 (CAII; CA II; Car-2; Ltw-5; Lvtw-5) – carbonic anhydrase 2	2.20	1.32	autoantigen; zinc metalloenzyme that catalyzes the reversible hydration-dehydration of CO2 and HCO3-
	IMAGE=6434790 (no BLAST match)	2.20	1.21	TGFbeta superfamily member
XM_230274	2310042M24Rik	2.20	1.24	Thioredoxin??
BC058467 (3)	Rpl10 (QM; QM16; D0HXS648; DXHXS648; DXHXS648E; 24.6kDa protein) – ribosomal protein L10	2.20	1.25	60S ribosomal subunit protein; translational regulator
M12516	Por (CPR; CYPOR; NADH cytochrome P450 oxydoreductase)	2.20	1.37	transfers electrons to microsomal cytochromes P450
XM_214583 (2)	Hspa9a (mot2; 74kDa; Hsc74; Hsp74; Hsp74a; mortalin) – similar to grp75	2.20	1.24	negative regulator of gene expression
NM_031114 (2)	S100a10 (p11; Cal1l) – S-100 related protein	2.20	1.34	Ca+ binding, perhaps involved in chronic inflammatory responses
XM_222000	Zipro1 (RU49; Zfp38; Zfp-38; Ctfin51) – zinc finger proliferation 1	2.20	1.35	transcription factor or coactivator
XM_230296	similar to endoplasmic reticulum membrane protein with at least 3 transmembrane domains of bilateral origin like (XB300)	2.19	1.20	????
XM_342383	Fbxw5 – WD repeat-containing F-box protein FBW5	2.18	1.25	component of ubiquitin ligase??
NG_004074	Rnf5 (NG2; 2410131O05Rik) – ing finger protein 5 pseudogene on chromosome 20	2.18	1.24	a RING finger protein that regulates cell motility by targeting paxillin ubiquitination and altered localization
XM_215710	Emcn-pending (0610012K22Rik) – endomucin-1	2.17	1.28	ligand for selectins; pro- or anti-cell adhesion
NM_133599	Cdc42ep1; Lgals2 (CEP1; Borg5; MSE55; 1810058K22Rik; 2200008F12Rik) – lectin, galactoside-binding, soluble, 2 (galectin 2)	2.17	1.34	a member of the Rho GTPase family that mediates actin cytoskeleton reorganization at the plasma membrane
XM_226240	similar to putative transcription factor Zfp319	2.16	1.28	involved in the induction of programmed cell death
XM_343103	2900070E19Rik (2310004O13Rik)	2.16	1.27	Glutaredoxin-related protein
NM_019365	Rassf5 (Rapl; Maxp1; Nore1A; Nore1B; 1300019G20Rik) – protein interacting with guanine nucleotide exchange factor	2.16	1.21	binds to active Ras; growth inhibitory activity
AB052085	delta-5 fatty acid desaturase	2.16	1.28	unsaturated fatty acid synthesis

AB004231	IMAGE=6430204 mid BLAST match w/ selenium-dependent glutathione peroxidase	2.15	1.33	????
XM_216323	Mrpl15 (Rpml7; MRP-L7; HSPC145) – mitochondrial ribosomal protein L15	2.15	1.24	60S ribosomal subunit protein
9755	IMAGE=613673_2 (no BLAST match)	2.15	1.50	????
BC058149 (4)	Rps6; Tcfcp2 – ribosomal protein S6 (Rps6)	2.15	1.32	Ribosomal protein S6 located in the mRNA binding site of the 40S subunit of cytosolic ribosomes
11232	Eif3s6ip (PAF67; HSP-66Y; MGC:37328; 0610011H21Rik) – not in database	2.15	1.38	Protein that is associated with the initiation-competent form of RNA polymerase I
XM_343670	Ubxdc2 (Ubxd1; 1200008L11Rik; 2210415J11Rik) – similar to UBX domain-containing protein 1	2.15	1.26	ubiquitin regulatory protein
XM_230861	IMAGE=6432362 – BLAST match w/ similar to sulfatase FP (LOC311642)	2.15	1.28	????
XM_340847	Tiaf1 – similar to myosin containing PDZ domain	2.14	1.23	TGF-beta 1-induced factor that protects L929 fibroblasts from TNF-mediated apoptosis
XM_343403	ribosomal protein, large, P1 (Rplp1)	2.14	1.40	60S Acidic ribosomal protein
NM_012655	Sp1 (Sp1-1) – Sp1 transcription factor	2.14	1.22	Transcription factor
XM_340785	Pank3 – pantothenate kinase 3	2.14	1.28	catalyzes the rate-controlling step in the coenzyme A (CoA) biosynthetic pathway
BC063162 (4)	Eef1a1 (MGC:7551; MGC:8115; MGC:8209; MGC:18758; MGC:27859) – eukaryotic translation elongation factor 1 alpha 1	2.13	1.32	key factor in protein synthesis, where it promotes the transfer of aminoacylated tRNAs to the A site of the ribosome
XM_346253	similar to RIKEN cDNA 1700001E04, low BLAST match w/ ubiquitin-conjugating enzyme E2 variant 1 isoform b	2.13	1.21	ubiquitin-conjugating enzyme??
NM_053611	Nupr1 (p8; Com1; 2310032H04Rik) – nuclear protein 1	2.13	1.25	transcription factor; anti-inflammatory
9756	IMAGE=613673_10 (no BLAST match)	2.13	1.47	????
629	8430436F23Rik (no BLAST match)	2.13	1.29	????
XM_017311	Atp5g1 – ATP synthase, H+ transporting, mitochondrial F0 complex, subunit c, isoform 1	2.13	1.35	mitochondrial ATP synthase
X71122	Ceacam2; Ceacam1 (Bgp2; Bgp; Bgp1; Cea1; MHVR; bb-1; C-CAM; CD66a; Cea-1; Cea-7; mCEA1; mmCGM1; mmCGM2; mmCGM1a; mouse hepatitis virus receptor) – cell adhesion molecule (C-CAM)	2.13	1.27	cell adhesion; receptor-mediated insulin endocytosis
XM_213769	BC026744 (2300007F24Rik) – similar to Hypothetical protein MGC25614	2.12	1.31	????
NM_012821	Adcy6 – adenylyl cyclase 6	2.12	1.30	cAMP generation
AY179603	IMAGE=5946728 – mid BLAST match w/ Kv3.3c voltage gated potassium channel subunit splice variant C	2.12	1.23	potassium channel
XM_215880	Raly (Merc) – RNA-binding protein Raly	2.12	1.40	ubiquitously expressed gene of unknown function, which encodes a new member of the heterogeneous nuclear ribonucleoprotein gene family
	IMAGE=5682240 (no BLAST match)	2.11	1.21	????
XM_232596 (2)	Tram1 (TRAMP; MGC:1174; 1810049E02Rik)	2.11	1.39	Transcriptional coactivator

BC059123 (3)	Ran – member RAS oncogene family	2.11	1.23	GTPase; control centrosome and spindle pole function and kinetochore function
BC058135 (3)	Rpl19 – ribosomal protein L19	2.10	1.24	ribosomal protein
NM_022498	Ppp1cc (dis2m1; PP1C gamma; PP1C gamma 1; PP1C gamma 2) – protein phosphatase 1, catalytic subunit, gamma isoform	2.10	1.30	protein phosphatase
U38370	Hap1 (HAP-1) – huntingtin associated protein (rHAP1-B)	2.10	1.28	endonuclease
NM_012801	Pdgfa – platelet derived growth factor, alpha	2.09	1.23	growth factor
XM_231280	Nyren18-pending (BS4; 6330412F12Rik) – similar to NEDD8 ultimate buster-1	2.09	1.24	Ubiquitin-like protein that controls vital biological events through its conjugation to target proteins
XM_343475	Nprl2-pending (2810446G01Rik) – similar to G21 protein	2.09	1.21	????
NM_053328	Bhlhb2 (CR8; Clast5; Stra13; Stra14; C130042M06Rik; cytokine response gene 8; eip1 (E47 interaction protein 1)) – basic helix-loop-helix domain containing, class B2	2.09	1.37	transcription factor
BC061580	basic leucine zipper and W2 domains 1	2.09	1.18	Translation factor??
NM_172023	Osbpl1a – oxysterol binding protein-like 1A	2.09	1.30	Cholesterol metabolism
6623	IMAGE=6432423 (no BLAST match)	2.09	1.26	????
X04310	low BLAST match w/ thymocyte mRNA for 37K chain of CD8 antigen	2.09	1.28	CD8??
	Hypothetical LOC315211, hypothetical LOC310330 (no BLAST match)	2.09	1.24	????
BC063174 (3)	Eno1 (Eno-1; MBP-1; alpha-enolase; 2-phospho-D-glycerate hydrolase)	2.08	1.38	glycolytic enzyme OR negatively regulates the transcription of the protooncogene Myc
2117	IMAGE=6436217 (no BLAST match)	2.08	1.17	????
NM_031130	Nr2f1 (EAR3; SVP44; Erbal3; COUPTFA; COUP-TF1; COUP-TFI; Tcfcoup1) – nuclear receptor subfamily 2, group F, member 1	2.08	1.21	Transcription factor
9282	Dtnbp1 (sdy; dysbindin; 5430437B18Rik) – no BLAST match	2.08	1.25	exocytosis??
XM_213779	ataxin-2	2.08	1.26	apoptosis
XM_341259	1110067D22Rik	2.08	1.35	????
XM_220885	similar to cDNA sequence BC034054 (LOC287637)	2.08	1.25	????
XM_213823	Rbms2 (Scr3) – similar to RNA binding motif, single stranded interacting protein 2	2.07	1.19	RNA binding protain
11182	IMAGE=5668123 (no BLAST match)	2.07	1.18	????
AB022701	Ak1 (Ak-1; B430205N08Rik) – adenylate kinase isozyme 1	2.07	1.23	adenine nucleotide metabolism
11795	IMAGE=5679974 (no BLAST match)	2.07	1.18	????
XM_343415 (2)	AA409661; Usp3 – ubiquitin specific protease 3	2.06	1.25	deubiquitination
10343	IMAGE=613673_6 (no BLAST match)	2.06	1.54	????
XM_342414	Sh2d3c (Chat; Nsp3; Shep1; Cas/HEF1-associated signal transducer; SH2-containing Eph receptor-binding protein 1)	2.06	1.21	adaptor proteins linking integrin and tyrosine kinase receptors to the c-Jun N-terminal kinase/stress-activated protein kinase signaling pathway
XM_345856	Gcat (Kbl; aminoacetone synthase) – 2-amino-3-ketobutyrate-coenzyme A ligase	2.06	1.42	threonine degradation
XM_218854	Mesdc2 (2210015O11Rik) – Mesoderm development candidate 2	2.06	1.22	????

11907	8430408O14Rik; 2900026A02Rik (no BLAST match)	2.06	1.23	????
XM_217601	Kcnd1 (Shal; Kv4.1; Kca2-1; mShal1; 1110037K09Rik) – similar to potassium channel protein Shal1 – mouse	2.06	1.26	potassium channel
AF121345	Phyh (PAHX; Lnap1) – peroxisomal phytanoyl-CoA hydroxylase	2.06	1.36	peroxisomal lipid metabolism
XM_222899	Copa – coatomer protein complex subunit alpha	2.06	1.24	ER/Golgi protein trafficking
XM_234468	ribosomal protein S6 kinase, polypeptide 4; mitogen- and stress-activated protein kinase-2	2.06	1.30	ribosomal protein; phosphorylated by mTOR; protein synthesis
XM_226369	Rbmxrt (Hnrpg; hnRNP G) – similar to heterogeneous nuclear ribonucleoprotein G (human)	2.06	1.42	mRNA synthesis and maturation
XM_235705	Coatomer zeta-1 subunit	2.06	1.28	ER/Golgi protein trafficking
9518	Vcl; Ap3m1 – not in database	2.05	1.22	????
4151	2810410A03Rik (no BLAST match)	2.04	1.21	????
XM_222624	R3H domain (binds single-stranded nucleic acids)	2.04	1.22	binds single-stranded nucleic acids
2393	IMAGE=5679847 (no BLAST match)	2.04	1.27	????
U05963	2700084A17Rik; Ptpn11 – Sprague Dawley protein tyrosine phosphatase, nonreceptor type 11	2.04	1.27	protein tyrosine phosphatase
8433	BB219131 (no BLAST match)	2.04	1.26	????
NM_020075	eukaryotic initiation factor 5 (eIF-5)	2.03	1.36	translation
AF148323	Arts1-pending (PILSAP) – aminopeptidase PILS	2.03	1.17	aminopeptidase
XM_215631	similar to hypothetical protein MGC11722	2.03	1.26	????
1132	blank1175 – not in database	2.03	1.28	????
NM_031515	Kras2 (K-ras; Ki-ras; Kras-2) – p21 (c-Ki-ras)	2.03	1.18	protooncogene
6823	Sox9; AV220920 (2010306G03Rik) – not in database	2.02	1.24	transcription factor
XM_345143	Col4a3bp (GPBP; 2810404O15Rik; 9230101K08Rik) – similar to Goodpasture antigen-binding protein	2.02	1.19	serine/threonine kinase; autoantigen
9229	IMAGE=6436172 – not in database	2.02	1.26	????
XM_214288	9430098E02Rik	2.02	1.22	????
AY071924	Ero1l – oxidoreductase ERO1-L	2.01	1.23	makes oxidative conditions in the ER; induced in the course of the unfolded protein response
NM_181637	CDC91 cell division cycle 91-like 1, PIG-U	2.01	1.22	oncogene; a transamidase complex unit in the glycosylphosphatidylinositol (GPI) anchoring pathway
4160	IMAGE=5679565 (no BLAST match)	2.01	1.25	????
U73458	protein tyrosine phosphatase (PTPNE6)	2.01	1.36	protein tyrosine phosphatase
XM_341577	LOC361292	2.00	1.32	????
13333	2410005K20Rik – not in database	2.00	1.26	????
10674	1110030L07Rik – not in database	2.00	1.20	????
NM_199102	2400001E08Rik – Unknown (protein for MGC:72560)	2.00	1.25	????
XM_216815	IMAGE=5680353 – similar to HCV NS3-transactivated protein 1	2.00	1.26	????

XM_233022	similar to U4/U6 small nuclear ribonucleoprotein Prp4 (U4/U6 snRNP 60 kDa protein) (WD splicing factor prp4)	2.00	1.29	mRNA splicing; assembly of an active spliceosome
XM_214067	Xbp1 (TREB5; TREB5; XBP-1; TREB-5; D11Ertd39e) – similar to hepatocarcinogenesis-related transcription factor	2.00	1.18	transcription factor; basic region leucine zipper protein which binds to a cyclic AMP responsive element (CRE)-like element
XM_228014	Bing4	2.00	1.28	????
NM_012766	Ccnd3; Ccnd3-ps – cyclin D3	2.00	1.30	cell cycle
NM_134382	Elovl5 (HELO1; 1110059L23Rik; - fatty acid elongase 1	2.00	1.22	PUFA elongation
XM_343537	similar to KIAA0792 gene product	1.99	1.23	????
XM_237056	Rw1-pending (Neg; CC28; YR-23; D1Bwg0491e; 2610524E03Rik) – no BLAST match	1.99	1.24	????
NM_012545	dopa decarboxylase (Ddc)	1.99	1.18	DOPA decarboxylase
XM_216479	D4Ertd765e (1300017C12Rik)	1.98	1.18	????
AY155572	Gpiap1 (MMGPIP137) – glycosyl-phosphatidyl-inositol-anchored protein p137-like	1.98	1.26	GPI-anchored protein
M12673 (2)	Gnas (P1; P2; P3; Gsa; Nesp; Gnas1; Gnasxl; Nesp55; Gs alpha; Gs-alpha; G alpha s; XL alpha s) – guanine nucleotide-binding protein G-s, alpha subunit	1.98	1.23	cAMP generation, GPCR
NM_022592 (3)	Tkt (TKT; p68) – transketolase	1.98	1.36	catalyzes the reversible transfer of a two-carbon ketol unit from xylulose 5-phosphate to an aldose receptor, such as ribose 5-phosphate, to form sedoheptulose 7-phosphate and 152ignaling152152g152es 3- phosphate; links glycolysis and pentose phosphate shunt
U35365	Fyn (Src Kinase p59) – proto-oncogene FYN	1.98	1.21	Src kinase
XM_342775	Hebp1 (p22 HBP) – heme binding protein	1.98	1.24	????
XM_213728	AI747533; Mcm7 – similar to mCDC47	1.97	1.25	Minichromosome maintenance
XM_341877	Iqgap1 (D7Ertd237e; D7Ertd257e) – similar to IQ motif containing GTPase activating protein 1	1.97	1.28	Regulates cytoskeletal dynamics through interactions with the Rho family GTPases
11373	D3Ertd330e (no BLAST match)	1.97	1.30	????
94	Cbx5 (HP1a; Hp1a; mHP1(alpha); 2610029O15Rik; heterochromatin protein 1 alpha) – NOT IN DATABASE	1.97	1.19	Protein involved in the packaging of chromosomal domains into a repressive heterochromatic state
XM_232991	coronin, actin binding protein, 2A	1.97	1.20	Actin binding protein
AF192757	ALG-2 interacting protein 1	1.97	1.21	Cell death and vesicular trafficking
XM_342024	Ddb1 (127kDa; p127-Ddb1; DNA repair protein) – DNA damage binding protein 1	1.97	1.26	DNA repair
NM_173145	BC024558 (MGC:37636) – disks large-associated protein 4 (Dap4)	1.96	1.19	Promotes MST1-induced apoptosis by enabling colocalization of MST with p53
XM_213385	Il2ra; Prpf8 (CD25; Il2r; Ly-43; p55 chain gene; IL-2R alpha chain; Prp8; Sfprp8l; DBF3/PRP8; D11Bwg0410e) – splicing factor Prp8	1.96	1.31	pre-mRNA splicing factor
X75307	Tap2 (Y1; HAM2; Ham2; MTP2; PSF2; ABC18; Abcb3; Ham-2; PSF-2; Tap-2; RING11)	1.96	1.31	peptide transporters; antigen processing
NM_053998	Mel – transforming oncogene (derived from cell line NK14)	1.95	1.21	oncogene

8417	Stratagene Control Human COT1 (Block 29) – not in database	1.95	1.18	????
AB016800	Dhcr7 – 7-dehydrocholesterol reductase	1.95	1.28	Cholesterol metabolism
NM_153303	Dyt1 (DQ2; Tor1a) – dystonia 1, torsion (autosomal dominant; torsin A)	1.95	1.23	AAA+ ATPase; membrane trafficking, organelle biogenesis, proteosome function, and microtubule regulation
AB116149	PGAP1 mRNA for GPI deacylase	1.94	1.30	Inositol deacylation
U49694	Bach-pending (2410041A17Rik) – brain cytosolic acyl coenzyme A thioester hydrolase	1.94	1.39	acyl coenzyme A thioester hydrolase
X92097	Rnp24-pending (Sid394; 1110032D12Rik; 1810020N21Rik) – transmembrane protein rnp21.4	1.94	1.30	RNA binding protein? OR 153ignali trafficking and protein transport
XM_224894	mCAF1 protein	1.94	1.19	mRNA processing; processive deadenylase/3'-5'-exonuclease
3026	IMAGE=6434608 (no BLAST match)	1.93	1.24	????
XM_343136	Atp5b – ATP synthase, H+ transporting, mitochondrial F1 complex, beta polypeptide	1.93	1.19	ATP synthase; correlated with mitochondrial damage and metabolic stress
	1810012I05Rik – no BLAST match	1.93	1.20	????
XM_223403	BC013481 (MGC:18900; 9530077J19Rik)	1.93	1.24	????
XM_342742	Mbd4 – methyl-CpG binding domain protein 4	1.93	1.21	DNA repair; remove thymines from T:G mismatches resulting from deamination of 5-methylcytosine
XM_221747	Gbe1 (D16Ertd536e; 2310045H19Rik; 2810426P10Rik) – glucan (1,4-alpha-), branching enzyme 1	1.92	1.22	Glycogen metabolism; catalyzes hydrolysis at the –(1,4)-glucosidic linkage
6267	Arih1 (ARI; HARI; HHARI; UIP77; UBCH7BP) – not in database	1.92	1.22	ubiquitin-conjugating enzyme
614	Rnf7 (SAG) (no BLAST match)	1.92	1.17	A novel zinc RING finger protein that protects cells from apoptosis induced by redox agents
X74593	Sdh1 (Sord; Sdh-1; Sodh-1) – sorbitol dehydrogenase	1.92	1.26	Sorbitol dehydrogenase
6380	1810043M15Rik (no BLAST match)	1.92	1.21	????
XM_213293	D11Ertd175e; 0610039K22Rik (2410130M07Rik) – BLAST match w/ nucleolar protein family A, member 2	1.92	1.37	????
BC061547 (3)	heat shock protein 8	1.91	1.27	protein chaperone
NM_053592	Dutp (dUTPase; D2Bwg0749e; 5031412I06Rik; 5133400F09Rik) – Deoxyuridinetriphosphatase (dUTPase)	1.91	1.32	dUTPase
XM_230292	GDP-fucose transporter 1, multispan transmembrane protein fuct1	1.90	1.34	GDP-fucose transporter 1
NM_031030	Gak (MGC:31204; D130045N16Rik) – cyclin G-associated kinase	1.90	1.22	Clathrin-mediated endocytosis; Ser/Thr protein kinase
BC058450	Prdx1 (PAG; Paga; Prxl; TDX2; MSP23; NkefA; OSF-3; TPx-A; Tdpx2; osteoblast specific factor 3; macrophage 23kDa stress protein; macrophase stress protein 22kDa; Trx dependent peroxide reductase 2; thioredoxin dependent peroxide reductase 2) – peroxiredoxin 1	1.90	1.24	peroxide metabolism
D17296	Ubc – polyubiquitin (ubiquitin C)	1.90	1.24	Ubiquitin-dependent degradation
AY156922	Cdc26-pending (2010012C09Rik) – similar to CDC26, but better BLAST match w/ BWK-2	1.90	1.16	Component of anaphase-promoting complex (APC) that is a cell cycle-regulated ubiquitin-protein ligase
NM_139082	Bambi (2610003H06Rik) – BMP and activin membrane-bound	1.89	1.31	An inhibitor of transforming growth factor-beta signaling

	inhibitor			
XM_343494	Pdcd6ip (AIP1; Alix; Eig2) – programmed cell death 6 interacting protein	1.89	1.18	Inhibitor of paroptosis
AF092445	low BLAST match w/ MIS type II receptor (MRII)	1.89	1.22	TGFbeta superfamily member
XM_342760	Cd9 – CD9 antigen (p24)	1.89	1.18	Tetraspanin; interacts with GPCRs
XM_213324	Shmt1; 2310076G09Rik (Shmt; mshmt; mshmt1; mshmt2) – serine hydroxymethyl transferase 1	1.89	1.27	folate metabolism; serine hydroxymethyl transferase
NM_031135	Tieg1 (mGIF; Gdnfif) – TGFB inducible early growth response	1.89	1.24	apoptosis; upregulated by NO
NM_017151 (2)	Rps15 (rig; insulinoma; rat insulinoma gene) – ribosomal protein S15	1.88	1.28	ribosomal protein
XM_216635	Cox7a2l (EB1; SIG81; COX7AR; COX7RP; SIG-81)	1.88	1.21	Cytochrome c oxidase subunit VIIa
XM_230734	H13 (H-13; 1200006O09Rik; 4930443L17Rik; 5031424B04Rik) – histocompatibility 13; presenilin-like protein 3	1.88	1.26	????
Z12651	Comt (D16Wsu103e) – catechol methyltransferase	1.88	1.30	Tyrosine and catecholamine metabolism
XM_213898 (2)	Ivns1abp; Acvrinp1 (ND1; NS-1; Nd1-L; Nd1-S; NS1-BP; HSPC068) – kelch family protein Nd1-L	1.88	1.26	notch 154ignaling??
XM_221955	IMAGE=6436228 – similar to hypothetical protein MGC9712	1.88	1.29	????
NM_031318	Tctex1 (Tctex-1) – t-complex testis expressed 1	1.87	1.31	Vesicle transport
XM_232413	Ppp2r5a – protein phosphatase 2A, 56 kDa regulatory subunit, alpha isoform	1.87	1.24	Protein phosphatase
NM_133293	Gata3 (Gata-3) – GATA-binding protein 3	1.87	1.25	Chromatin 154ignaling154; master transcription factor
XM_343499	Simp-pending (1300006C19Rik) – similar to Oligosaccharyl transferase 3 CG7748-PA	1.87	1.26	Protein modification
XM_342299	Txnip (THIF; VDUP1; mVDUP1) – upregulated by 1,25-dihydroxyvitamin D-3	1.87	1.35	Increases oxidative stress; thioredoxin-interacting protein
NM_134378	sulfatase 1	1.87	1.20	endosulfatase
XM_213518	Gprc5c (1110028I06Rik; 3200002M13Rik) – retinoic acid inducible protein 3	1.87	1.29	GPCR
AB121446	mast cell surface antigen-1 (masa-1)	1.87	1.23	????
XM_213602	2010319C14Rik – similar to hypothetical protein FLJ20522	1.86	1.16	????
XM_229231	Sqrdl (154ignal-binding protein) – sulfide 154ignali reductase-like	1.86	1.26	Sulfide oxidation
NM_053512	Lamp2 (CD107b) – BLAST match w/ peroxiredoxin 4	1.86	1.20	Lysosome-associated membrane glycoprotein; cell adhesion
10650	Wdfy3 (ALFY; Ggtb3; ZFYVE25; 2610509D04Rik) – no BLAST match	1.85	1.20	????
XM_215134	Cars (CA3) – cysteine-tRNA ligase isoform b, cysteine translase; cysteine-tRNA synthetase	1.85	1.21	Protein synthesis
XM_340920	IMAGE=5668735 – similar to KIAA1267 protein	1.85	1.28	????
XM_225997	protocadherin 1 isoform 2 precursor, protocadherin 42, cadherin-like protein 1	1.85	1.25	Ca2+-mediated cell-cell adhesion
BC058460	ribosomal protein S12	1.85	1.29	ribosomal protein
9191	IMAGE=6434300 – not in database	1.85	1.17	????

NM_139081	Oaz1 (AZ-1; Antizyme; antizyme 1) – ornithine decarboxylase antizyme 1	1.84	1.38	negative regulator of cellular polyamines
NM_030861	Mgat1 (Mgat-1) – N-acetylglucosaminyltransferase I	1.84	1.36	diacylglycerol synthesis
7545	IMAGE=6437216 (no BLAST match)	1.84	1.16	????
AY291063	Hs1bp1 (HAX-1; mHAX-1s) – HS1 binding protein variant	1.84	1.25	anti-apoptotic
U42627	Dusp6 (MKP3; MKP-3; PYST1; 1300019I03Rik) – dual-specificity protein tyrosine phosphatase (rVH6)	1.84	1.25	protein tyrosine phosphatase
AY123225	Atf5 (AFTA; Atf7; Atfx; ODA-10) – NEDH activating transcription factor 5	1.83	1.21	Transcription factor
XM_345867	4432409D24Rik – similar to hypothetical protein (LOC366980)	1.83	1.15	????
BX883049	low BLAST match w/ chromosome 20, major histocompatibility complex	1.83	1.21	regulates endosomal function/trafficking
NM_017321 (2)	Aco1 (Irp1; Aco-1; Irebp) – BLAST match w/ iron-responsive element-binding protein	1.83	1.21	A cytosolic protein that binds a highly conserved sequence in the untranslated regions of mRNAs involved in iron metabolism including ferritin, 155ignaling155155g receptor, and erythroid 5-aminolevulinate acid synthase
XM_233838	AW548124 (ESTM17) – similar to AI115348 protein (LOC313860)	1.83	1.29	????
XM_219411	Pign – phosphatidylinositolglycan class N	1.82	1.16	Transfers phosphoethanolamine to the first mannose of the glycosylphosphatidylinositol
XM_213927	5630401D24Rik	1.82	1.17	????
U84402	AU041277; Smo – smoothened	1.82	1.36	receptor for Indian Hedgehog; involved in pancreas development and beta cell function
XM_342542	Brain glycogen phosphorylase (Pygb)	1.82	1.29	Glycogen metabolism
	Zfp278 (MAZR; PATZ; 8430401L15Rik; POZ-AT hook-zinc finger protein) – no BLAST match	1.82	1.23	Transcriptional coactivator??
3623	1110020C13Rik (2010324E22Rik) – not in database	1.82	1.18	????
BC061873	Slc2a1 (Glut1; Glut-1) – solute carrier family 2, member 1	1.82	1.24	GLUT1
U92699	Rps2 (Rps2; Llrep3) – ribosomal protein S2	1.82	1.26	ribosomal protein
8794	Stra13 (no BLAST match)	1.81	1.22	Transcription factor
NM_212519	D16Ium22e (D16Ium22) – TOM70 protein	1.81	1.27	mitochondrial import receptor
BC070880	Mfn2 (Fzo; D630023P19Rik; hypertension related protein 1) – mitofusin 2	1.81	1.25	Determines mitochondrial network architecture and mitochondrial metabolism
AF273024	Slc38a2 (5033402L14Rik) – amino acid system A transporter	1.81	1.22	Sodium-coupled neutral amino acid transporters
9856	A730055F12Rik (Tom1l2; 2900016I08Rik; myb1-like protein 2) – no BLAST match	1.81	1.24	????
XM_234810	Gsta4 (GST 5.7) – blast match similar to GLUTATHIONE S-TRANSFERASE 8 (GST 8-8)	1.81	1.31	GLUTATHIONE S-TRANSFERASE; PPARgamma target??
XM_342095	Retinoic acid receptor RXR-beta	1.80	1.16	RXR
XM_233533	Rpl37a – 60S ribosomal protein L37a	1.80	1.27	ribosomal protein
XM_343984	Kctd2 (2310012I15Rik) – similar to KCTD2 protein (LOC363705)	1.80	1.32	????

13257	IMAGE=5939509 (no BLAST match)	1.80	1.23	????
XM_215992	Npdc1 (NPDC-1) – neural proliferation, differentiation and control gene 1	1.79	1.22	inhibits cell proliferation
XM_216062	Smarcd3 (1500001J14Rik) – SWI/SNF-related matrix-associated actin-dependent regulator of chromatin d3, Rsc6p, mammalian chromatin remodeling complex BRG1-associated factor 60C	1.79	1.20	Actin-dependent regulators of chromatin
5940	IMAGE=6434889 – not in database	1.79	1.15	????
XM_216327	AL024076; Rps20 – 40S ribosomal protein S20	1.79	1.29	ribosomal protein
XM_217041 (2)	Fkbp11 (1110002O23Rik) – FK506 binding protein 11	1.79	1.19	Posttranslational modification, protein turnover, chaperone
XM_213330	Fliih; Llglh (Fli1; 3632430F08Rik; Mgl1) – Fliih protein	1.78	1.26	cell polarity
8587	Tera-pending (no BLAST match)	1.78	1.21	????
Y17295	Prdx6 (GPx; Aop2; CP-3; Ltw4; Ltw-4; ORF06; Brp-12; Lvtw-4; aiPLA2; 1-Cys Prx; acidic calcium-independent phospholipase A2) – thiol-specific antioxidant protein (1-Cys peroxiredoxin)	1.78	1.18	antioxidant
6864	Adnp (no BLAST match)	1.78	1.17	Activity-dependent neuroprotective protein??
AJ312934	Itga6 (Cd49f; 5033401O05Rik) – integrin alpha 6 subchain	1.77	1.27	laminin receptor
XM_225919	very long-chain acyl-CoA synthetase homolog 1	1.77	1.13	FA synthesis
8820	IMAGE=5945360 (no BLAST match)	1.76	1.22	????
XM_213217	1110025F24Rik; AI256624	1.76	1.22	????
XM_215182	Fmo5 – BLAST match w/ Splicing factor 3b, subunit 2	1.76	1.15	Flavin-containing monooxygenase??
3625	Pcdh18 (PCDH68L) – not in database	1.76	1.18	Calcium-dependent cell-cell adhesion
AF156878	Ogfr (2010013E17Rik) – opioid growth factor receptor	1.76	1.28	A tonic inhibitory peptide that modulates cell proliferation and tissue organization during development, cancer, cellular renewal, wound healing, and angiogenesis
XM_217347	DNA-directed RNA polymerase I 40 kDa polypeptide (RPA40)	1.76	1.15	RNA polymerase
BC060572	alpha-tubulin	1.75	1.21	Microtubule formation
XM_214924	retinoic acid inducible in neuroblastoma cells 1 (Rainb1)	1.75	1.30	Ca2+-binding/actin-bundling protein
674	Brd8 (SMAP; p120; 2610007E11Rik) – not in database	1.75	1.18	A specific coactivator for 9-cis-retinoic acid receptor (RXR) on peroxisome proliferator-activated receptor-gamma/RXR heterodimers
2399	IMAGE=6435586 (no BLAST match)	1.75	1.14	????
10596	6330576B01Rik (no BLAST match)	1.75	1.22	????
XM_213800	Tpst2 – tyrosylprotein sulfotransferase-2	1.75	1.21	post-translational modification
AJ428514	IMAGE=6430146 – BLAST match w/ rat complete mitochondrial genome (NOT IN DATABASE)	1.74	1.38	????
XM_215889	AA986553; AU045326 – Transmembrane 9 superfamily protein member 4	1.74	1.20	beta-adrenergic receptor??
NM_031668	Mybbp1a (P160) – MYB binding protein 1a	1.74	1.22	A nucleocytoplasmic shuttling protein that utilizes CRM1-dependent and independent nuclear export pathways
13319	IMAGE=6435054 – not in database	1.74	1.25	????

XM_344537	Rps28 – 40S ribosomal protein S28	1.73	1.33	ribosomal protein
XM_221775	D030053O22Rik – no BLAST match	1.73	1.27	????
2431	IMAGE=6435538 (no BLAST match)	1.73	1.20	????
BX883042	chromosome 20, major histocompatibility complex	1.73	1.16	????
NM_144730	Gata4 (Gata-4) – GATA binding protein 4	1.72	1.18	transcription factor
XM_345930	guanine nucleotide exchange factor	1.72	1.20	guanine nucleotide exchange factor
XM_228273	Rev3l (Sez4) – DNA polymerase zeta catalytic subunit	1.72	1.29	Error-prone DNA polymerase
XM_223786	B830002A16Rik – zinc-finger protein NOLZ1	1.72	1.18	????
NM_022264	Kit (W; Bs; Fdc; Ssm; CD117; c-KIT; Tr-kit; belly-spot; dominant spotting; spotted sterile male; Steel Factor Receptor; Dominant white spotting) – c-kit receptor tyrosine kinase	1.72	1.24	Receptor tyrosine kinase
XM_217272	Scotin-pending (2310008D10Rik; 6430628I05Rik)	1.72	1.18	A novel p53-inducible proapoptotic protein located in the ER and the nuclear membrane
AC096051	IMAGE=5666953 – 10 BAC CH230-21G1	1.72	1.23	????
XM_228047	Fgd2 – faciogenital dysplasia homolog 2	1.71	1.26	A guanine nucleotide exchange factor (GEF) that specifically activates the Rho GTPase Cdc42
XM_340850	60S ribosomal protein L23a	1.71	1.19	ribosomal protein
XM_341815	Rps16 – ribosomal protein S16	1.71	1.29	ribosomal protein
X68199	Myo1b – myosin I heavy chain	1.71	1.22	Membrane dynamics and transport
NM_053884	Atp6v1f (1110004G16Rik) – ATPase, vacuolar, 14 kD	1.71	1.28	ATPase
XM_342249	Eif3s2 (36kDa; D4Ertd632e) – Eukaryotic translation initiation factor 3 subunit 2	1.71	1.28	translation initiation
BC060541	Apacd-pending – blast match w/ ES cell-related protein	1.71	1.14	ATP binding protein associated with cell differentiation
XM_341854	similar to RIKEN cDNA 2410004H02 (LOC361571)	1.71	1.26	????
XM_231116	2900073H19Rik	1.70	1.16	????
XM_241622	1300018I05Rik	1.70	1.15	????
BC061877	Got1 (Got-1; cAspAT; cytosolic aspartate aminotransferase) – glutamate oxaloacetate transaminase 1	1.70	1.17	ER-Golgi transport
M75168	liver nuclear protein p47	1.70	1.24	ER-Golgi assembly??
2941	IMAGE=5945659 (no BLAST match)	1.70	1.19	????
XM_216400	protein translocation complex beta, protein transport protein SEC61 beta subunit	1.70	1.21	Protein transport
BC060556	Rpn2; Rpn2-rs1 (Rpn-2; 1300012C06Rik) – ribophorin 2	1.70	1.18	Glycan Biosynthesis and Metabolism
XM_215924	ubiquitin-conjugating enzyme E2C	1.70	1.23	ubiquitin-conjugating enzyme
XM_214958	Rpl44 (L44L) – large subunit ribosomal protein L36a	1.70	1.22	ribosomal protein
XM_228783	Wdr13 (5730411P10Rik) – WD-repeat protein	1.70	1.30	Regulatory role in nuclear function??
D00675	Serpina1a; Serpina1b (PI1; Spi1-1; PI2; Spi1-2; D12Ucla2) – alpha-1-protease inhibitor	1.70	1.18	alpha-1-protease inhibitor

NM_031335	polymerase II	1.69	1.30	polymerase II
NM_152935	Tomm20-pending (1810060K07Rik) – outer mitochondrial membrane receptor rTOM20	1.69	1.17	Mitochondrial import receptors; chaperone-like activity
BC062061	Hmox2 (HO-2) – heme oxygenase 2	1.69	1.20	Heme oxygenase
NM_139114	ribosomal protein L15 (Rpl15)	1.69	1.26	ribosomal protein

NEGATIVE SIGNIFICANT GENES (833/15 v 834/40)

NM_012707 (6)	Gcg (GLP-1; glucagon-like peptide I) – glucagon gene	-12.51	0.18	Glucagon Gene (Ggn, GLP-1, GLP-2)
NM_031831	Rtn4 (ASY; NOGO; NSP-CL; 1110020G17Rik) – Reticulon 4	-9.25	0.40	Apoptosis?? May interact with Bcl-2 and Bcl-xL
	Sara (1600019H17Rik) – no BLAST match	-8.03	0.54	
XM_342136	Sara (1600019H17Rik) – similar to SAR1a gene	-7.92	0.54	GTPase for 158ignali budding/ER export – 158ignali trafficking
J04807 (4)	Ins2 (Mody; Ins-2; Mody4)	-7.19	0.39	Insulin Gene
AF022088	Gng3 (83) – guanine nucleotide binding protein gamma 3 subunit	-6.94	0.34	GPCR subunit – Ca++ ??, involved in Ca++ content?
V01242 (5)	Ins1 (Ins-1; Ins2-rs1)	-6.45	0.47	Insulin Gene
XM_216043	Gsn – similar to Gelsolin precursor, plasma (Actin-depolymerizing factor) (ADF) (Brevin) (LOC296654)	-6.34	0.56	Gelsolin prevents apoptosis by depolymerizing actin – cytoskeleton assembly/disassembly
X15906	Fn1 (Fn-1) – fibronectin 1	-6.13	0.48	Structural protein
XM_344618 (2)	similar to endoplasmic oxidoreductase 1 beta (LOC364755)	-5.92	0.53	endoplasmic reticulum membrane-associated protein involved in disulfide bond formation [Posttranslationalmodification, protein turnover, chaperones /Intracellular trafficking and secretion]"
XM_216841	IMAGE=5679970 similar to RIKEN cDNA 5730519E19 gene	-5.91	0.53	????
BC061549	Scg2 (Chgc; SgII) – secretogranin 2	-5.70	0.54	Found in secretory granules; regulates sorting – 158ignali trafficking
NM_031628	AI573420 – nuclear receptor subfamily 4, group A, member 3 (Nr4a3); NOR-1	-5.70	0.54	Nuclear hormone receptor
XM_342415	Tor2a – torsin family 2, member A (LOC362112)	-5.43	0.52	Salusin gene; increase intracellular Ca++ and mitogenesis
	AI158848 (no BLAST match)	-5.40	0.57	????
M28216	Cycs – cytochrome c, nuclear gene for mitochondrial product	-5.39	0.57	Forms apoptosome
NM_012586 (2)	Iapp (amylin) – islet amyloid polypeptide	-5.14	0.51	Cosecreted with Ins; amyloid deposits correlated with beta cell loss and T2DM
XM_227104	similar to putative transcription factor ZNF131 (LOC310375)	-5.13	0.68	*Transcription factor??*
	IMAGE=5662002 (no BLAST match)	-4.89	0.57	????
	Ha1r-pending – not in database	-4.89	0.59	*Human adenosine receptor??*
AF388527 (2)	Pairbp1-pending (1200009K13Rik) – hypothetical RNA binding protein RDA288	-4.82	0.55	*RNA binding protein or coreceptor??*
AF419341	Sytl4 (Slp4; granuphilin-a; granuphilin-b)	-4.78	0.55	Inhibits dense core vesicle exocytosis
XM_220013	Smbp-pending (1810073M23Rik; 2810031D16Rik) – similar to transmembrane protein TM9SF3 (LOC309475)	-4.62	0.71	Binds SM-1044; 9 transmembrane protein; may serve as channel, small molecule transporter, or receptor
NM_012614	Npy (0710005A05Rik) – Neuropeptide Y	-4.51	0.42	Autocrine inhibition of insulin secretion

	IMAGE=6432981 (no BLAST match)	-4.35	0.61	????
AB008752	Catnd2 (Nprap; Ctnnd2; neurojungin) – delta-catenin, partial match	-4.31	0.62	Scaffolding protein involved in signal transduction
	IMAGE=573245 (no BLAST match)	-4.30	0.70	????
	3830421F13Rik (2010005M05Rik; 3830411L18Rik; 4933423A17Rik) – no BLAST match	-4.25	0.62	????
XM_235269	IMAGE=5682030 similar to RIKEN cDNA C820002P14 (LOC299903)	-4.17	0.63	????
	IMAGE=5939637 (no BLAST match)	-4.09	0.64	????
NM_212531	HLA-B associated transcript 5	-4.06	0.61	HLA-B associated transcript 5
9736	IMAGE=5678862 (no BLAST match)	-4.04	0.60	????
XM_341574	Colec12 (SRCL; CL-P1; Scara4) – similar to collectin placenta 1	-4.02	0.69	Binds carbohydrate antigens – cell recognition
AY149902	Elac2 (D11Wsu80e; 1110017O07Rik)	-3.90	0.51	DNA repair
XM_343649	similar to tubulin-specific chaperone d	-3.90	0.57	Chaperonoe – protein processing
XM_217048	Mcrs1 – similar to microspherule protein 1	-3.89	0.70	Modulates Daxx localization – gene transcription
BC060547 (2)	Sep15-pending (15kDa) – selenoprotein	-3.86	0.70	Related to apoptosis; when down regulated by RNAi, increases proliferation
XM_341867	similar to ubiquitin protein ligase E3A isoform 1; human papilloma virus E6-associated protein	-3.84	0.64	ubiquitin protein ligase
NM_022397 (3)	ribonucleoprotein F (Hnrpf)	-3.68	0.70	Pre-mRNA processing
BC062032 (2)	Ctsd (CD; CatD) – cathepsin D	-3.68	0.74	Activated by ceramides; activates tBid by proteolysis of Bid; leads to caspase activation (apoptosis)
XM_342679	Cul1 – similar to SCF complex protein cul-1	-3.67	0.75	Cullin-based E3 ligases target substrates for ubiquitin-dependent degradation by the 26S proteasome
12482	IMAGE=6433212 (no BLAST match)	-3.62	0.69	????
XM_215054 (2)	Bag3 (Bis; Bcl-2-interacting death 159ignaling159)	-3.60	0.69	Apoptosis; binds to HSP70, Bcl-2, PLCgamma; inhibits cytochrome c release
235	AI929937; 4930469P12Rik (no BLAST match)	-3.57	0.72	????
XM_343192 (3)	Tra1 (gp96; Erp99; GRP94; Tra-1; tumor rejection antigen (gp96) 1)	-3.57	0.64	Chaperone; antigen presentation, protein processing
XM_216634	D030028O16Rik (MGC:7980) – similar to RIKEN cDNA D030028016 (LOC298771)	-3.57	0.65	????
U56241	transcription factor Maf1 – Mafb (Kreisler)	-3.56	0.63	Transcriptional coactivator
X14833 (2)	Insulin-like growth factor 2	-3.54	0.56	Growth Factor
	Sfrs1; 5730507C05Rik (1110054N12Rik; 6330415C05Rik)	-3.49	0.70	mRNA splicing factor
NM_017064	Stat5a	-3.48	0.69	Signal transducer and activator of transcription; increases Bcl-xL
7961	AI854107 (no BLAST match)	-3.47	0.63	????
XM_342200	AI842364; Rpo2tc1 – BLAST match w/ pR-ET2 encoded oncodevelopmental protein (LOC192269)	-3.40	0.73	Transcriptional coactivator
NM_017226	Padi2 (Pdi; Pdi2; PAD type II) – peptidyl arginine deiminase, type 2	-3.37	0.74	Peptidyl arginine deiminase; arginine to citrulline; antibody for autoimmune diseases
NM_199085	Serpinb6a (Spi3; ovalbumin) – serine (or cysteine) proteinase inhibitor, clade B (ovalbumin), member 6	-3.33	0.73	Serine protease inhibitor
XM_238596	IMAGE=6432798 – low BLAST match w/ hypothetical LOC301598	-3.33	0.42	????

AY122323	Ppp1r14b (Png; PHI-1; AOM172; PLCB3N) – PKC-potentiated PP1 inhibitor (Phi1)	-3.32	0.59	PP1 inhibitor; Integrates PKC and PKA signalling
NM_057104	Enpp2 (ATX; Npps2; Pdnp2; Autotaxin; PD-Ialpha) – ectonucleotide pyrophosphatase/phosphodiesterase 2	-3.31	0.69	Extracellular PDE; down-regulated by IL1 – signal transduction
NM_175761	Hspca (hsp4; 86kDa; 89kDa; Hsp89; Hsp90; Hsp86-1) – heat shock protein 1 alpha	-3.28	0.68	Chaperone – protein processing
XM_214963	similar to U2 small nuclear ribonucleoprotein polypeptide A (LOC292997)	-3.26	0.70	Pre-mRNA splicing
5025	IMAGE=5939954 (no BLAST match)	-3.26	0.69	????
AY325226	Ba1-643	-3.25	0.75	????
BC063162 (2)	A330103N21Rik, 2610509H23Rik – low BLAST match w/ eukaryotic translation elongation factor 1 alpha	-3.23	0.70	translation elongation
AF227741	protein kinase WNK1	-3.23	0.68	Activated by Akt; activates ERK5
3044	IMAGE=6436528 (no BLAST match)	-3.23	0.72	????
8924	IMAGE=6436436 (no BLAST match)	-3.22	0.71	????
XM_340998	3830421F13Rik (2010005M05Rik; 3830411L18Rik; 4933423A17Rik) – similar to putative 40-2-3 protein (LOC360726)	-3.21	0.64	????
NM_019271	stress 70 protein chaperone, microsome-associated (Stch)	-3.20	0.74	Calcium-inducible, microsome-associated ATPase – protein processing
XM_341266	1200003F12Rik – similar to polynucleotide phosphorylase-like protein (LOC360992); also similar to RNA-binding protein EWS	-3.16	0.70	mRNA processing
NM_057198	phsophoribosyl pyrophosphate amidotransferase (Ppat)	-3.16	0.74	Purine biosynthesis
12436	Luc7a-pending (3300001P08Rik) – not in database	-3.13	0.67	RNA splicing; interacts with SF2/ASF
12128	IMAGE=6434406 (no BLAST match)	-3.10	0.75	????
AC095491	IMAGE=2182581 (mid BLAST match w/ 4 BAC CH230-7L13)	-3.10	0.73	????
466	IMAGE=6431573 (no BLAST match)	-3.08	0.63	????
XM_343891	Tcf7 (Tcf1; TCF-1; T cell factor-1; T-cell factor 1)	-3.07	0.72	Transcription factor; SNP for T1DM
X83750	Cbx3 (M32; HP1g; heterochromatin protein 1 gamma) – low BLAST match w/ 5S rRNA gene (clone pRA5S5)	-3.05	0.68	Chromobox protein; binds to methylated DNA in telomeres
1449	IMAGE=620282 (no BLAST match)	-3.03	0.67	????
	IMAGE=6436822 no BLAST match	-3.02	0.81	????
	8030460C05Rik (MGC:7474) – no BLAST match	-3.01	0.75	????
BC059124	Mor2; D17921 (Mor-2) – malate dehydrogenase 1	-2.99	0.79	malate dehydrogenase
NM_022598	cellular nucleic acid binding protein	-2.98	0.70	transcription factor
D37934	mid BLAST match w/ 5E5 antigen	-2.96	0.68	DNA-binding protein
XM_217326	Plcl2 (Plce2) – phospholipase C-like 2	-2.96	0.70	Phospholipase C
995	1110057H19Rik – not in database	-2.92	0.74	????
BC061979	Gilz – glucocorticoid-induced leucine zipper	-2.92	0.76	inhibits apoptosis via down-regulation of Fas/FasL; inhibits nuclear factor B (NF B) activation and nuclear translocation by interacting directly with p65/p52

AY336973	AI642085; Zfp191 – zinc finger protein 191	-2.89	0.79	Kruppel-like transcription factor
XM_233872	2810405J04Rik	-2.87	0.73	????
NM_024374 (2)	Mtpn (V1; Gcdp) – myotrophin, V1 protein	-2.87	0.76	Protection against cell death induced by NO at low levels by promoting the synthesis of BH(4)
XM_341215	Tbc1d1 (1110062G02Rik) – no BLAST match	-2.87	0.77	Differentiation and cell growth
AF482705	Uxs1 (1600025I13Rik) – UDP-glucuronate decarboxylase	-2.86	0.81	Converts UDP-glucuronic acid to UDP-xylose
J05148	Gc; AL024368; Snrpb (DBP; vitamin D binding protein)	-2.86	0.75	Protein involved in the actin-scavenger system
XM_218425	fukutin-related protein	-2.85	0.78	Cell envelope biogenesis, outer membrane
BC060575 (5)	Psma5 (ZETA) – proteasome (prosome, macropain) subunit, alpha type 5	-2.81	0.70	component of 20S proteosome
XM_225396	similar to Vps41 protein	-2.81	0.74	Protein transport
13895	AI316828 (no BLAST match)	-2.80	0.75	????
BC062017	Hspa5 (Bip; Sez7; 78kDa; Grp78; SEZ-7; Hsce70; D2Wsu17e; D2Wsu141e) – heat shock 70kD protein 5	-2.80	0.68	Chaperone
AY217032	Wdr5 (Big-3; 2410008O07Rik) – brain and heart protein NDRG4-C1	-2.80	0.65	????
7675	C79468 (no BLAST match)	-2.79	0.74	????
AB118026	Unc5h3 (rcm; rostral cerebellar malformation)	-2.79	0.77	Induces apoptosis via its juxtamembrane region through an interaction with NRAGE
XM_213403 (2)	0610009H04Rik – hypothetical LOC287541	-2.79	0.76	????
XM_224630	similar to RIKEN cDNA 2310034K10 (LOC306222)	-2.78	0.68	????
XM_241375	Narg1 (mNAT1; Tbdn-1; tubedown; 5730450D16Rik) – similar to N-terminal aceyltransferase 1	-2.77	0.76	Acetyltransferase
4159	IMAGE=5679272 (no BLAST match)	-2.76	0.82	????
9745	IMAGE=6435775 (no BLAST match)	-2.75	0.75	????
NM_012963 (4)	Hmgb1 (Hmg1) – high mobility group box 1	-2.73	0.75	Transcription factor/enhancer
NM_019379	Vdp-pending (TAP; 115kDa; transcytosis associated protein p115) – vesicle docking protein	-2.73	0.74	Vesicle docking
XM_343002	Cebpa-rs1 (Cbf) – CCAAT binding factor 1	-2.70	0.75	Transcription factor
XM_225033	Cln8 (mnd; motor neuron degeneration) – neuronal ceroid lipofuscinosis type 8	-2.68	0.76	Lipid-sensing transmembrane protein?
XM_342350	Transmembrane 4 superfamily, member 8; Tetraspanin 5 (Tspan-5)	-2.67	0.68	Cell adhesion
1871	IMAGE=6436457 – not in database	-2.67	0.69	????
XM_213726	1110007L15Rik	-2.65	0.79	????
U03416	Olfm1 (AMY; Noelin 1; Noelin 2; Pancortin 1-4) – neuronal olfactomedin-related ER localized protein	-2.63	0.80	Secreted glycoprotein
XM_343128	P5-pending (CaBP5; 1700015E05Rik) – protein disulfide isomerase-related protein (P5)	-2.62	0.75	Disulfide isomerase and chaperone activities
XM_215236	5033414D02Rik (1110007H22Rik)	-2.62	0.77	????
NM_030873 (2)	Pfn2 (Pfn) – 161ignaling 2	-2.61	0.74	Actin binding protein; cytoskeleton assembly

	Gcap4; Peg3 – no BLAST match	-2.59	0.64	Zinc-finger protein involved in TNF-NFkB signaling and p53-mediated apoptosis
XM_240329 (2)	Pb1-pending (2610016F04Rik) – polybromo-1D	-2.55	0.76	protein-protein interaction; chromatin 162ignaling162; gene transcription
13676	Usp1 (no BLAST match)	-2.55	0.70	Ubiquitin-specific protease
AF253473	D16Ertd803e - diphosphoinositol polyphosphate phosphohydolase type II (Nudt4)	-2.51	0.75	Inositol phosphate metabolism
6562	IMAGE=6433791 (no BLAST match)	-2.51	0.80	????
XM_344567	heterogeneous nuclear ribonucleoprotein A0	-2.50	0.75	mRNA stability
L09752	Ccnd2 (Vin1; Vin-1) – G1/S-specific cyclin D2 (VIN-1 proto-oncogene)	-2.50	0.64	Cell Growth and Death; Cell cycle; signal transduction
XM_216578	Tardbp	-2.49	0.75	RNA-binding protein that functions in mammalian cells in transcriptional repression and exon skipping
BC061833	Nbl1 (DAN; NO3; Dana; D4H1S1733E) – neuroblastoma, suppression of tumorigenicity 1	-2.49	0.72	Antagonise different TGF beta ligands
BC072508	BLAST match w/ phosphoribosylaminoimidazole carboxylase	-2.49	0.77	????
NM_133590	Rab7l1 (MGC:28972; MGC:36539) – Ras-related GTP-binding protein Rab29	-2.48	0.78	Purine nucleotides synthesis
XM_242556	Np220 (mNP220) – nuclear protein, NP220	-2.47	0.66	Transcription factor
13518	IMAGE=5945372 (no BLAST match)	-2.44	0.80	????
AF442357	reticulon 3 protein isoform a	-2.44	0.75	ER-associated protein complexes
AF110732	Prdx5; 0610038D11Rik (AOPP; PrxV; PrxV; PMP20; Prdx6; AOEB166; peroxiredoxin V) – antioxidant enzyme B166, peroxiredoxin 5	-2.44	0.76	Antioxidant
XM_215278	Wbp5 – pp21 homolog	-2.43	0.77	Cell 162ignaling (phosphorylated 162ignaling TCR activation and Calcium increase)??
XM_240072	Spnb2 (elf1; elf3; Spnb-2; spectrin G; beta fodrin; 9930031C03Rik; brain spectrin; non-erythrocytic) – spectrin beta 2	-2.42	0.69	Structural protein
XM_340880	Luc7a-pending (3300001P08Rik) – similar to cisplatin resistance-associated overexpressed protein	-2.40	0.77	5'-splice site recognition
XM_341337	Ddx26 (HDB; DICE1; Notch2l; 2900075H24Rik) – DEAD/H (Asp-Glu-Ala-Asp/His) box polypeptide 26	-2.39	0.75	Helicase
NM_053974 (4)	Eif4e (lf4e; eIF-4E) – eukaryotic translation initiation factor 4E	-2.39	0.75	translation initiation
NM_017187	Hmgb2 (Hmg2; HMG-2) – high mobility group box 2	-2.37	0.80	Transcription regulation, DNA repair, recombination
NM_021655	Chga – chromogranin A	-2.37	0.82	Vessicle trafficking
13191	IMAGE=477834 (no BLAST match)	-2.37	0.68	????
NM_031090 (2)	Rab1 (Gtbp; Ypt1; Rab-1; Rab1A; ras-related YPT1 protein) – ras-related protein	-2.37	0.76	Small monomeric GTPase
NM_030835	Serp1-pending (D3Ucla1) – BLAST match w/ ribosome associated membrane protein 4 (RAMP4) and SERP1	-2.37	0.71	Stabilizes membrane proteins during stress and facilitates subsequent glycosylation
XM_213619	Ndufb4 (0610006N12Rik) – NADH:ubiquinone oxidoreductase B15 subunit	-2.36	0.84	Component of mitochondrial complex I

6085	IMAGE=6430251 (no BLAST match)	-2.36	0.79	????
BC070919	Rps27a (0610006J14Rik) – BLAST match w/ both polyubiquitin and ribosomal protein S27a	-2.35	0.68	Ubiquitin-80-amino-acid fusion-protein gene
NM_031355	Vdac3 – mitochondrial voltage dependent anion channel 3	-2.35	0.82	Mitochondrial voltage dependent anion channel 3
XM_217454 (2)	Cul3 – cullin 3	-2.34	0.77	proteasomal degradation of the topoisomerase I-DNA covalent complex
AF399643	Coil (Cln80; p80-coilin) – p80 coilin	-2.34	0.77	Cajal body formation
AY083459	kidney-type glutaminase GAC isoform	-2.34	0.80	Glutamine metabolism
BC058139 (3)	AI788777, Rps27a (0610006J14Rik) – BLAST match w/ ribosomal protein S27a	-2.33	0.77	ribosomal protein
XM_342687	D6Ertd772e (D6Ertd404e; 1200003J11Rik) – Tax1 binding protein 1 homolog	-2.33	0.75	Transcriptional coactivator
XM_234904	similar to Protein Sck (LOC314612)	-2.33	0.77	????
D79221	vesicle transport-related protein (RA410)	-2.31	0.79	Vesicle transport-related protein
U78105	latrophilin-1 precursor (CL1AA)	-2.31	0.79	Component of calcium-permeable membrane pores
1258	IMAGE=6435509 (no BLAST match)	-2.31	0.80	????
XM_223190	Hnrpdl (JKTBP; D5Wsu145e; D5Ertd650e) – heterogeneous nuclear ribonucleoprotein D-like; A+U-rich element RNA binding factor	-2.31	0.77	mRNA biogenesis and mRNA metabolism
XM_342252	Mbnl – muscleblind-like	-2.30	0.78	mRNA splicing??
AB033713 (2)	IMAGE=6430085 – BLAST match w/ mitochondrial gene for cytochrome b	-2.29	0.77	Mitochondrial electron transport
NM_017339 (2)	Isl1 (Islet 1) – ISL1 transcription factor, LIM/homeodomain 1	-2.29	0.80	Transcription factor
XM_345656	IMAGE=5680664 – BLAST match w/ similar to synaptophysin-like protein; pantophysin; Pan I; DNA segment, Chr 12, ERATO Doi 446	-2.28	0.79	Vessicle/membrane docking
XM_220748	AL022898; AI851076 – similar to hypothetical protein DKFZp434K1421	-2.28	0.75	????
12947	4921520P21Rik (no BLAST match)	-2.28	0.74	????
NM_022616	CDC10 (cell division cycle 10)	-2.28	0.67	Cell division cycle
XM_224611	2410004H05Rik (5430414N14Rik) – similar to Da2-24	-2.27	0.69	Glycosyl transferase
NM_013113	Atp1b1 (Atpb; Atpb-1; sodium/potassium ATPase beta subunit) – ATPase Na+/K+ transporting beta 1 polypeptide	-2.27	0.83	Sodium/potassium ATPase
XM_214242	2410174K12Rik	-2.26	0.78	????
NM_012746	Pcsk2 (PC2; Nec2; SPC2; Nec-2; Phpp-2; prohormone convertase 2) – proprotein convertase subtilisin/kexin type 2	-2.25	0.78	Pro-hormone processing
X62908	Cfl1 (Cof; cofilin)	-2.24	0.62	Actin cytoskeleton organization and biogenesis
BC061532 (2)	C76540; Trnt1; AI987788; Amd1 – S-adenosylmethionine decarboxylase 1	-2.24	0.78	Polyamin biosynthesis
5979	Kif5b (KHC; Khc; kinesin heavy chain) – no BLAST match	-2.24	0.75	mRNA localization and/or transport of endoplasmic reticulum-derived vesicles
9222	2900024N03Rik – no BLAST match	-2.24	0.83	????

XM_225453	Rab18	-2.24	0.74	Small GTPase
NM_031616	zinc finger protein 265 (Znf265),	-2.23	0.69	Regulation of transcription and/or mRNA splicing
M63574 (2)	Sepp1 (Se-P; selp; D15Ucla1) – selenoprotein P	-2.23	0.65	Responsible for antioxidant properties of selenium??
AF209196	calcium transporter CaT2	-2.22	0.78	Calcium transport
AJ002967	Utrn (DRP; Dmdl; G-utrophin) – utrophin	-2.22	0.78	Structural protein
9313	IMAGE=6430502 (no BLAST match)	-2.22	0.69	????
XM_344744	glutaryl-CoA dehydrogenase precursor	-2.21	0.74	Lysine degradation; tryptophan metabolism
BC070948	Silg41 (SIG-41) – splicing factor, arginine/serine-rich (transformer 2 Drosophila homolog) 10	-2.21	0.81	mRNA splicing
XM_216361	Wwp1-pending (8030445B08Rik) – similar to WW domain-containing protein 1, Nedd-4-like ubiquitin-protein ligase; atrophin-1 interacting protein 5, also similar to vesicle associated protein 1 (VAP1)	-2.20	0.76	Ubiquitin ligase
AF452728	Ppp3ca (CN; I; Caln; Calna; PP2BA alpha; PP2B alpha 1; 2900074D19Rik) – synaptogenesis-related mRNA sequence 7, 3’	-2.20	0.81	protein phosphatase
XM_340798	P4ha2 (P4hl) – Prolyl 4-hydroxylase alpha Iia subunit	-2.19	0.84	synthesis of the extracellular matrix
XM_223680	Activator of 90 kDa heat shock protein ATPase homolog 1 (AHA1) (p38) (HSPC322)	-2.18	0.74	Chaperone
8535	IMAGE=5668672 (no BLAST match)	-2.18	0.83	????
3782	IMAGE=890391 (no BLAST match)	-2.18	0.76	????
NM_019242	Ifrd1 (PC4; Ifnl; Tis7) – interferon-related developmental regulator 1	-2.17	0.80	Cell 164ignaling (GLP2 or IGF)
XM_224617	AU022319; Capn7 – similar to PalBH	-2.17	0.77	Cysteine protease
XM_344524	pericentriolar material 1 (Pcm1)	-2.17	0.71	Centrosomal protein and microtubule organization
NM_053580	Slc27a1 (Fatp; FATP1) – fatty acid transport protein	-2.17	0.81	FA transporter
XM_345633	Msh6 (GTBP; Msh6) – mismatch repair protein MSH6	-2.16	0.78	DNA mismatch repair
	IMAGE=5661578 (no BLAST match)	-2.16	0.80	????
K01880	cytochrome c pseudogene, clone Ch4A-RC5, also match w/ cytochrome c	-2.15	0.80	Mitochondrial electron transport
XM_224241	Zfp198 (FIM; MYM; RAMP; SCLL; MGC:51607) – Zfp198 protein	-2.15	0.73	????
XM_238731	Chd1 (4930525N21Rik) – Chromodomain-helicase-DNA-binding protein 1	-2.15	0.76	Chromatin remodelling
XM_344241	1110003E01Rik; 5430439C17Rik (MGC:7185; 1700127H04Rik)	-2.14	0.84	????
819	2700008G24Rik (no BLAST match)	-2.14	0.78	????
BC058485	Calm2 – calmodulin 2	-2.14	0.74	signal transduction
XM_213540	Fgf22; 2610010O19Rik (2210414E06Rik) – similar to gamma actin-like protein	-2.14	0.84	Actin-like protein
XM_214331	Klhl2 (Mav; ABP-KELCH; 6030411N21Rik)	-2.14	0.80	Actin-binding protein
XM_228829	Morf4l2 (MRGX; Sid393p; 2410017O14Rik) – Sid393p	-2.14	0.77	Transcription factor

M55601	Ptn (OSF; HARP; HBBN; HBNF; Osf1; Osf-1; HB-GAM; HBGF-8; osteoblastic cell factor; heparin-binding brain mitogen; heparin-binding growth factor 8; heparin affin regulatory peptide; heparin-binding neurotorphic factor; heparin-binding neurite promoting factor; heparin-bindin) – heparin-binding growth associated molecule	-2.13	0.77	Growth Factor
XM_344048	Mcm4 (Cdc21; Mcmd4; mcdc21) – Mini chromosome maintenance deficient 4 homolog	-2.13	0.85	Subunit of a putative replicative DNA helicase
NM_017058	Vdr (Nr1i1) – vitamin D receptor	-2.12	0.68	Vitamin D receptor
XM_217614	Syap1 (2010110O17Rik) – synapse associated protein 1	-2.12	0.73	Transcription factor??
NM_031123	Stc (STC1) – stanniocalcin 1	-2.12	0.71	calcium regulatory hormone
AB105193	Xpo1 (Crm1) – nuclear export factor CRM1, exportin 1	-2.11	0.80	mediates the nuclear export of a variety of protein and RNA substrates
XM_217560	Ap3s1 ([s]3A) – adaptor-related protein complex 3, sigma 1 subunit	-2.11	0.85	Adapter protein; binds to IRS-1
10449	IMAGE=6432949 (no BLAST match)	-2.11	0.78	????
XM_221656	AA409051 – similar to SON protein (LOC304092)	-2.11	0.83	????
X69489	beta-chimaerin	-2.11	0.81	Rac-GTPase-activating protein; non-PKC phorbol ester receptor
XM_344911	similar to KIAA1794 protein (LOC365292)	-2.11	0.81	????
NM_138823	protein phosphatase 1, regulatory (inhibitor) subunit 2 (Ppp1r2)	-2.11	0.73	protein phosphatase
D17469	AI844915 – thyrotropin releasing hormone receptor	-2.10	0.76	Increases insulin production in cultured beta-cells
8318	1810011E08Rik – not in database	-2.10	0.70	????
2908	IMAGE=1248131 (no BLAST match)	-2.09	0.81	????
XM_218820	Prc1 (MGC:6745) – Protein regulator of cytokinesis 1	-2.09	0.79	mitotic spindle-associated cyclin-dependent kinase substrate
11908	1110033J02Rik (no BLAST match)	-2.09	0.84	????
XM_213676	Usp16 (UBP-M; 1200004E02Rik; 2810483I07Rik) – ubiquitin specific protease 16	-2.08	0.77	ubiquitin specific protease
BC060536	Rabggtb – Rab geranylgeranyl transferase componenet, subunit beta	-2.08	0.81	post-translational modification
BC058143	ribosomal protein L13	-2.08	0.84	ribosomal protein
XM_341670 (2)	Cbfb (PEA2; Pebp2; PEBP2b; Pebpb2) – similar to PEBP2b2 protein	-2.08	0.80	transcription factor
BC060572	2810410D24Rik – BLAST match w/ alpha-tubulin	-2.08	0.76	Microtubule formation
XM_214886	1200009C21Rik	-2.07	0.74	????
XM_341945	2010208K18Rik – similar to CG9643-PA (LOC361664)	-2.07	0.77	????
AF305713	Tgfbi (68kDa; big-h3) – beta ig-h3	-2.06	0.81	Cell adhesion??
XM_215815	Srp14 (14kDa; (homologous Alu RNA binding protein)) – signal recognition particle 14K chain	-2.06	0.80	Cotranslational targeting of proteins to the endoplasmic reticulum
XM_215524	similar to PC-1 (LOC294900)	-2.05	0.84	????
5061	IMAGE=6436587 – not in database	-2.05	0.77	????
XN_342265	Ppid (CYP-40; 4930564J03Rik) – BLAST match w/ peptidylprolyl isomerase D (cyclophilin D)	-2.05	0.79	Heat shock protein; folding of outer membrane proteins

NM_152935	GENBANK=BM876088 - outer mitochondrial membrane receptor rTOM20	-2.05	0.82	Mitochondrial import receptors; chaperone-like activity
4094	IMAGE=761010 (no BLAST match)	-2.05	0.81	????
BC062406	Ilk – integrin-linked kinase	-2.04	0.79	Serine/threonine protein kinase
NM_053440	stathmin-like 2 (Stmn2), Scg10	-2.04	0.77	Microtubule destabilizing protein
1664	Lt1 (no BLAST match)	-2.03	0.83	????
	AA407235 – no BLAST match	-2.03	0.74	????
NM_032072	Appbp1 (59kDa; MGC:29435; MGC:36437; MGC:36630) – APP-binding protein 1	-2.02	0.76	Ubiquitin conjugation
XM_215769	similar to mitochondrial carrier homolog 2	-2.02	0.75	????
2155	IMAGE=6435113 – not in database	-2.01	0.85	????
XM_341538	Kif5b (KHC; Khc; kinesin heavy chain) – kinesin family member 5B	-2.01	0.57	mRNA localization and/or transport of endoplasmic reticulum-derived vesicles
XM_230478	similar to nucleolar protein ANKT	-2.01	0.86	nucleolar protein??
XM_230584	similar to Na-Ca exchanger 5	-2.00	0.76	Na-Ca exchanger??
XM_213651	Gart (Gaps; Prgs) – glycinamide ribonucleotide sythetase (GARS), aminoimidazole ribonucleotide synthetase (AIRS), glycinamide ribonucleotide formyltransferase (GART)	-1.99	0.85	De novo purine biosynthesis
XM_213967	similar to rab3 GTPase-activating protein, non-catalytic subunit	-1.99	0.76	GTPase activating protein
NM_020081	Cd86 (B70; MB7; B7-2; CLS1; Ly-58; Cd28l2; TS/A-2) – CD86 antigen	-1.99	0.79	cytokine co-stimulatory molecule
9604	IMAGE=6436951 – not in database	-1.98	0.77	????
XM_225606 (2)	Bmi1 (Bmi-1) – transforming protein bmi1	-1.98	0.75	Preservation of gene silencing
	B230219D22Rik; D530037I19Rik – no BLAST match	-1.98	0.73	????
XM_215927	6430706C13Rik	-1.98	0.72	????
XM_342143	similar to p30 DBC protein; deleted in breast cancer 1	-1.98	0.82	DNA-binding (bihelical) motif predicted to be involved in chromosomal organisation
XM_341360	Wbp4 (FBP21) – WW domain binding protein 4 (formin binding protein 21)	-1.98	0.80	Pre-mRNA splicing
NM_144730	Gata4 (Gata-4) – GATA binding protein 4	-1.98	0.79	transcription factor
436	Cugbp2 (ETR-3; Napor-2) – no BLAST match	-1.98	0.82	Stabilizes mRNA but inhibits its translation
NM_019259	AA407365; C1qbp – complement component 1, q subcomponent binding protein	-1.97	0.79	Endogenous substrate for MAP kinase and is translocated to the nucleus upon mitogenic stimulation
1327	IMAGE=6437069 (no BLAST match)	-1.97	0.86	????
Y00697	Ctsl (fs; MEP; nkt; Cat L; nackt; furless; major excreted protein) – cathepsin L	-1.96	0.86	Cysteine proteinase
XM_218703	Hnrph2 (Ftp3) – similar to Murine homolog of human ftp-3	-1.96	0.82	mRNA processing
NM_024373	Galnt1 (ppGaNTase-T1) – polypeptide GalNAc transferase T1	-1.96	0.75	O-glycosylation of proteins

APPENDIX B
Qiagen Rat Operon 1.1 Microarray analysis of INS-1_{res} 833/15 vs. INS-1 832/13 + 834/40 cell lines

Data Set 1 – Brent Presley

POSITIVE SIGNIFICANT GENES (833/15 v 832/13 + 834/40)

Genebank ID (rat) – if available	Description	Fold Change	Spot Value – Cytokine Resistant	Spot Value – Cytokine Sensitive	Common Abreviation	Map Location
CAB	CAB	5.619	0.702	0.125		
NM_012849	Gastrin	4.986	14.891	2.986	Gast	10q32.1
NM_017207	Vanilloid receptor-like protein 1	4.499	0.396	0.088	Vrl1	10q23
NM_019622	Jerker, deafness locus – actin-binding protein of ectoplasmic specialization	4.307	1.802	0.418	Espn	5q36
RCP1	RCP1	4.303	2.161	0.502		
NM_031056	Matrix metalloproteinase 14, membrane-in	4.268	0.125	0.029	Mmp14	15p13
NM_012627	Protein kinase inhibitor beta, cAMP dependent	3.732	11.409	3.057	Pkib	20q12-q13
PRKase	PRKase	3.720	0.201	0.054		
AB062758	carbonyl reductase	3.677	0.798	0.217	LOC266686	15p13
NM_031239	SH3-domain GRB2-like 1	3.630	3.638	1.002	LOC81922	
NM_022711	Steroid 5-alpha-reductase 2	3.599	10.506	2.919	Srd5a2	6q13
NM_012558	Fructose-1,6-bisphosphatase 1	3.373	6.941	2.058	Fbp1	17p14
TIM	TIM	3.342	0.203	0.061		
NM_031645	Receptor (calcitonin) activity modifying	2.839	1.102	0.388	Ramp1	9q36
U66322	Dithiolethione-inducible gene-1	2.828	0.322	0.114	Dig1	5q24
NM_012825	Aquaporin 4	2.655	17.165	6.466	Aqp4	18p13
NAC1	NAC1	2.633	2.785	1.058		
M91235	Rat VL30 element mRNA	2.633	0.317	0.121		
NM_024390	NAD-dependent 15-hydroxyprostaglandin	2.569	11.208	4.363	Hpgd	16p11
X73371	R.norvegicus mRNA for Fc gamma receptor	2.538	0.219	0.086	Fcgr2	13q24
NM_017098	Fatty acid binding protein 6 (bile acid-binding protein)	2.379	3.368	1.416	Fabp6	10q21
NM_016996	Calcium-sensing receptor	2.369	6.130	2.588	Casr	11q22
AJ312934	Integrin, alpha 6	2.313	0.383	0.165	Itga6	3q21-q22
L29427	Glutathione S-transferase, pi 2	2.182	0.027	0.012	Gstp1; GST-P	1q43

NM_031766	Carboxypeptidase Z	2.138	2.022	0.946	Cpz	14q21
X02904	Glutathione S-transferase, pi 2	2.123	0.038	0.018	Gstp2	1q42
NM_012620	Serine (or cysteine) proteinase inhibitor	2.120	0.063	0.030	Serpine1	12q11-q12
Z27087	R.norvegicus (F344	2.094	0.679	0.324		17
NM_022685	Ras-related GTP-binding protein of the R	1.989	4.529	2.277	Rem2	15p13
rbcL	rbcL	1.962	0.905	0.461		
NM_022647	Histone 2b	1.958	1.085	0.554	H2b	17q11
RCA	RCA	1.956	0.597	0.305		
LTP6	LTP6	1.922	2.633	1.370		
L09216	Pancreatic lipase-related protein 2 – triacylglycerol acyl-hydrolase	1.901	2.583	1.359	Pnliprp2	1q55
NM_013086	CAMP responsive element modulator – transcription factor	1.895	2.676	1.412	Crem	17q12.1
AY017337	Expressed in non-metastatic cells 3, pro	1.861	3.545	1.905	Nme3	10q12
NM_031800	Death effector domain-containing protein	1.860	2.401	1.291	Dedd	13q24
J05132	UDP glycosyltransferase 1 family, polype	1.859	0.314	0.169	Ugt1a6	9q35
L40364	Rattus norvegicus MHC class I RT1.O type	1.841	0.641	0.348	LOC360231	20
LTP4	LTP4	1.841	0.758	0.412		
NM_022224	Phosphotriesterase related	1.837	0.870	0.474	Pter	17q12.3
Y09164	Sodium channel, voltage-gated, type 6, a	1.813	1.679	0.926	Scn6a	3q21
NM_031971	Heat shock protein 70-1	1.784	0.083	0.046	Hspa1a	20p12
M60616	Matrix metalloproteinase 13	1.755	0.318	0.181	Mmp13	8q11
AW915000	ESTs, Highly similar to PIGC_HUMAN Phosp	1.728	2.482	1.436		
U20286	Lamina-associated polypeptide 1C	1.704	0.671	0.394	Lap1c	13q21
BI296335	ESTs, Moderately similar to hypothetica	1.700	3.551	2.089		
AB025017	Rattus norvegicus gene for TIS11, comple	1.692	0.173	0.102	Zfp36; Tis11	1q21
NM_012868	Natriuretic peptide receptor 3	1.683	1.995	1.186	Npr3	2q16
NM_030827	Low density lipoprotein receptor-related	1.678	1.047	0.624	Lrp2	3q21
NM_021845	Xlas protein	1.673	6.959	4.159		
AF134054	Protein kinase Chk2	1.645	1.635	0.994	Rad53	12q16
NM_017259	B-cell translocation gene 2	1.631	0.650	0.398	Btg2	13
Y17295	Peroxiredoxin 5	1.616	0.295	0.183	Prdx6	13q22
NM_130421	lymphocyte cytosolic protein 2 (SH2 doma	1.613	0.476	0.295	Lcp2	10q12
AW919878	ESTs, Moderately similar to T cell acti	1.603	1.169	0.729		
NM_019132	Guanine nucleotide-binding protein G-s,	1.556	6.140	3.947	Gnas	3q42
NM_017014	Glutathione-S-transferase, mu type 2 (Yb	1.550	0.833	0.538	Gstm1	2q34
NM_032075	growth hormone secretagogue receptor	1.541	1.514	0.983	Ghsr	2q24
NM_012570	Glutamate dehydrogenase	1.518	1.920	1.265	Glud1	16p16
NM_017136	Squalene epoxidase	1.511	0.812	0.537	Sqle	7q33

BG665058	ESTs, Moderately similar to tyrosinase-	1.494	0.935	0.626		
U19614	Lamina-associated polypeptide 1C	1.492	0.721	0.483	Lap1c	13q21
U41803	Mitofusin 2	1.489	2.054	1.380	Mfn2	5q36
U90725	Lipoprotein-binding protein	1.478	0.449	0.304	Hdlbp	9q36
BF524208	ESTs, Highly similar to TYRO_MOUSE Tyros	1.475	1.094	0.742	Tyr	1q32
NM_021577	Argininosuccinate lyase	1.436	0.774	0.539	Asl	Y
NM_032063	Delta-like 1 (Drosophila)	1.431	1.217	0.850	Dll1	1q12
AJ300162	Uncoupling protein UCP-4	1.395	1.505	1.079	Ucp4	9q12
NM_017030	Propionyl Coenzyme A carboxylase, beta p	1.390	1.631	1.173	Pccb	8q31
BG662979	ESTs, Moderately similar to PFD3_HUMAN P	1.390	1.037	0.746		
M22030	ESTs, Highly similar to A31568 electron	1.356	0.581	0.429	Etfa; ETF	
AB000489	Solute carrier family 20 (phosphate tran	1.356	0.593	0.438	Slc20a1	3q36
NM_031969	Calmodulin 1 (phosphorylase kinase, delt	1.353	2.371	1.752	Calm1	6q31-q32
AF168795	Schlafen 4	1.352	0.096	0.071	Slfn4	10q26
NM_031579	Protein tyrosine phosphatase 4a1	1.344	1.060	0.789	Ptp4a1	9q21
M28671	ESTs, Highly similar to GCB_RAT IG GAMMA	1.328	0.856	0.645	IgG-2b	
AW141869	ESTs, Highly similar to cellular repres	1.321	0.614	0.465		13q23
NM_012621	6-phosphofructo-2-kinase/fructose-2,6-bi	1.318	1.445	1.097	Pfkfb1	Xq22-q31
NM_031334	Cadherin 1	1.308	5.662	4.330	Cdh1	19q12
NM_134346	RAP1B, member of RAS oncogene family	1.294	0.896	0.692	Rap1b	7q22
AF306546	Solute carrier family 21 (organic anion	1.276	1.600	1.255	Slc21a14	4q44
NM_013224	Ribosomal protein S26	1.259	1.104	0.877	Rps26	7q11
AF145050	Translation elongation factor 1-delta su	1.259	0.780	0.620	Eef1d	
U53512	Mitochondrial ribosomal protein L17	1.258	1.755	1.395	Mrpl17	1q33
AF366369	GCIP-interacting protein p29	1.238	0.990	0.800	P29	5q36
AW915528	ESTs, Highly similar to RIKEN cDNA 3110	1.228	0.887	0.722		5q36
BG668294	ESTs, Moderately similar to ESTD_HUMAN E	1.222	0.721	0.590	Esd	15q11
NM_031103	Ribosomal protein L19	1.199	1.038	0.866	Rpl19	10q31
NM_017201	S-adenosylhomocysteine hydrolase	1.187	1.235	1.040	Ahcy	3q42

NEGATIVE SIGNIFICANT GENES (833/15 v 832/13 + 834/40)

AF018261	Epsin 1	0.860	1.584	1.841	Epn1	1q12
NM_019201	C-terminal binding protein 1	0.857	1.521	1.776	Ctbp1	14q21
D16554	Polyubiquitin	0.814	1.556	1.912	Loc192255	10q23
NM_019249	Protein tyrosine phosphatase, receptor t	0.790	1.581	2.001	Ptprf	1q22
NM_031749	Glucosidase 1	0.776	1.077	1.387	Gcs1	4q34
AW918154	ESTs, Highly similar to mitochondrial r	0.759	1.035	1.364		4q42

BG663014	ESTs, Moderately similar to selenoprote	0.758	1.436	1.895		
NM_032615	Membrane interacting protein of RGS16	0.751	2.670	3.553	Mir16	
AF311311	Rho interacting protein 3	0.742	0.913	1.231	Rhoip3	10q22
BF566725	ESTs, Highly similar to suppressor of T	0.736	1.137	1.544		9q12
NM_031797	Kangai 1	0.727	0.328	0.450	Kai1	3q24
NM_017344	Glycogen synthase kinase 3 alpha	0.717	2.001	2.791	Gsk3a	1q21
AF277892	Guanine nucleotide binding protein, beta	0.709	1.571	2.214	Gnb2	12q12
BG665004	ESTs, Moderately similar to VATE_MOUSE V	0.706	2.189	3.103	MGC72933	4q42
AJ249240	Cytokine inducible SH2-containing protein	0.704	0.392	0.557	socs-3	
AJ400732	Tap-binding protein	0.697	0.883	1.267	Tapbp	20p12
M83681	RAB3D, member RAS oncogene family	0.693	4.461	6.433	Rab3d	8q13
NM_031711	ADP-ribosylation-like 2	0.692	1.291	1.865	Arl2	1q43
BQ782968	ESTs, Highly similar to adaptor-related	0.686	1.059	1.542		7q11
NM_012747	Signal transducer and activator of trans	0.685	0.699	1.020	Stat3	10q32.1
NM_031337	Sialyltransferase 9 (CMP-NeuAc:lactosylc	0.684	0.581	0.850	Siat9	4q33
NM_019231	Mitogen activated protein kinase 13	0.671	0.903	1.347	Mapk13	20p12
NM_017231	Phosphatidylinositol transfer protein	0.667	0.690	1.035	Pitpn	10q24
NM_021692	Smad5	0.664	1.248	1.879	Madh5	17p14
U52103	Collapsin response mediator protein 3	0.661	1.423	2.154	Dpys4	1q41
M99567	Phospholipase C, beta 3	0.658	0.396	0.603	Plcb3	1q43
AJ230617	Protein kinase C, delta	0.653	0.486	0.744	Prkcd; Pkcd	16p16
AF154572	Interferon, alpha-inducible protein 27-l	0.646	1.484	2.298	Ifi27l	6q32
NM_138916	protein kinase for splicing component	0.637	0.726	1.139	Pksc	10q31
NM_017048	Solute carrier family 4, member 2, anion	0.632	1.273	2.015	Slc4a2	4q11
NM_017206	Solute carrier family 6, member 6	0.628	2.043	3.251	Slc6a6	4q34
Y16774	Dri 27/ZnT4 protein – zinc transporter	0.627	0.690	1.100	Slc30a4	
AF065438	Peptidylprolyl isomerase C-associated pr	0.626	1.120	1.789	Ppicap	10q32.3
BG672730	ESTs, Highly similar to hypothetical pr	0.617	0.535	0.866		18p12
AB027143	Peanut (Drosophila)-like 1	0.614	1.747	2.843	Gp1bb	11q23
NM_152242	G protein-coupled receptor 56	0.603	4.482	7.431	Gpr56	19p13
BF524262	ESTs, Highly similar to HLA-B associate	0.598	1.399	2.338		
AF186469	Fasting-inducible integral membrane prot	0.597	0.877	1.470	Tm6p1	4q33
NM_016989	Adenylate cyclase activating polypeptide	0.583	0.698	1.198	Adcyap1	9q37
AJ293697	Nasal embryonic LHRH factor	0.572	1.747	3.057	Nelf	3p13
NM_030995	Microtubule-associated protein 1a	0.571	0.847	1.481	Mtap1a	3q36
NM_021869	Syntaxin 7	0.560	1.175	2.098	Stx7	1p12
NM_022715	Major vault protein	0.552	0.491	0.889	Mvp	1q36

NM_147137	cystatin SC	0.551	0.730	1.325	LOC257643	3q41
NM_016990	Adducin 1, alpha – spectrin actin interaction	0.550	0.675	1.227	Add1	14q21
NM_031511	Insulin-like growth factor II (somatomed	0.546	26.687	48.865	Igf2	1q41
NM_031747	Calponin 1	0.537	0.629	1.170	Cnn1	8q13
AF388527	hypothetical RNA binding protein RDA288	0.527	0.392	0.744	Rda288	4q31
NM_022673	Methyl CpG binding protein 2	0.522	1.767	3.386	Mecp2	Xq37
M15883	Clathrin, light polypeptide (Lcb)	0.508	3.124	6.146	Cltb	17p14
NM_023982	RhoGEF glutamate transport modulator GTR	0.502	0.401	0.799	Gtrap48	2q34
NM_080905	seven in absentia 1A	0.497	0.944	1.899	Siah1a	19p11
NM_031518	Antigen identified by monoclonal antibod	0.485	2.626	5.420	Mox2	11q21-q22
AF098301	Neural F box protein NFB42	0.482	2.896	6.010	Fbxo2	5q36
NM_031967	Development-related protein	0.463	3.158	6.821	AF045564	19p13
NM_022669	Secretogranin II	0.451	14.246	31.600	Scg2	9q34
AF375462	Synaptotagmin 8	0.450	0.736	1.634	Syt8	1q41
NM_019152	Calpain 1	0.448	0.226	0.505	Capn1	1q43
NM_012688	Cholecystokinin A receptor	0.446	1.423	3.191	Cckar	14q11
BF567990	ESTs, Highly similar to lymphocyte anti	0.401	2.551	6.358		
U54807	RAB3C, member RAS oncogene family	0.301	2.055	6.828	Rab3c	2q14
AB040468	Exchange factor for ARF6	0.269	1.483	5.504	EFA6	1q54
NM_023099	G protein-coupled receptor 27	0.251	1.350	5.379	Gpr27	4q34
NM_130410	Interleukin 23, alpha subunit p19	0.220	3.798	17.293	Il23a	7q11
AY125814	Rattus norvegicus Na+-dependent amino ac	0.176	0.147	0.834	Slc1a7	
NM_022929	Kv channel-interacting protein 1	0.171	3.258	19.029	Kcnip1	10q12
X89603	Metallothionein 3	0.086	0.572	6.638	Mt3	19p12
NM_030999	Corticotropin releasing hormone 1	0.031	0.070	2.272	Crhr1	10q32.1

Data Set 2 – Lisa Poppe (analyzed by Brent Presley)

POSITIVE SIGNIFICANT GENES (833/15 v 832/13 + 834/40)

Genebank ID (rat) – if available	Description	Fold Change	Spot Value – Cytokine Resistant	Spot Value – Cytokine Sensitive	Common Abreviation	Map Location
M31109	Rat UDP-glucuronosyltransferase mRNA, complete cds	9.382	4.959	0.529	Udpgt	14p21
M83143	Sialyltransferase 1 (beta-galactoside alpha-2,6-sialytransferase)	9.316	4.258	0.457	Siat1	11q23
AB001075	Lectin, galactoside-binding, soluble, 2 (galectin 2)	8.673	22.361	2.578	Lgals2	7q34

NM_012849	Gastrin	7.986	26.080	3.266	Gast	10q32.1
NM_022284	Uroguanylin	7.231	10.427	1.442	Guca2b	5q36
NM_020101	Centaurin-alpha2 protein	6.375	6.600	1.035	LOC56826	10q26
M27440	Apolipoprotein B	6.333	5.634	0.890	Apob	6q14
BQ196534	ESTs, Moderately similar to thioesterase, adipose associated [Mus musculus] [M.musulus]	5.736	6.792	1.184		
AF370882	TORID	5.527	4.767	0.863	TORID	4q24
NM_021854	Tuberous sclerosis 1	5.052	9.111	1.803	Tsc1	3p12
NM_019622	Jerker, deafness locus	5.041	2.765	0.549	Espn	5q36
NM_130756	4,8-dimethylnonanoyl-CoA thioesterase	5.022	38.339	7.634	Pte1	3q42
NM_031045	Inositol 1,4,5-triphosphate 3-kinase	4.874	5.374	1.103	Itpka	3q35
NM_031070	Nel-like 2 homolog (chicken)	4.699	15.088	3.211	Nell2	7q35
NM_022950	Core1 UDP-galactose:N-acetylgalactosamine-alpha-R beta 1,3-galactosyltransferase (C1galt1)	4.401	1.282	0.291	C1galt1	4q21
NM_012619	Phenylalanine hydroxylase	4.326	0.171	0.039	Pah	7q13
AF273025	Solute carrier family 38, member 3	3.636	4.093	1.125	Slc38a3	8q32
NM_021594	ERM-binding phosphoprotein	3.488	6.473	1.856	LOC59114	Y
NM_012489	Acetyl-CoA acyltransferase, 3-oxo acyl-CoA thiolase A	3.462	3.502	1.012	Acaa1	8q32
NM_020098	Presynaptic cytomatrix protein	3.283	3.347	1.019	Pclo	4q12
AB000215	Sphingomyelin phosphodiesterase 3, neutral	3.242	15.667	4.833	Smpd3	19q12
NM_022936	Cytosolic epoxide hydrolase	3.151	6.568	2.084	Ephx2	15p12
U25282	Rattus norvegicus brush border myosin I (BBMI) mRNA, alternatively spliced testis-specific variant,	2.996	3.571	1.192	BBMI	
AW918850	ESTs, Moderately similar to regulator of G-protein signaling 19 [Rattus norvegicus] [R.norvegicus]	2.917	10.205	3.499		2q45
NM_012624	Pyruvate kinase, liver and RBC	2.912	11.922	4.093	Pklr	2q34
BE099884	ESTs, Moderately similar to RIKEN cDNA 4930548G07 [Mus musculus] [M.musulus]	2.696	1.312	0.487		
NM_012901	Alpha-1 microglobulin	2.637	93.059	35.294	Ambp	5q24
NM_138914	rapostlin	2.533	0.464	0.183	Rnd2	3p12
AF063890	Rattus norvegicus focal adhesion kinase-related protein (PYK2) mRNA, complete cds	2.508	4.729	1.885	Ptk2b	15p12
NM_021754	Nopp140 associated protein	2.487	1.055	0.424	Nap65	9q31
AB062758	carbonyl reductase	2.461	0.825	0.335	LOC266686	15p13
NM_017207	Vanilloid receptor-like protein 1	2.459	0.565	0.230	Vrl1	10q23
NM_031130	Nuclear receptor subfamily 2, group F, member 1	2.440	1.108	0.454	Nr2f1	2q11
NM_017096	C-reactive protein	2.412	4.948	2.051	Crp	13q24
J03867	Diaphorase (NADH) (cytochrome b-5 reductase)	2.404	1.563	0.650	Dia1	7q34
NM_017007	Glutamate decarboxylase 1 (brain)	2.398	17.719	7.388	Gad1	3q21

AB075607	Rattus norvegicus mRNA for PSD-Zip70, complete cds	2.367	4.163	1.759	Psdzip70	16p14
NM_017136	Squalene epoxidase	2.354	1.440	0.612	Sqle	7q33
AB049189	Suppression of tumorigenicity 14	2.343	4.195	1.790	St14	8q13
NM_022866	Solute carrier family 13 (sodium-dependent dicarboxylate transporter), member 3	2.337	12.085	5.171	Slc13a3	3q42
U82591	Putative c-Myc-responsive	2.335	2.558	1.095	Rcl	9q12
U62315	Rattus norvegicus alpha-globin (GloA) gene, complete cds	2.330	1.463	0.628	GloA	
NM_031645	Receptor (calcitonin) activity modifying protein 1	2.310	1.039	0.450	Ramp1	9q36
NM_080891	Fas death domain-associated protein	2.310	9.536	4.129	Daxx	20p12
NM_031641	Sulfotransferase family 4A, member 1	2.290	22.587	9.863	Sult4a1	
AF461734	UDP-glucuronosyltransferase 1 family, member 1	2.260	0.966	0.427	Ugt1a1	9q35-q36
AF057564	Rattus norvegicus putative retrovirus-related gag protein mRNA, complete cds	2.230	0.630	0.283	LOC286991	
NM_022695	Neurotensin receptor 2	2.217	2.308	1.041	Ntsr2	
U75903	UDP glycosyltransferase 1 family, polypeptide A7	2.206	0.758	0.343	Ugt1a7	9q35
U49099	Golgi SNAP receptor complex member 1	2.153	1.851	0.860	Gosr1	10q26
NM_022924	Coagulation factor 2	2.151	0.210	0.098	F2	3q24
NM_021576	5 nucleotidase	2.129	0.664	0.312	Nt5	8q31
NM_013219	Ca2+-dependent activator protein	2.129	6.198	2.911	Caps	15p16
X13309	Extracellular proteinase inhibitor	2.091	0.134	0.064	Expi	10q26
NM_031726	Secretory carrier membrane protein 5	2.055	6.273	3.053	Scamp5	8q24
M95578	Interleukin 1 receptor, type I	2.028	1.434	0.707	Il1r1	9q21
L31883	Tissue inhibitor of metalloproteinase 1	2.007	0.087	0.043	Timp1	Xq12
NM_031050	Lumican	1.999	0.073	0.037	Lum	7q13
NM_022215	Glycerol 3-phosphate dehydrogenase	1.991	6.443	3.236	Gpd3	7q36
AA957762	ESTs, Highly similar to pleckstrin homology domain interacting protein [Homo sapiens] [H.sapiens]	1.985	1.343	0.676		
U66470	Cell growth regulatory with EF-hand domain	1.985	1.103	0.556	Cgr11	6q14
AB026841	Neuraminidase 3	1.980	3.748	1.893	Neu3	1q32
NM_022949	Ribosomal protein L14	1.977	0.592	0.299	Rpl14	8q32
NM_012603	V-myc avian myelocytomatosis viral oncogene homolog	1.975	1.214	0.615	Myc	7q33
NM_031119	Sjogren syndrome antigen B	1.970	0.979	0.497	Ssb	3q24-q32
NM_031840	Farensyl diphosphate synthase	1.942	0.899	0.463	Fdps	2q34
AF336041	Cysteine desulfurase	1.933	3.551	1.837	Nifs	3q42
AF136601	DNA fragmentation factor, alpha subunit	1.918	8.775	4.575	Dffa	5q36
AF079864	Rattus norvegicus putative G-protein coupled receptor RA1c mRNA, complete cds	1.907	2.478	1.299	Olfr78	1q32
J05132	UDP glycosyltransferase 1 family, polypeptide A6	1.881	0.731	0.389	Ugt1a6	9q35
NM_012718	Androgen regulated 20 kDa protein	1.832	45.447	24.803	Andpro	3q41

X87157	Neurolysin (metallopeptidase M3 family)	1.830	1.874	1.024	Nln	2q13
AF327562	Protein that interacts with C kinase 1	1.776	7.807	4.395	Pick1	7q34
NM_012733	Retinol-binding protein 1	1.774	4.545	2.562	Rbp1	8q31
NM_012819	Acyl Coenzyme A dehydrogenase, long chain	1.763	0.710	0.403	Acadl	9q32
NM_012945	Diphtheria toxin receptor (heparin binding epidermal growth factor – like growth factor)	1.763	1.603	0.909	Dtr	18p11
AF062594	Nucleosome assembly protein 1-like 1	1.745	0.791	0.453	Nap1l1	7q21
NM_012992	Nucleoplasmin-related protein (Nuclear protein B23	1.741	0.917	0.526	Npm1	10q12
U01914	A kinase anchor protein 8	1.729	1.869	1.081	Akap8	7q11
NM_024133	Huntingtin-associated protein 1	1.722	3.791	2.202	Hap1	10q32.1
AF097887	Rho family GTPase	1.719	2.416	1.406	Arhv	3q35
NM_130399	Adenosine deaminase	1.705	1.152	0.676	Ada	3q42
BE100543	ESTs, Highly similar to NCR1_MOUSE Nuclear receptor co-repressor 1 (N-CoR1) (N-CoR) (Retinoid X receptor interacting protein 13) (RIP13) [M.musulus]	1.694	2.117	1.250		
NM_012578	Histone H1-0	1.688	0.348	0.206	H1f0	7q34
X53003	Rat mRNA for acetyl-coenzyme A carboxylase (EC 6.4.1.2.) 3' untranslated region	1.688	1.694	1.004	Acac	10q26
NM_031714	Heat-responsive protein 12	1.672	1.926	1.152	Hrsp12	7q22
Y00826	Nuclear pore membrane glycoprotein 210	1.669	2.661	1.594	Pom210	4q34
NM_031053	Mismatch repair protein	1.664	0.983	0.591	Mlh1	8q32
NM_017271	Nuclear distribution gene C homolog (Aspergillus)	1.646	0.804	0.489	Nudc	5q36
AF378332	BCL2-related protein A1	1.641	0.270	0.165	Bcl2a1	8q31
NM_031101	Ribosomal protein L13	1.622	0.807	0.498	Rpl13	19q12
NM_031334	Cadherin 1	1.615	6.952	4.304	Cdh1	19q12
AF069525	Ankyrin 3 (G)	1.615	7.712	4.776	Ank3	20p11
NM_022586	Neuropeptide FF-amide peptide precursor	1.612	2.444	1.516	Npff	
AB032178	Cytochrome c oxidase, subunit XVII assembly protein homolog (yeast)	1.608	3.535	2.198	Cox17	11q21
L13445	Sialyltransferase 8 (alpha-2, 8-sialytransferase) B	1.605	3.015	1.879	Siat8b	1q31
M89902	3-hydroxybutyrate dehydrogenase (heart, mitochondrial)	1.597	2.744	1.719	Bdh	11q22
AW915955	ESTs, Moderately similar to T13963 formin related protein, lymphocyte specific – mouse [M.musulus]	1.586	0.702	0.443		10q32.1
BE117828	EST, Moderately similar to S12207 hypothetical protein (B2 element) – mouse [M.musulus]	1.575	1.530	0.972		
NM_133296	X transporter protein 3	1.572	21.919	13.942	Xtrp3	8q32
AI102591	ESTs, Highly similar to SMD1_HUMAN Small nuclear ribonucleoprotein Sm D1 (snRNP core protein D1) (Sm-D1) (Sm-D autoantigen) [M.musulus]	1.546	2.110	1.365		9q13
NM_019124	Rabaptin 5	1.534	1.842	1.200	LOC54190	10q24
D50559	Sterol-C4-methyl oxidase-like	1.531	0.653	0.426	Sc4mol	16p13

L22655	Rat anti-acetylcholine receptor antibody gene, kappa-chain, VJC region, complete cds	1.524	0.027	0.018		
AF095576	Adaptor protein with pleckstrin homology and src homology 2 domains	1.479	3.260	2.204	Aps	12q12
NM_012709	Cytochrom P450 15-beta gene	1.476	7.120	4.825	Cyp2c12	1q53
NM_013224	Ribosomal protein S26	1.467	1.131	0.771	Rps26	7q11
NM_022506	Ribosomal protein L31	1.455	1.116	0.767	Rpl31	9q21
AJ298278	Poly(A) binding protein, cytoplasmic 1	1.453	0.770	0.530	Pabpc1	7q22
M34136	Tropomyosin 1, alpha	1.449	0.192	0.133	Tpm1	8q24
AF192757	Programmed cell death 6 interacting protein	1.446	1.024	0.708	Pdcd6ip	
NM_012570	Glutamate dehydrogenase	1.444	1.633	1.131	Glud1	16p16
NM_022515	Ribosomal protein L24	1.442	1.045	0.725	Rpl24	11q12
NM_022504	Ribosomal protein L36	1.440	1.262	0.876	Rpl36	9q11
NM_019259	Complement component 1, q subcomponent binding protein	1.438	1.400	0.974	C1qbp	10q24
NM_139080	Ku70 DNA-binding component of DNA-dependent proteinkinase complex (thyroid autoantigen 70 kDa)	1.428	2.022	1.416	G22p1	7q34
BF567456	ESTs, Moderately similar to RIKEN cDNA 2810036K01 [Mus musculus] [M.musulus]	1.423	1.248	0.877		18q11
NM_017160	Ribosomal protein S6	1.422	0.469	0.330	Rps6	5
NM_031779	Amyloid beta (A4) precursor protein-binding, family A, APBA1: amyloid beta (A4) precursor protein-bi	1.414	1.542	1.091	Apba1	1q51
NM_019277	SEC15 homolog (S. cerevisiae)	1.407	3.913	2.780	Sec15	1q54
NM_022510	Ribosomal protein L4	1.407	0.508	0.361	Rpl4	8q24
NM_031773	RNA polymerase I (127 kDa subunit)	1.404	1.204	0.858	Rpo1-2	3q36
NM_017340	Acyl-coA oxidase	1.402	1.541	1.099	RATACOA1	10q32.3
M91234	Rat VL30 element mRNA	1.400	0.324	0.231		
NM_017306	Dodecenoyl-coenzyme A delta isomerase	1.390	0.894	0.643	Dci	10q12
AW143206	ESTs, Moderately similar to RIKEN cDNA 1110029G07 [Mus musculus] [M.musulus]	1.349	1.299	0.963		15p13
AF248543	Igb3 synthase	1.346	2.069	1.537	LOC171553	5q36
M17412	Growth and transformation-dependent protein	1.340	0.568	0.424	LOC60380	11q11
NM_031709	Ribosomal protein S12	1.332	0.702	0.527	Rps12	1p12
NM_017030	Propionyl Coenzyme A carboxylase, beta polypeptide	1.328	1.745	1.314	Pccb	8q31
NM_017264	Protease (prosome, macropain) 28 subunit, alpha	1.320	0.853	0.646	Psme1	15p13
AF327513	Smhs2 protein	1.316	0.877	0.666	Smhs2	
NM_031570	Ribosomal protein S7	1.312	0.543	0.414	Rps7	6q16
AW435190	ESTs, Highly similar to RIKEN cDNA 2610529H08 [Mus musculus] [M.musulus]	1.310	0.834	0.636	c3orf6	11q22
U41803	Mitofusin 2	1.309	2.459	1.879	Mfn2	5q36
NM_031043	Glycogenin	1.304	0.928	0.712	Gyg	2q24
AF000578	Cell division cycle 5-like (S. pombe)	1.290	1.597	1.238	Cdc5l	9q12
X78167	Ribosomal protein L15	1.290	1.267	0.982	Rpl15	15p16

BF555394	ESTs, Highly similar to A54691 octamer-binding protein NonO – mouse [M.musulus]	1.269	1.384	1.091		
NM_022514	Ribosomal protein L27	1.264	1.041	0.824	Rpl27	10q32.1
AB041723	Programmed cell death 8 (apoptosis-inducing factor)	1.262	1.240	0.983	Pdcd8	Xq35
AF121265	Beta-catenin	1.257	0.605	0.481	Catnb	8q32
NM_012963	High mobility group box 1	1.252	1.060	0.847	Hmgb1	1q51
NM_022799	Nuclear ubiquitous casein kinase 2	1.249	1.310	1.048	Nucks	
U42209	Sec22 homolog	1.239	1.478	1.193	Sec22a	
AB073550	Tmprss2 Transmembrane protease, serine 2	1.238	1.907	1.540	Tmprss2	11q12
U58857	Serine (or cysteine) proteinase inhibitor, clade B, member 5	1.233	4.808	3.899	Serpinb5	13p13
NM_031106	Ribosomal protein L37	1.232	0.720	0.584	Rpl37	2q16
NM_022291	Rfamide-related peptide receptor	1.225	1.906	1.556	OT7T022	20q11
NM_021661	Regulator of G-protein 176ignaling 19	1.223	1.015	0.831	Rgs19	3q43
NM_019213	Jumping translocation breakpoint	1.180	1.216	1.030	Jtb	2q34
BG668035	ESTs, Moderately similar to RL34_RAT 60S RIBOSOMAL PROTEIN L34 [R.norvegicus]	1.175	0.840	0.715		Xq31
NM_031772	RNA polymerase I (194 kDa subunit)	1.174	1.184	1.008	Rpo1-4	4q33
NM_017236	Phosphatidylethanolamine binding protein	1.163	2.526	2.172	Pbp	12q16

NEGATIVE SIGNIFICANT GENES (833/15 v 832/13 + 834/40)

NM_031723	Signal peptidase complex 18kD	0.847889	1.4709939	1.7348887	Spc18	1q31
NM_080689	dynamin 1	0.83579	0.9697405	1.1602683	Dnm1	3p11
NM_134399	Mk1 protein	0.8306	0.6709063	0.80773723		
NM_017005	Fumarate hydratase	0.818651	1.141721	1.3946375	Fh1	13q25
AW918154	ESTs, Highly similar to mitochondrial ribosomal protein 64 [Mus musculus] [M.musulus]	0.807144	0.78305656	0.9701577		4q42
NM_019222	Coronin, actin-binding protein, 1B	0.80111	1.1035433	1.3775172	Coro1b	1q42
NM_017041	Protein phosphatase 3, catalytic subunit, alpha isoform	0.792734	0.96630555	1.2189538	Ppp3ca	15
AB032828	Erythrocyte protein band 4.1-like 3	0.792385	3.4416764	4.343441	Epb4.1l3	9q38
BG671325	ESTs, Highly similar to synapse associated protein 1 [Mus musculus] [M.musulus]	0.790532	0.63854414	0.8077396		
AF285078	Quiescin Q6	0.785861	0.95221204	1.2116792	Qscn6	13q21
NM_031749	Glucosidase 1	0.781929	1.2228185	1.5638493	Gcs1	4q34
NM_080899	inhibitor of kappa light polypeptide enhancer in B-cells, kinase complex-associated protein	0.78055	0.26759174	0.34282467	Ikbkap	5q22
NM_031035	GTP-binding protein (G-alpha-i2)	0.775449	1.4322488	1.8469921	Gnai2	8q32
NM_019144	Acid phosphatase 5, tartrate resistant	0.773289	0.49999917	0.64658767	Acp5	8
M19262	Clathrin, light polypeptide (Lcb)	0.771247	3.1918929	4.1386156	Cltb	17p14
NM_017135	Adenylate kinase 4	0.759952	0.829082	1.090966	Ak4	5q33
AI412631	ESTs, Moderately similar to GDS1_HUMAN RAP1 GTPASE-GDP DISSOCIATION STIMULATOR 1 (SMG P21 STIMULATORY GDP	0.75886	0.59348756	0.7820774		

Y13336	Defender against cell death 1	0.754795	1.0585425	1.4024233	Dad1	15p13
NM_031594	Purinergic receptor P2X, ligand-gated ion channel, 4	0.750806	1.3147234	1.7510817	P2rx4	12q16
NM_013067	Ribophorin I	0.747807	1.3828696	1.8492343	Rpn1	4
BF407789	ESTs, Highly similar to GDP-mannose pyrophosphorylase B, isoform 1; mannose-1-phosphate guanylyltransferase [Homo sapiens] [H.sapiens]	0.747755	0.8310104	1.1113403		
NM_022546	Death-associated like kinase	0.747517	0.8219788	1.099612	Dapkl	7q11
NM_012857	Lysosomal associated membrane protein 1 (120 kDa)	0.74293	0.96347684	1.2968614	Lamp1	16q12.5
BQ205309	ESTs, Highly similar to CBX3_MOUSE Chromobox protein homolog 3 (Heterochromatin protein 1 homolog gamma) (HP1 gamma) (Modifier 2 protein) (M32) [M.musulus]	0.738949	1.1678807	1.5804611		
NM_019220	Amino-terminal enhancer of split	0.738059	1.392859	1.8871908	Aes	7q11
AJ243123	Cytokine inducible SH2-containing protein 1	0.73776	0.6967023	0.9443483	Cish1	10q11
NM_031589	Glucose-6-phosphatase, transport protein 1	0.736184	1.4942305	2.0296974	G6pt1	8q22
NM_022507	Protein kinase C, zeta	0.733638	1.1713353	1.5966116	Prkcz	5q36
D10874	ATPase, H+ transporting, lysosomal (vacuolar proton pump) 16 kDa	0.733075	1.7826074	2.431685	Atp6l	10q12
M75148	kinesin light chain 1	0.732363	1.2378587	1.6902257	Klc1	6q32
BG671427	ESTs, Highly similar to VA0D_MOUSE Vacuolar ATP synthase subunit d (V-ATPase d subunit) (Vacuolar proton pump d subunit) (V-ATPase AC39 subunit) (V-ATPase 40 kDa accessory protein) (P39) (Physophilin) [M.musulus]	0.732293	1.824135	2.490992		19q11
NM_031575	Thymoma viral proto-oncogene 3	0.724322	0.71337664	0.98488855	Akt3	13
NM_017018	Histamine receptor H1	0.721588	0.91738963	1.2713474	Hrh1	4q42
AJ007704	Malonyl-CoA decarboxylase	0.719969	1.2006056	1.6675807	Mlycd	19q12
AW141364	ESTs, Highly similar to SIN1_HUMAN Stress-activated map kinase interacting protein 1 (SAPK interacting protein 1) (Putative Ras inhibitor JC310) [H.sapiens]	0.718991	0.60984033	0.8481891		3p11
NM_012690	P-glycoprotein 3/ multidrug resistance 2	0.717049	0.13239978	0.18464532	Abcb4	4q11-q12
AF367467	Rattus norvegicus Ratsg2 mRNA, complete cds	0.711405	1.2310572	1.7304602	Ratsg2	1q22
AB002466	Lamin B receptor	0.709032	0.31363273	0.44233942	Lbr	13q26
NM_138549	synaptic glycoprotein SC2	0.708082	1.0335138	1.4595966	SC2	19q11
U20525	Tumor protein, translationally-controlled 1	0.703987	0.3217014	0.4569709	Tpt1	15q11
NM_012564	Group-specific component (vitamin D-binding protein)	0.694821	15.4465	22.230904	Gc	14p22
AF061947	Cain	0.694402	1.0487348	1.5102711	Cain	20p12
BF552567	ESTs, Highly similar to pleckstrin homology domain-containing, family A (phosphoinositide binding specific) member 3 [Mus musculus] [M.musulus]	0.693792	0.4114972	0.59311354		
NM_031685	Golgi SNAP receptor complex member 2	0.693788	1.9621491	2.8281662	Gosr2	10q32.1
D21095	Cytokine-induced neutrophil chemoattractant-2	0.692132	1.3538741	1.956091	Cinc2	14p22
NM_031774	Rab acceptor 1 (prenylated)	0.692104	1.2826827	1.8533089	Rabac1	1q21
AJ004912	Integral membrane protein Tmp21-I (p23)	0.69158	0.42989525	0.6216134	Tmp21	6q31
X89968	N-ethylmaleimide sensitive fusion protein attachment protein alpha	0.689446	1.091086	1.5825553	Napa	1q21

NM_031698	Ribophorin 2	0.687737	1.9429737	2.8251686	Rpn2	3q42
AF526268	Rattus norvegicus T-cell activation protein-related protein mRNA, partial cds	0.686905	1.7119557	2.492275	Pgr1	
NM_031711	ADP-ribosylation-like 2	0.686792	1.0397607	1.5139371	Arl2	1q43
NM_023092	Unconventional myosin Myr2 I heavy chain	0.6819	0.29558802	0.43347704	Myr2	10q24
NM_019331	Proprotein convertase subtilisin/kexin type 3 (paired basic amino acid cleaving enzyme, furin, membr	0.679296	0.5987333	0.8814033	Pcsk3	1q22
Z49761	R.norvegicus mRNA for RT1.Ma	0.678543	0.46122453	0.6797275	Hla-dma	20p12
M76591	Phosphoglycerate mutase 1	0.669937	0.4195903	0.6263135	Pgam1	1
AY029283	Caspase 11	0.66779	0.23752195	0.3556838	Casp11	8q11
AA800019	ESTs, Highly similar to T9S3_MOUSE Transmembrane 9 superfamily protein member 3 precursor [M.musulus]	0.661567	0.57087964	0.862921		
BQ211483	EST, Moderately similar to RIKEN cDNA 1110007A14 [Mus musculus] [M.musulus]	0.661344	0.639052	0.9662922		1q22
U78977	ATPase, Class II, type 9A	0.659488	7.8885064	11.96157	Atp9a	
NM_031154	Glutathione S-transferase, mu type 3 (Yb3)	0.65509	4.5844903	6.99826	Gstm3	2q34
NM_019355	CPG2 protein	0.654646	1.2543876	1.9161308	CPG2	1p11
NM_033021	Vesicle associated protein	0.652418	0.4876086	0.7473867	VAP1	14p22
NM_023976	Periaxin	0.650923	0.5462317	0.8391646	Prx	1q21
NM_080787	diacylglycerol kinase, alpha (80 kDa)	0.646719	0.26649958	0.41207922	Dgka	7q11
NM_022605	Heparanase	0.635671	0.81109685	1.2759693	Hpse	14p22
NM_012747	Signal transducer and activator of transcription 3	0.630179	0.674054	1.0696235	Stat3	10q32.1
NM_012673	Thymus cell surface antigen	0.622179	0.5586495	0.897892	Thy1	8q22
AF419333	gamma-aminobutyric acid A receptor, theta	0.620848	1.0226886	1.6472452	Gabrq	Xq37
U92010	Lin-10 protein homolog	0.620817	1.3575761	2.186756	Lin10	19q11
NM_022523	CD151 antigen	0.620522	0.99145186	1.5977706	Cd151	1q41
NM_031975	Parathymosin	0.620237	1.5884438	2.561026	Ptms	4q42
NM_019208	Multiple endocrine neoplasia 1	0.610138	1.2166766	1.9941006	Men1	1q43
AW142290	ESTs, Highly similar to poly(rC) binding protein 1; poly(rC)-binding protein 1 [Mus musculus] [M.musulus]	0.609376	0.46478358	0.76272017		
M15327	Alcohol dehydrogenase (class I), alpha polypeptide	0.608676	0.09471444	0.15560745	Adh1	2q44
AJ303374	ATP-binding cassette, sub-family G (WHITE), member 1	0.607036	1.0990101	1.8104519	Abcg1	20p12
AY030278	Wnt inhibitory factor 1	0.604899	3.1627738	5.2286005	Wif1	7q22
NM_012494	Angiotensin receptor 2	0.603756	0.77678746	1.2865918	Agtr2	Xq34
NM_012824	Apolipoprotein C1	0.596742	0.6605867	1.1069887	Apoc1	1q21
D30040	V-akt murine thymoma viral oncogene homolog 1	0.594706	0.4519033	0.75987697	Akt1	6q32
NM_138917	F-box only protein 6 b	0.594518	0.4906156	0.82523227	Fbxo6b	5q36
AF015953	Aryl hydrocarbon receptor nuclear translocator-like	0.588301	0.96543306	1.6410515	Arntl	1q34
U44129	Lectin, mannose-binding, 1	0.58325	0.98159456	1.6829736	Lman1	18q12.1
AB011529	Cadherin EGF LAG seven-pass G-type receptor 2	0.58251	1.2883531	2.2117262	Celsr2	2q34

NM_017081	Hydroxysteroid dehydrogenase, 11 beta type 2	0.582308	0.38447008	0.66025215	Hsd11b2	19q11-q12
NM_031635	Fucosyltransferase 2	0.581342	0.83462995	1.4356949	Fut2	1
BF553584	ESTs, Moderately similar to heterogeneous nuclear ribonucleoprotein H1; heterogeneous nuclear ribonucleoprotein H [Mus musculus] [M.musulus]	0.580874	0.23230723	0.39992717	Hnrpf	6q11
AI556487	ESTs, Highly similar to TIAM_MOUSE T-lymphoma invasion and metastasis inducing protein 1 (TIAM1 protein) [M.musulus]	0.57912	0.3668556	0.6334705		
NM_012667	Tachykinin 1 receptor	0.577286	1.0722415	1.857384	Tacr1	4q34
NM_022217	Amphiphysin	0.577184	0.89562476	1.5517154	Amph1	17q11
NM_024394	5-hydroxytryptamine (serotonin) receptor 3a	0.575431	0.5955098	1.0348928	Htr3a	8q23
U08290	Neuronatin	0.574156	72.035515	125.46328	Nnat	3q42
AI236618	ESTs, Moderately similar to hypothetical protein LOC51257 [Homo sapiens] [H.sapiens]	0.573966	0.42123252	0.73389864		
M25732	Peptidylglycine alpha-amidating monooxygenase	0.57382	4.357863	7.5944715	Pam	9q36
J00750	Metallothionein	0.568411	0.58888847	1.0360254	Mt1a	19p12
M94040	Branched chain keto acid dehydrogenase E1, beta polypeptide	0.567584	0.43336493	0.7635261	Bckdhb	8q31
X89603	Metallothionein 3	0.563038	0.5236163	0.92998457	Mt3	19p12
NM_013087	CD81 antigen (target of antiproliferative antibody 1)	0.561123	1.2700762	2.2634537	Cd81	1q41
M88469	F-spondin	0.559993	0.41632825	0.7434522	Sponf	1q34
BF523209	ESTs, Highly similar to fatso [Mus musculus] [M.musulus]	0.558961	0.56679314	1.0140119		
NM_017178	Bone morphogenetic protein 2	0.557569	0.6157308	1.1043124	Bmp2	3q36
NM_013007	Prepronociceptin (neuropeptide nociceptin) (N23K)	0.55691	0.8798441	1.5798676	Pnoc	15p12
M86870	Protein disulfide isomerase related protein (calcium-binding protein, intestinal-related)	0.554121	0.6809977	1.22897	Erp70	4q24
X79328	R.norvegicus (Wistar) CaBP1 mRNA	0.552394	0.46869332	0.8484771	P5	6
NM_012836	Carboxypeptidase D precursor	0.549971	0.90757364	1.6502218	Cpd	10q26
AY122323	protein phosphatase 1, regulatory (inhibitor) 5 subunit 14B	0.549515	0.9604096	1.747742	Ppp1r14b	1q43
NM_012918	Calcium channel alpha 1A	0.548903	3.1605713	5.757981	Cacna1a	19q11
NM_031242	CDP-diacylglycerol synthase (phosphatidate cytidylyltransferase) 1	0.54721	6.0555897	11.066298	Cds1	14p22
AA944531	ESTs, Highly similar to beta-catenin-interacting protein ICAT [Mus musculus] [M.musulus]	0.545285	0.44322315	0.8128277		
NM_022854	Testis lipid binding protein	0.542329	1.9172498	3.535217	Tlbp	2q23
X01153	Whey acidic protein	0.538206	1.0054725	1.8681943	WAP	
NM_024128	Brain specific mRNA B (clone p1a75)	0.538041	9.663679	17.960861	Bsmrb	14q21
Y15054	Tumor specific antigen 70 kDa	0.537647	0.61966854	1.1525564	Loc192276	10q12
NM_053299	ubiquitin D	0.536851	1.0400598	1.9373333	Ubd	20
U22830	P2 purinoreceptor subclass 2Y	0.536395	1.5067072	2.8089533	P2ry1	2q31
NM_022715	Major vault protein	0.533588	0.65745944	1.2321485	Mvp	1q36

X53565	Trans-golgi network protein 1	0.532191	0.85878944	1.6136874	Ttgn1	4q33
NM_012998	Protein disulfide isomerase (Prolyl 4-hydroxylase, beta polypeptide)	0.53184	0.79947525	1.5032264	P4hb	10q32.3
NM_017355	Ras-related GTP-binding protein 4b	0.530959	1.0081213	1.8986816	Rab4b	1q21
NM_017249	Membrane bound C2 domain containing protein	0.526496	0.3645764	0.6924585	Mbc2	7q11
NM_024146	Fibroblast growth factor receptor 1	0.521225	1.104516	2.1190782	Fgfr1	16q12.4
AI602689	ESTs, Highly similar to RIKEN cDNA 1500031O19 [Mus musculus] [M.musulus]	0.520747	1.0871496	2.0876727		1p13
NM_017310	Semaphorin 3a	0.511467	0.4431587	0.86644655	Sema3a	4q12
NM_145084	hypothetical protein RMT-7	0.510497	0.6201889	1.2148738	Rmt7	4q33
M81786	Syndecan 4	0.504297	0.18402897	0.36492157	Sdc4	3q42
BG663014	ESTs, Moderately similar to selenoprotein SelM [Homo sapiens] [H.sapiens]	0.503516	1.4921536	2.963471		
M11794	ESTs, Highly similar to SMRT2 metallothionein II – rat [R.norvegicus]	0.492955	0.1583173	0.32116008		
NM_031091	Rab3B protein	0.489403	6.3038783	12.880747	Rab3b	5q35
X63574	Somatostatin receptor 28	0.486135	4.7421794	9.754852	Smstr28	7q34
NM_031803	Glucocorticoid modulatory element binding protein 2	0.478082	1.146161	2.3974128	Gmeb2	3q43
AF336828	Nucleobindin	0.468621	0.46266097	0.9872814	Nucb	1q22
NM_022232	Protein kinase inhibitor p58	0.460073	0.5050191	1.0976927	LOC63880	15q24
NM_022669	Secretogranin II	0.44628	14.383671	32.23015	Scg2	9q34
NM_012595	Lactate dehydrogenease B	0.444638	0.40505022	0.91096723	Ldhb	4q44
NM_013080	Protein tyrosine phosphatase, receptor-type, zeta polypeptide	0.437911	4.9439993	11.289959	Ptprz1	4q22
NM_022282	Discs, large (Drosophila) homolog 2 (chapsyn-110)	0.421773	0.8286208	1.9646128	Dlg2	1q32
NM_012580	Heme oxygenase	0.41058	0.13078111	0.31852755	Hmox1	19p11
NM_019221	Tumor protein 63 kDa	0.409475	0.23182377	0.5661484	Trp63	11q23
NM_022534	Transcobalamin II precursor	0.395962	0.7463514	1.8849076	Tcn2p	14q21
BF405195	ESTs, Highly similar to hypothetical protein, 2-65 [Mus musculus] [M.musulus]	0.387076	1.6137246	4.1690154		
NM_031691	Integrin alpha X	0.378801	1.195826	3.1568675	Itgax	1q36
NM_022538	Phosphatidate phosphohydrolase type 2a	0.377419	0.8034959	2.1289253	Ppap2a	2q14
M35266	Cytosolic cysteine dioxygenase 1	0.369721	0.4880167	1.3199595	Cdo1	18q11
AF239157	DEXRAS1 (Dexras1)	0.360498	1.8649737	5.1733284	Rasd1	10q22
NM_133621	global ischemia induced protein GIIG15B	0.35682	0.23145595	0.64866245	GIIg15b	14p11
BF403302	ESTs, Highly similar to NOGG_RAT Noggin precursor [R.norvegicus]	0.328249	1.9372208	5.901675		
NM_017337	Phosphodiesterase 3A	0.327884	2.4317167	7.41639	Pde3a	4q44
NM_012512	Beta-2-microglobulin	0.321981	0.07525718	0.23373188	B2m	3q35
NM_012656	Secreted acidic cystein-rich glycoprotein (osteonectin)	0.293077	0.011340673	0.03869519	Sparc	10q22
NM_031358	Potassium inwardly rectifying channel, subfamily J, member 11	0.292789	8.124335	27.748058	Kcnj11; Kir6.2	1q22
NM_019248	Neural receptor protein-tyrosine kinase	0.289728	1.6547801	5.7114935	Ntrk3	1q31
NM_012688	Cholecystokinin A receptor	0.272417	1.1602921	4.2592516	Cckar	14q11
NM_012614	Neuropeptide Y	0.25437	2.5549357	10.044193	Npy	4q24

U22520	Small inducible cytokine B subfamily (Cys-X-Cys), member 10	0.238754	0.43920743	1.8395779	Cxcl10	14p22
NM_057199	lymphocyte antigen 94 (mouse) homolog (activating NK-receptor; NK-p46)	0.221471	3.1016126	14.004624	Ly94	1q12
NM_031967	Development-related protein	0.168384	1.8061222	10.726204	AF045564	19p13
L29232	Insulin-like growth factor 1 receptor	0.150921	0.570226	3.7783017	Igf1r	1q22
NM_031123	Stanniocalcin 1	0.140934	2.070083	14.688329	Stc1	15p11
AF036537	Homocysteine respondent protein HCYP2	0.120781	0.18105148	1.4990056	Hcyp2	15p13
NM_012707	Glucagon	0.072337	8.080022	111.700294	Gcg	3q22-q24

APPENDIX C

Quantitative PCR Primer Probe Sets

Quantitative PCR Probe Sets	ABI Assay ID
Gastrin (5’ set)	Rn01644838_m1
Gastrin (3” set)	Rn00563373_g1
TGFa	Rn00562037_m1
Cholecystokinin	Rn00563215_m1
Trefoil Factor 3	Rn00564851_m1
CCK2R	Rn00565867_m1
Cyclin A2	Rn01493717_g1
Cyclin B1	Rn00596848_m1
Cyclin B2	Rn02346769_g1
Cyclin E1	Rn01457762_m1
Cdk1	Rn00570728_m1
Ribosomal 18S	4319413E

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VITAE

Brent Kevin Presley was born in Nashville, Tennessee, on February 28, 1976, the son of Gerald Franke Presley and Joan Hampton Presley. After graduating from The McCallie School in Chattanooga, Tennessee in 1994 he entered Duke University in Durham, North Carolina as an undergraduate chemistry student. He was fortunate enough to study under Drs. David and Jane Richardson in the Department of Biochemistry where he not only learned a great deal of science, but played with computers all day as well. He graduated with a B.S. in Chemistry with Honors in 1998 and entered the M.D./Ph.D. program at The University of Texas Southwestern Medical Center at Dallas in 1999 where, after 2 years of medical school, he joined the lab of Dr. Chris Newgard and promptly moved back to Durham, North Carolina in a wry twist of fate. The greatest day of his life was when he married his lovely wife, Jaime Ai-Khanh Le at a sunset ceremony in Honolulu, Hawaii on September 9, 2005.

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