NEW ROLES OF THE TRANSCRIPTION FACTOR NKX6.1 IN BETA CELL BIOLOGY

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DEDICATION

To Stacy Lynn,

You have supported me always, in all ways.

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NEW ROLES OF THE TRANSCRIPTION FACTOR NKX6.1 IN BETA CELL BIOLOGY

by

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Pancreatic islet beta cells play a critical role in the maintenance of metabolic fuel homeostasis. Type 1 diabetes results from autoimmune destruction of the beta cells, whereas type 2 diabetes involves loss of beta cell function, particularly glucose-stimulated insulin secretion (GSIS), and a gradual diminution of beta cell mass. To identify genes that are involved in GSIS and contribute to the mature beta cell phenotype, a panel of beta cell lines with varying capacities for GSIS was employed for a candidate gene screen. Expression of the homeodomain transcription factor Nkx6.1 was found to be positively correlated with the capacity for GSIS in these lines. Although previously identified as an essential factor for beta cell differentiation, little was known about the function of Nkx6.1 in mature beta cells.

The purpose of this dissertation research was to investigate the role of Nkx6.1 in the biology of mature beta cells via manipulation of its expression in beta cell lines and primary pancreatic islets and measurement of a variety of functional outcomes. These studies led to identification of three novel roles of Nkx6.1 in the mature beta cell. First, Nkx6.1 was found to suppress expression of the glucagon gene via a direct interaction with its promoter, although the studies also demonstrate a role for Pdx1 and MafB in mediating this suppression. Second, the silencing of Nkx6.1 expression in pancreatic beta cells results in a severe impairment in GSIS, suggesting that Nkx6.1 target genes are critical for robust GSIS. Third, Nkx6.1 is shown to regulate beta cell proliferation, in part via direct interaction with the cyclin B1 gene and stimulation of its expression. Thus, whereas the majority of primary beta cells are quiescent, overexpression of Nkx6.1 is sufficient to initiate DNA synthesis and cell division. Importantly, and different from the common experience, this manipulation also enhances GSIS. These studies suggest that Nkx6.1 should be further investigated as a gene involved in development of diabetes and as a potential new therapeutic target.

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Acetyl Co-A – acetyl coenzyme A

ACh – acetylcholine

ADP, ATP – adenosine diphosphate, adenosine triphosphate

AEC - 3-amino-9-ethylcarbazole

βgal – beta-galactosidase

BrdU – 5'-bromo-2'-deoxyuridine

cAMP – cyclin adenosine monophosphate

ChIP – chromatin immunoprecipitation

Ckd – cyclin-dependent kinase

CMV - cytomegalo virus (promoter)

DAB – 3,3'-diaminobenzidine-tetra-hydrochloride

DAG – diacylglycerol

E'#' – days of gestation

EDTA – ethylene diamine tetraacetate

EMSA – electromobility shift assay

GDP, GTP – guanidine diphosphate, guanidine triphosphate

GFP – green fluorescent protein

γINF – interferon gamma

GK – glucokinase, hexokinase IV

GLP-1 – glucagon-like peptide 1

GLUT2 – glucose transporter 2

GSIS – glucose-stimulated insulin secretion

HGF – hepatocyte growth factor

HIT – hamster insulinoma transformed cell line

HNF – hepatocyte nuclear factor

ICDc – cytosolic isocitrate dehydrogenase

IDDM – insulin-dependent diabetes mellitus

Il-1β – interleukin 1-beta

INS-1 – insulinona-derived beta cell line

K^+_{ATP} channel – ATP-dependent potassium channel

MODY – maturity onset diabetes of the young

MTS - (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium

NADH – nicotinamide adenine dinucleotide

NADPH – nicotinamide adenine dinucleotide phosphate

Ngn3 – neurogenin 3

NIDDM – non-insulin-dependent diabetes mellitus

PBE – proximal beta cell enhancer

PBS – phosphate buffed saline

PCNA – proliferating cell nuclear antigen

Pdx1 – pancreatic duodenal homeobox factor-1

PI-3/Akt – phosphatidylinositol 3-kinase, protein kinase B, Akt

RIA – radio-immuno assay

RT-PCR – reverse-transcriptase polymerase chain reaction

SCID mice – immunodeficient mouse strain

TCA – trichloroacetic acid

TCA cycle – tricarboxylic acid cycle, Krebs cycle

Tris – tris (hydroxymethyl) aminomethane

ZDF – Zucker diabetic fatty

CHAPTER ONE

INTRODUCTION

History and etiology of diabetes mellitus

The critical role of the hormone insulin in diabetes mellitus or 'sugar disease' was uncovered in 1920 by Frederick Banting and Charles Best (Banting 1924). This finding arose from their observation that administration of a pancreatic extract to diabetic dogs eliminated symptoms associated with the disease. As a result, administration of exogenous insulin was quickly adopted as a therapy for insulin deficiency, continuing to this day. Despite this important breakthrough, nearly a century ago, diabetes mellitus remains a major health concern in the United States.

Insulin dependent diabetes mellitus

Diabetes mellitus is caused by a number of metabolic derangements which center on deficiencies in insulin secretion and insulin action and can be further divided into two categories that arise from distinct etiologies (Association 2005). Type 1, or insulin-dependent diabetes mellitus (IDDM), results from the specific destruction of the insulin-secreting beta cells located in the Islets of Langerhans (Gepts 1965). The loss of beta cells is mediated through an auto-immune response which creates a condition of insulin deficiency (Atkinson and Maclaren 1994). The major function of insulin is to promote uptake of glucose into peripheral tissues. Therefore, in insulin deficient states glucose uptake is

impaired and elevated blood glucose levels persist. This hyperglycemic state eventually leads to ketoacidosis, and results in a myriad of health problems such as cardiovascular disease and renal failure.

Type 1 diabetes has traditionally been treated by insulin injection therapy to compensate for the loss of endogenous insulin production. This approach is not without shortcomings, since maintenance of euglycemia involves constant monitoring of blood glucose levels and careful administration of insulin (DCCTRG 1993). In addition, hypoglycemic episodes are common and can lead to a multitude of disabling symptoms and can be fatal if not treated appropriately (Fanelli *et al.* 2004).

The combination of automated islet isolation (Ricordi *et al.* 1988) and advances in use of immunosuppressive drugs has resulted recently in a dramatically improved success rate of human islet transplant studies (Shapiro *et al.* 2000). Despite these advances, the shortage of donor pancreases and the long-term side effects of the immunosuppressive drugs limit the utility of this approach for human diabetes therapy. Additionally, even with the improved methods, nearly 25% of transplant recipients fail to maintain independence from exogenous insulin and require additional transplantation (Ryan *et al.* 2002). In spite of these limitations, the recent work has provided new hope that beta cell replacement can become a bona fide therapy for type diabetes. Since the primary defect in type 1 diabetes is the loss of beta cell mass, it is vital to understand the genes and factors involved in beta cell growth and proliferation. Identification of these genes and the pathways that they regulate may lead to new strategies for development of expandable populations of beta-cells with well regulated insulin secretion that can be used for therapy of type 1 diabetes.

Non-insulin dependent diabetes mellitus

Type 2, or non-insulin dependent diabetes mellitus (NIDDM) results from the combination of insulin resistance and derangements in beta cell function. In response to increased demand on the beta cell, as commonly seen in obesity, there is a compensatory increase in beta cell mass and insulin output (Polonsky *et al.* 1988; Butler *et al.* 2003). If the demand on beta cells is not reduced, beta cell failure occurs, involving both a loss of beta cell function and a decrease in beta cell mass. One early indicator of decreased beta cell function is the loss of the first phase of glucose-stimulated insulin secretion (GSIS) (Mitrakou *et al.* 1992). Changes in gene expression also accompany the early defects in GSIS, as shown in the Zucker Diabetic Fatty (ZDF) rat, a rodent model of type 2 diabetes (Tokuyama *et al.* 1995). The dynamics of beta cell mass in type 2 diabetes was demonstrated by analysis of islets from both obese and lean subjects with either a non-diabetic or type 2 diabetic diagnosis (Butler *et al.* 2003). Beta cell mass increased with obesity in subjects with a non-diabetic background as a normal physiological response to the increased metabolic demand. However, islets from obese type 2 diabetic subjects showed a dramatic decrease in beta cell volume compared to non-diabetic obese subjects. A similar decrease also occurred in lean type 2 diabetic subjects compared to non-diabetic lean subjects. In both obese and lean diabetic subjects, the apparent decline in beta cell mass could be attributed to an increase in beta cell apoptosis. Eventually, beta cell mass decreases ~50% compared to non-diabetics accompanied by worsening of insulin resistance and progression to hyperglycemia (Rossetti 1996).

Early stages of type 2 diabetes can effectively be treated with diet and exercise, resulting in improved insulin sensitivity and a decreased demand on the beta cell. Remarkably, human subjects in the early stages of diabetes (exhibiting impaired glucose tolerance) can significantly lower their probability of progressing to full-blown type 2 diabetes simply by adopting changes in diet and lifestyle (Tuomilehto *et al.* 2001).

Several classes of drugs are used to treat type 2 diabetes (Larsen 2003; Lebovitz 2004). Sulfonylureas and meglitinides function to stimulate insulin secretion via modulation of the activity of ATP-sensitive K^+ channels (K_{ATP} channels) in the beta cell plasma membrane (Saloranta *et al.* 2002). Unfortunately, these drugs loose efficacy over time and similar to insulin therapy, cause hypoglycemia and weight gain (Kabadi and Kabadi 2003; Lebovitz 2004). Another therapeutic approach is to increase insulin sensitivity in peripheral tissues such as liver, muscle, and fat. This can be facilitated by the drug metformin and a class of compounds known as thiazolidinediones, which principally target the liver and muscle, respectively; both have been effective therapies in the management of type 2 diabetics (Knowler *et al.* 2002; Knowler *et al.* 2005). These drugs do not increase insulin levels, and therefore decrease the risk of hypoglycemia. However, side effects such as adverse gastrointestinal function and lactic acidosis have been associated with metformin therapy, whereas weight gain and hepatotoxicity are the most common side effects seen with the use of thiazolidinediones. Understanding the mechanisms behind the deteriorating beta cell function seen in type 2 diabetes will provide other avenues for intervention in disease progression.

In sum, both major forms of diabetes involve impairment in beta cell function and mass. Identification of the genes involved in beta cell growth, proliferation and function will be required in order to develop improved diabetes therapies.

Pancreatic beta cells and glucose-stimulated insulin secretion

Glucose-stimulated insulin secretion (GSIS)

The primary role of the pancreatic beta cell is to synthesize insulin and secret the hormone in response to changes in the levels of glucose and other metabolic signals (Grodsky *et al.* 1963). The direct relationship between extracellular glucose and the rate of glucose metabolism in beta cells was described in 1968 (Matschinsky and Ellerman 1968) and the tight relationship between the rate of glucose metabolism and insulin secretion was reported shortly thereafter (Malaisse *et al.* 1979b).

Insulin secretion in response to glucose is a biphasic event (Curry *et al.* 1968). The first phase comprises the initial acute release of insulin which begins 1-2 minutes after stimulation with high glucose and peaking in approximately 4 minutes, followed by a return to baseline levels. A second phase follows, characterized by a sustained period of insulin release. Figure 1-1 outlines the critical components of glucose-stimulated insulin secretion (reviewed in (Newgard and McGarry 1995)). Extracellular glucose is transported into the cell through the high affinity transporter GLUT2. After entry into the cell, glucose is phosphorylated by the enzyme glucokinase (GK, or hexokinase IV) and traverses glycolysis

to form pyruvate, which subsequently enters the TCA cycle as acetyl Co-A. The combination of glycolytic and mitochondrial metabolism results in an increase in ATP synthesis. An increase in the ATP to ADP ratio causes the closure of the K^+_{ATP} channel (Kir6.2/Sur1) resulting in membrane depolarization and triggers the opening of voltage-gated calcium channels (Misler *et al.* 1986). The influx of extracellular calcium and rise in intercellular free calcium triggers a cascade of signaling events that leads to the fusion of insulin-containing granules to the plasma membrane and insulin granule exocytosis. The cascade described above is referred to as K^+_{ATP} channel-dependent glucose stimulated insulin secretion and is primarily responsible for the first phase of insulin exocytosis.

In recent years, signaling events other than closure of K^+_{ATP} channels have been shown to be important components of GSIS, especially for the second phase of insulin release. Beta cells were shown to secrete insulin in response to glucose, even when closure of K^+_{ATP} channels was prevented by a combination of diazoxide and high K^+ conditions (Gembal *et al.* 1992; Sato *et al.* 1992; Taguchi *et al.* 1995). Therefore, signaling events other than glucose-induced changes in the ATP to ADP ratio play an important role in GSIS. Several mechanisms have been proposed to explain the K^+_{ATP} channel-independent signaling mechanism (reviewed in (Straub and Sharp 2002)). Such factors include malonyl Co-A and long-chain acyl CoA esters (Corkey *et al.* 1989; Prentki *et al.* 1992), glutamate (Maechler and Wollheim 1999), and changes in various nucleotides such as ADP, ATP, GDP, GTP, and NADPH (Detimary *et al.* 1996; Detimary *et al.* 1998; Ivarsson *et al.* 2005; Ronnebaum *et al.* 2006). Although the relative contributions of each of these potential second messengers

remain to be fully clarified, recent evidence suggests that non-oxidative metabolism of pyruvate in the mitochondria and the generation of NADPH are key events in GSIS.

Pyruvate can enter the TCA cycle either as acetyl-CoA (catalyzed by pyruvate dehydrogenase), or as oxaloacetate (catalyzed by pyruvate carboxlyase). In the latter case, pyruvate can be regenerated through a variety of pathways, both mitochondrial and cytosolic, through a process known generally as pyruvate cycling (Lu *et al.* 2002). A common byproduct of recycling oxaloacetate to pyruvate is the generation of the reduced nucleotides NADH and NADPH, which were originally shown to be important in GSIS through their coupling with calcium transport (Malaisse *et al.* 1979a). Similar to the increase in the ratio of ATP to ADP, the ratio of NADPH to NADP increases in parallel with increasing extracellular glucose concentrations and GSIS (Ivarsson *et al.* 2005; Ronnebaum *et al.* 2006). In addition, NADPH has been shown to directly stimulate exocytosis of insulin granules (Ivarsson *et al.* 2005). Further evidence linking NADPH and pyruvate cycling to GSIS has been demonstrated in studies involving siRNA-mediated silencing of cytosolic isocitrate dehydrogenase (ICDc). ICDc catalyzes the conversion of isocitrate to alpha-ketoglutarate, in an NADPH-generating reaction. Silencing of ICDc reduces the NADPH to NADP ratio, lowers pyruvate cycling, and potently impairs GSIS in both cell lines at primary rat islets.

In addition to the metabolic effects described above, GSIS can be further modulated through secondary messengers such as diacylglycerol (DAG) and cyclic adenosine monophosphate (cAMP), which increase in concentration within beta cells via classical G-protein coupled signaling, such as the G_s-coupled GLP-1-receptor and the G_q- and G_{11}-coupled acetylcholine receptor (reviewed in (Laychock 1990; Gilon and Henquin 2001; Holz

2004)). As illustrated in Figure 1-1, in the presence of stimulatory glucose concentrations, these signaling pathways enhance the release of intercellular stores of calcium and contribute to insulin exocytosis. Thus, glucose serves as a primary regulator of insulin secretion, whereas other agents such as free fatty acids (Stein *et al.* 1996; Koyama *et al.* 1997), GLP-1 (Montrose-Rafizadeh *et al.* 1994), and acetylchonline (Nakano *et al.* 2002) tend to potentiate the glucose response.

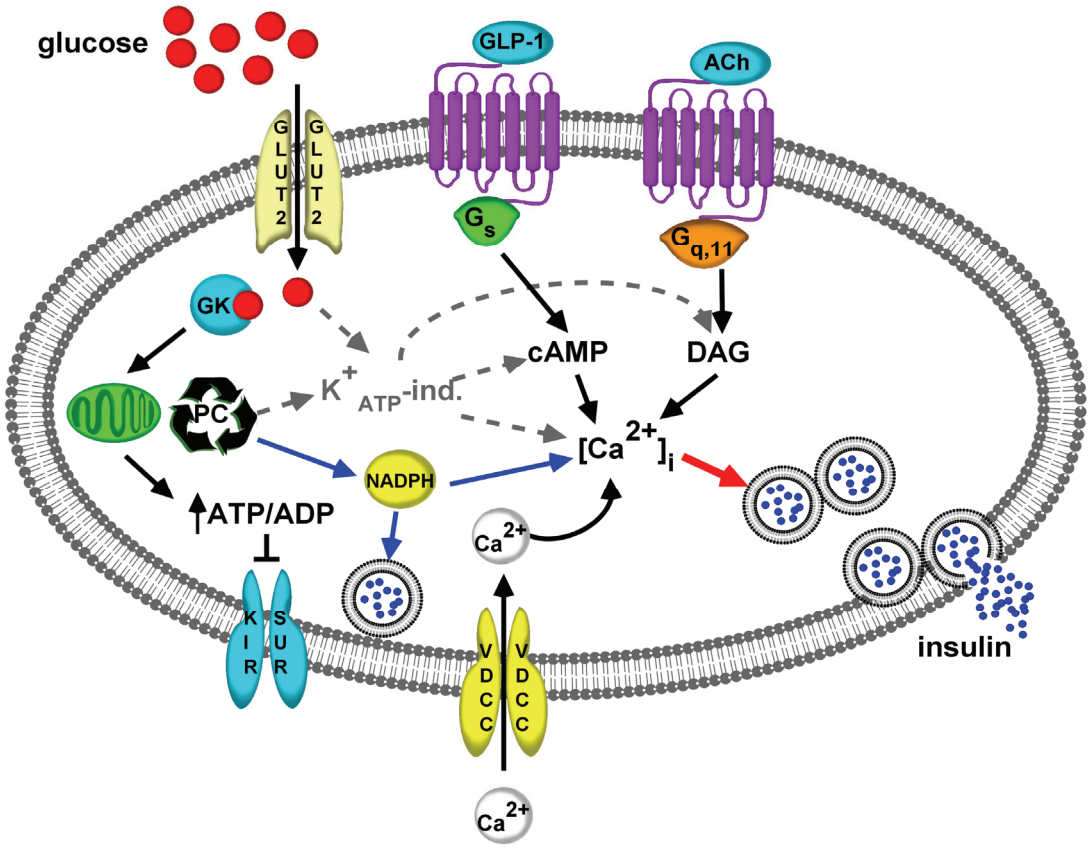


Figure 1-1. Glucose-stimulated insulin secretion.
This figure highlights the key factors and mechanisms involved in GSIS. **Glucose** is taken up via the high affinity glucose transporter (**GLUT2**) where it is subsequently phosphorylated by the enzyme glucokinase (**GK**) the rate-limiting step of beta cell glucose metabolism. Increased glucose metabolism results in an increase in the **ATP/ADP** ratio. This results in the closure of K^+_{ATP} channels, comprised of Kir6.2 and Sur1 (**KIR, SUR**), causing the plasma membrane to depolarize. Voltage dependent calcium channels (**VDCC**) open in response to the membrane depolarization, allowing an influx of calcium. The increase in intracellular levels of calcium triggers exocytosis of **insulin** granules Pyruvate cycling (**PC**) contributes to GSIS through generation of **NADPH** which can affect exocytosis as well as calcium transport. Further modulation of GSIS can occur through ligand-dependent effects, as seen with glucagon-like-peptide 1 (**GLP-1**) and acetylcholine (**ACh**), via G-protein coupled receptor signaling. The secondary messengers **cAMP** and **DAG** are downstream signals of the $\mathbf{G_s}$-coupled glucagon-like-peptide 1 receptor (GLP-1-R) and the $\mathbf{G_q}$- or $\mathbf{G_{11}}$-coupled acetylcholine receptor (AChR).

Beta cell transcription factors

Transcription factors are a class of proteins that function to regulate gene expression. Several transcription factors have been implicated in beta cell differentiation and function, and expression of these factors can be restricted to beta cells, or to islets more generally, and neuronal cells. Maturity onset diabetes of the young (MODY) is a monogenetic form of diabetes mellitus that occurs as a result of defects in beta cell function, a condition usually seen in later stages of type 2 diabetes. With the exception of MODY2, which is due to mutations in an important glucose metabolizing enzyme, glucokinase, MODY1 and MODY3-6 are due to deficiencies in beta cell transcription factors, including HNF-4α, HNF-1α, Pdx1, HNF-1β, or NeuroD (Hansen and Pedersen 2005). Hence, mutations in a single transcription factor gene can have deleterious effects on beta cell function and glucose homeostasis and can cause human disease.

The function of a transcription factor is typically mediated by its ability to bind to a DNA target in the promoter region of a gene. This ability to recognize and bind to a target sequence can be dependent on multiple factors: 1) nuclear localization of the transcription factor (Hagman *et al.* 2005); 2) post-translational modifications of the transcription factor, such as phosphorylation of NeuroD1 and Pdx1 (Khoo *et al.* 2003); and 3) access to the target DNA mediated by competing factors or chromatin structure (Chakrabarti *et al.* 2003; Francis *et al.* 2005). The net effect on transcription can either be positive or negative based on the ability of the transcription factor to recruit either co-activators or co-repressors. Although most factors are commonly characterized as activators or repressors, there are a few examples of proteins with both activities.

The role of transcription factors in beta cell biology has been most intensively investigated in the context of islet development. Histological analysis of embryos at different stages in development has revealed a temporal pattern of transcription factor expression. Knockout mice generated through targeted disruption of islet transcription factors have been used to better understand the hierarchy of these factors and the functional consequence of the knockout on beta cell differentiation. The transcription factor Pdx1 is critical in the formation of the pancreas and represents one of the earliest pancreatic transcription factors necessary for both exocrine and endocrine pancreatic development. Glucagon producing alpha cells and insulin producing beta cells appear to arise from a common precursor cell that expresses the transcription factor neurogenin3 (Schwitzgebel *et al.* 2000) and subsequently Beta2 (Huang *et al.* 2000). The subsequent expression of various transcription factors results in the differentiation of alpha and beta cells, as summarized in Figure 1-2.

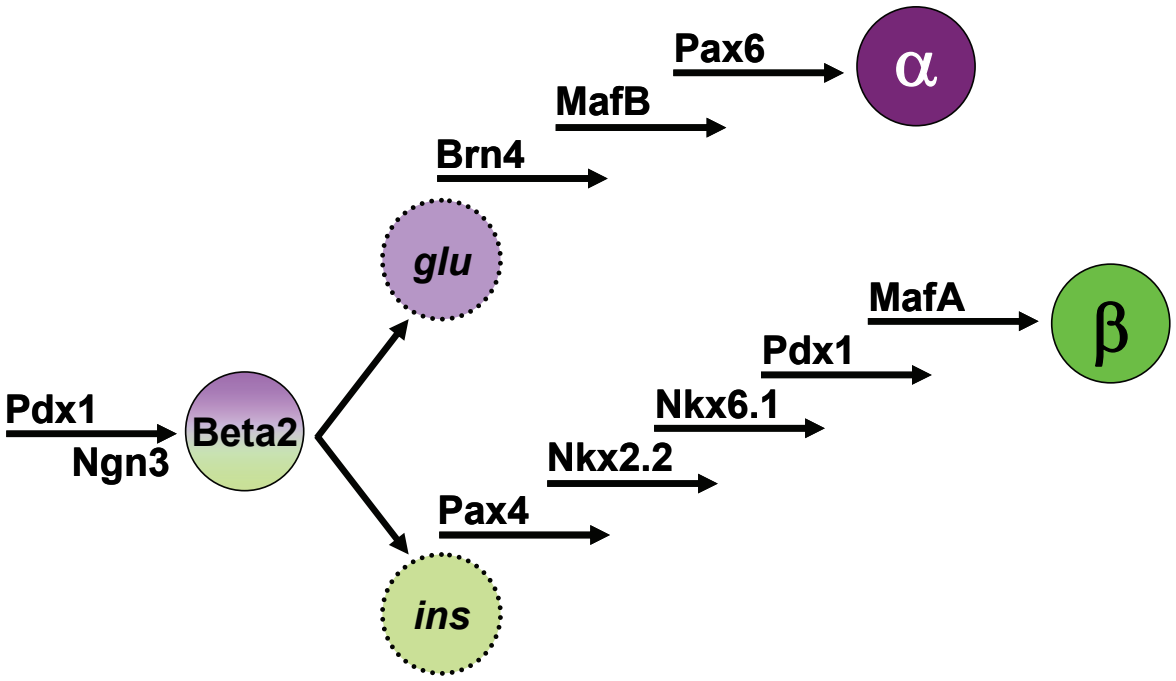


Figure 1-2. Pattern of alpha and beta cell transcription factor expression in differentiation.
During islet differentiation, expression of various transcription factors is required for the proper development of mature alpha and beta cells. The hierarchy of expression has been determined via immunocytochemical analysis of these various factors throughout differentiation as well as analysis of transgenic mice (adapted from (Habener *et al.* 2005)).

In some instances, knock-out mice have been used to gain insight into the role of individual transcription factors in control of beta cell function. This is dependent on the knockout being non-lethal. Alternatively, animals with only one disrupted allele, or ideally a conditional knockout, can be studied. Insights gained from knock-out mice must be viewed with caution for multiple reasons: 1) there may be compensatory responses that occur upon loss of a transcription factor during development that can mask a loss-of-function effect seen in mature beta cells; 2) the effect of losing expression during development may have different functional consequences than the loss of expression (or a transient change in

expression) in a terminally differentiated beta cell; 3) islet structure is crucial to beta cell function; therefore, derangements in beta cell architecture caused by the genetic manipulation may lead to erroneous conclusions about the role of a transcription factor in a mature beta cell. For example, disruption of the transcription factor MafA results in mice with dramatically impaired GSIS independent of a change in insulin content. However, islet morphology is grossly disorganized in these mice, with a large increase in the number of glucagon positive cells scattered throughout the islet, as opposed to their normal distribution on the periphery of the islet (Zhang *et al.* 2005). Derangements in islet architecture have also been observed in other animals such as the GLUT2 and glucokinase knockout mice (Terauchi *et al.* 1995; Guillam *et al.* 1997) and in mice with a beta cell-specific knockout of Pdx1 (Ahlgren *et al.* 1998). Due to the complications described, one of the founding tenants of this dissertation is that complete understanding of the function of specific beta cell transcription factors requires analysis of function in mature beta cells in conjunction with studies during islet development.

The homeodomain transcription factor, Nkx6.1

Discovery of Nkx6.1 and initial characterization

Initially described in *Drosophila* in 1978 by Lewis *et al.* (Lewis 1978) homeobox genes are found in all eukaryotes and are involved in differentiation and development of many organs and tissues such as the central nervous system, mammary gland, eye, and

pancreas (Lewis 2000; Lupo *et al.* 2000; Ashizawa *et al.* 2004; Akin and Nazarali 2005). Additionally, homeobox genes have been implicated in mature cell function as well as oncogenesis (Cillo *et al.* 2001; Del Bene and Wittbrodt 2005). The defining characteristic of homeobox gene products is the homeodomain, a 60 residue polypeptide that functions as a DNA recognition motif (McGinnis *et al.* 1984a; McGinnis *et al.* 1984b).

The transcription factor Nkx6.1, described by Rudnick *et al.* in 1994, was isolated from a screen for homeodomain-containing transcripts in a hamster insulinoma cDNA library obtained from HIT cells (Rudnick *et al.* 1994). Nkx6.1 expression was detected in multiple beta cell lines and subsequently found to be expressed only in insulin-positive cells in rat pancreatic sections as well as in dispersed newborn rat islet cells (Rudnick *et al.* 1994; Jensen *et al.* 1996). The beta cell-specific expression of Nkx6.1 suggested that this transcription factor may play an important role in defining beta cell-specific characteristics such as insulin expression and secretion (Jensen *et al.* 1996).

Evidence of a role for Nkx6.1 expression in beta cell differentiation was first provided via immunocytochemical analysis of developing mouse pancreas (Oster *et al.* 1998; Sander *et al.* 2000). Nkx6.1 expression was first detected at low levels at embryonic day 10.5 (days of gestation, or E10.5) in epithelial cells of the dorsal and ventral buds of the developing pancreas. Significant numbers of insulin-positive cells can first observed at E13.5, marking the onset of the "secondary transition", the second phase of endocrine pancreas differentiation (Pictet 1972). At this point, many insulin-positive cells express Nkx6.1; conversely, Nkx6.1 cannot be detected in any glucagon-positive cells. Approaching E15.5, two distinct pools of Nkx6.1-expressing cells can be seen: insulin-positive/Pdx1-

positive and insulin-negative/neurogenin3-positive cells. Interestingly, only the latter pool expressed proliferating cell nuclear antigen (PCNA), an indicator of proliferating cells (Sander *et al.* 2000). At E15.5 there is a dramatic expansion of insulin-expressing cells, the majority of which (>95%) co-express Nkx6.1 (Oster *et al.* 1998; Sander *et al.* 2000). Subsequently, all Nkx6.1-expressing cells are insulin-positive, a characteristic of beta cells maintained through the end of gestation and in mature beta cells.

Nkx6.1 knockout animals – a role for Nkx6.1 in beta cell differentiation and proliferation

Using targeted gene disruption, mice with two non-functional alleles for the gene Nkx6.1 ($Nkx6.1^{-/-}$) were generated by Sander and Sussel *et al.* (Sander *et al.* 2000). The loss of Nkx6.1 expression resulted in lethality days after birth, due to severe hypoglycemia. To understand how the disruption in Nkx6.1 expression could lead to such a severe phenotype, as well as understand its role in beta cell differentiation, histological analysis of developing endocrine pancreas from $Nkx6.1^{-/-}$ mice was carried out at various time points in embryogenesis.

Analysis of $Nkx6.1^{-/-}$ mice revealed a pronounced defect in the secondary transition with little to no expansion of beta cell mass. At time points after the start of the secondary transition (>E14.5) there was a >90% loss in the number of insulin-positive cells. One day prior to birth, at E18.5, insulin content in $Nkx6.1^{-/-}$ pancreases was reduced by 98% compared to the levels found in wild-type pancreases (Sander *et al.* 2000). Strikingly, despite the profound effect on beta cell differentiation, there was no effect on the other islet cell types (alpha, delta, and poly-peptide) determined by histological analysis. Islets in

Nkx6.1$^{-/-}$ mice were smaller than in wild-type animals, but the remaining islet cell types were retained in islet-like clusters (Sander *et al.* 2000). The beta cell-specific effect of Nkx6.1 on islet differentiation was further confirmed through real-time PCR analysis of insulin and glucagon mRNA (Henseleit *et al.* 2005). At E18.5, pancreases from Nkx6.1$^{-/-}$ mice had only 6% of insulin transcript compared to wild-type controls. Conversely, there was no effect on glucagon transcript levels in Nkx6.1$^{-/-}$ mice. Therefore, Nkx6.1 is critical to the development of normal beta cell mass and does not affect the differentiation of other islet cell types.

To understand the role of Nkx6.1 in regulating the *proliferation* of developing beta cells, BrdU labeling experiments were performed on Nkx6.1$^{-/-}$ and wild type animals. BrdU is a nucleotide analog (bromo-deoxyuridine) that is incorporated into genomic DNA during mitosis, detected through immunohistochemistry, and can be used as a quantitative estimation of actively proliferating cells. During the secondary transition (E14.5-E16.5) there was a 10-fold decrease in insulin and BrdU co-positive cells, comparing wild-type to Nkx6.1$^{-/-}$ animals (Sander *et al.* 2000). The rate of proliferation of pre-existing beta cells during this period is very low, suggesting that beta cells arise from Ngn3-positive, insulin-negative expressing precursors (Herrera 2000), and that expansion of this pool may be dependent on Nkx6.1 expression. Prior to birth and at the beginning of postnatal life, there is a third wave of beta cell expansion from preexisting beta cells. The rate of proliferation in the few remaining beta cells in the Nkx6.1$^{-/-}$ mice in this phase is equal to the rate seen in wild-type mice (Sander *et al.* 2000). Therefore, the authors of this study concluded that Nkx6.1 acts late in pancreatic development and is necessary for proliferation and differentiation of Ngn3-positive, insulin-negative precursors into functional beta cells.

However, this study did not assess the role of Nkx6.1 in maintaining differentiated function of mature beta cells, as there were inadequate numbers of beta cells available from these animals to allow for functional studies.

Functional analysis of Nkx6.1

Despite the critical role of Nkx6.1 in beta cell development, target genes of Nkx6.1 regulation have been poorly described. Initially the homeodomain of Nkx6.1 was shown to bind to TAAT- or ATTA-containing promoter elements (Jorgensen *et al.* 1999; Mirmira *et al.* 2000). This core element has also been identified as a DNA-binding element for other mammalian homeobox proteins, such as the transcriptional repressor Gtx (Awatramani *et al.* 2000), the LIM transcription factor LHX3 (Yaden *et al.* 2005), the mammalian family of Hox proteins (Catron *et al.* 1993) and Pdx1 (Peers *et al.* 1994). Further specificity for Nkx6.1 binding to DNA is derived from flanking nucleotides, which extends the core motif to CATTTAATTACCCT (Mirmira *et al.* 2000). Mutations within this core ablate binding of the Nkx6.1 homeodomain, and mutations to flanking regions can decrease homeodomain binding up to 75% (Mirmira *et al.* 2000).

The homeodomain of Nkx6.1 (residues 229-305), when fused to the VP16 activation domain, was shown to activate reporter constructs containing multiple copies of the TAAT-containing consensus sequence (Jorgensen *et al.* 1999). However, using the same assay system, full length Nkx6.1 was shown to be a potent transcriptional repressor (Mirmira *et al.* 2000), demonstrating that the homeodomain is necessary for DNA recognition but other domains of Nkx6.1 dictate the effects on transcription. Mutational analysis of Nkx6.1

revealed that the COOH-terminal region flanking the homeodomain (residues 306-364) acts as a binding interference domain (BID), the presence of which decreases the binding affinity of Nkx6.1 to its DNA target, and subsequently, decreases the level of transcriptional repression. The ability of Nkx6.1 to repress transcription requires an N-terminal domain (residues 91-268) which includes the NK decapeptide (Figure 1-3).

Figure 1-3. The functional domains of Nkx6.1.
Nkx6.1 is a 364 residue polypeptide with 4 distinct domains: **Repressor Domain** (91 – 268), **Homeodomain** (229 – 305), **Activation Domain** (306-322), and the **Binding Interference Domain** (BID 306 – 364).

Studies from the Mirmira and German labs have identified the insulin gene as a potential target gene regulated by Nkx6.1. Multiple TAAT-containing elements are conserved in mouse, rat, and human insulin promoters (Taylor *et al.* 2005). Nkx6.1 was found to bind to a TAAT-containing rat insulin promoter fragment *in vitro* (Mirmira *et al.* 2000). Moreover, Nkx6.1 overexpression in both NIH3T3 and mouse beta cell lines resulted in a 5- and 15-fold decrease in activity of an insulin promoter/reporter gene, respectively, compared to control promoters (Mirmira *et al.* 2000). More recently, an *in vivo* interaction between Nkx6.1 and the insulin promoter was confirmed via chromatin immunoprecipitation (ChIP) assays (Iype *et al.* 2004). Interestingly, there are no reports of the effects of Nkx6.1 to modulate endogenous insulin mRNA levels in beta cell lines or in primary rat islets.

Therefore, despite physical interaction between Nkx6.1 and the insulin promoter, there is no evidence that manipulation of Nkx6.1 levels modulates insulin transcript levels.

Moreover, the idea that Nkx6.1 suppresses insulin expression is counter to the analysis of Nkx6.1$^{-/-}$ mice, which were found to be severely deficient in insulin expression. Islets from Nkx6.1$^{-/-}$ mice also lack expression of the insulin activator, MafA, another gene expressed late in beta cell differentiation (Matsuoka *et al.* 2004). Hence, the lack of insulin expression in Nkx6.1$^{-/-}$ mice may be due to the failure to activate MafA expression and not to a direct effect of Nkx6.1 on the insulin promoter. In addition, Nkx6.1 has been proposed to have different roles in differentiating beta cells versus mature beta cells (Schisler *et al.* 2005; Taylor *et al.* 2005) such as functioning to suppress glucagon expression (see below).

Although initially characterized as a transcriptional repressor, Nkx6.1 has recently been reported to function as a potent transcriptional activator and can stimulate transcription of its own gene, unveiling Nkx6.1 as a bi-functional transcription factor (Iype *et al.* 2004). A G/C-rich region of the Nkx6.1 promoter (-157 to -30, relative to a cluster of transcriptional start sites) was sufficient to confer beta cell-specific expression of a reporter gene and represents a proximal beta cell enhancer (PBE) element. Iype *et al.* showed that the core element ATTT was necessary for Nkx6.1-mediated transcriptional activation of reporter genes and for the *in vitro* interaction between Nkx6.1 and the PBE. Furthermore, chromatin immunoprecipitation analysis demonstrated that Nkx6.1 interacts with the PBE *in vivo*. A portion of the COOH-terminus (residues 306-338) was determined to be necessary and sufficient for Nkx6.1-mediated activation and was identified as the activation domain of Nkx6.1. A model of the bi-functional activities of Nkx6.1 is shown in Figure 1-4.

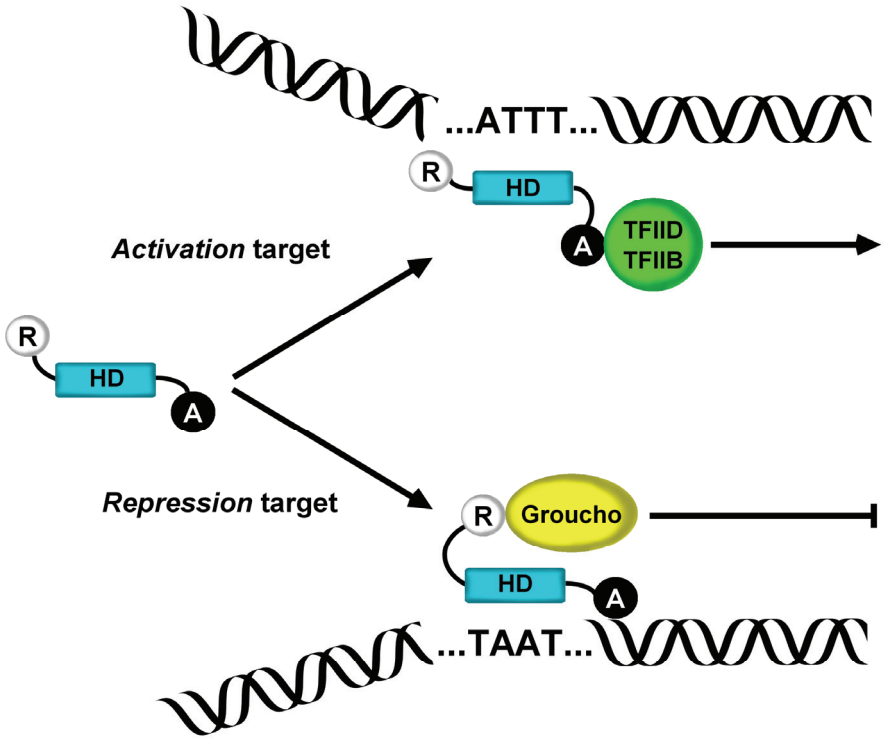


Figure 1-4. Model of the bi-functional activities of Nkx6.1.
Nkx6.1 contains an N-terminal repressor domain (**R**) and C-terminal activation domain (**A**). Nkx6.1 promotes activation of target genes that contain the DNA target sequence ATTT located in G/C-rich promoter regions. Subsequent recruitment of transcriptional co-activators such as TFIID and TFIIB promote transcriptional activation. Nkx6.1 can also bind to TAAT elements which results in recruitment of transcriptional repressors such as Groucho. As modeled here, changes in the tertiary structure of Nkx6.1 upon target recognition may promote either activation or repression. Not shown here are potential interactions of Nkx6.1 with other co-factors, or post-translational modification of Nkx6.1 – other mechanisms that may influence Nkx6.1 transcriptional activity.

Based on the studies described above, Nkx6.1 has the ability to directly activate or repress target genes. Other transcription factors with bi-functional activities have been described, including the glucocorticoid receptor (Sakai *et al.* 1988), YY1 (Bushmeyer *et al.* 1995), Sp3 (Majello *et al.* 1997), and Ets-1 (Petersen *et al.* 1995). The activity of these transcription factors and of Nkx6.1 appear to be dependent on the context of the DNA sequence in the promoter regions of target genes (Figure 1-4). In addition, activity of bi-functional transcription factors can be modulated via protein-protein interactions (Lee *et al.* 1993; Shrivastava *et al.* 1993) and post-translational modifications (Pufall *et al.* 2005). Therefore, yet to be defined modulators and co-factors of Nkx6.1 may play a critical role in dictating the function of this transcription factor.

Regulation of mature beta cell mass

Dynamic changes in beta cell mass are seen in the pathologies of both type 1 and type 2 diabetes; however, little is known about the mechanisms that control these events. As shown in Figure 1-5, a change in mature beta cell mass can occur via some combination of four mechanisms: 1) differentiation of stem cells or beta cell precursors, *neogenesis*; 2) proliferation from preexisting beta cells, *hyperplasia*; 3) increase in beta cell volume, *hypertrophy*; and 4) changes in beta cell death, *apoptosis* (Swenne 1992; Rane and Reddy 2000). The relative contribution of these mechanisms in normal and disease physiology is

incompletely understood. Therefore, elucidation of fundamental mechanisms involved in control of beta cell mass is of critical importance.

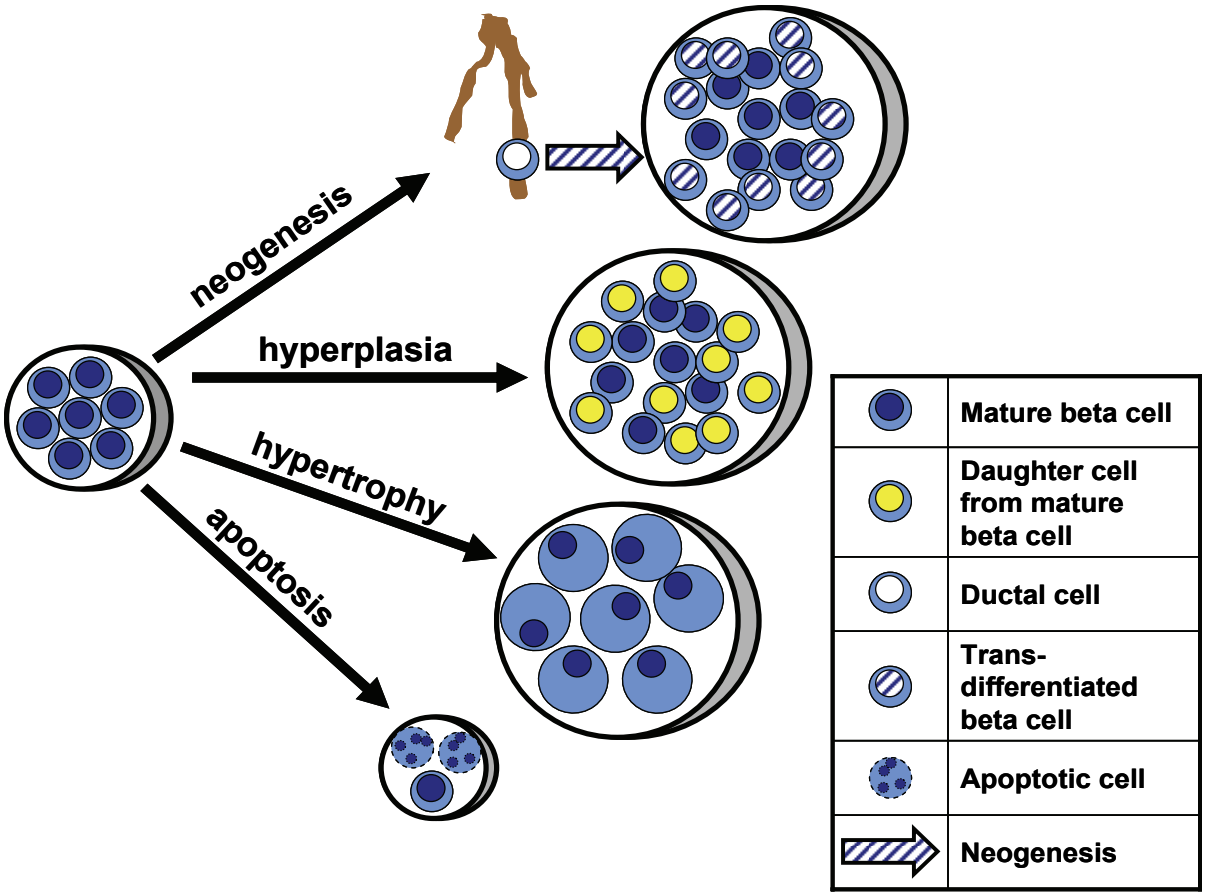


Figure 1-5. Mechanisms of changes in beta cell mass.
Islet cross-section diagrams demonstrate how changes in beta cell mass can occur through multiple mechanisms. In addition, islet mass can change due to a combination of these events, such as combined hyperplasia and hypertrophy in pregnancy.

The initial identification of potential beta cell precursors in the pancreatic ducts (Bonner-Weir *et al.* 1993) and the observation of duct- to islet-cell differentiation (Wang *et al.* 1995) has focused attention on better defining these cells and the role they have in maintaining beta cell mass. In contrast, a recent study using a transgenic labeling approach suggests that new beta cell mass derives only from preexisting beta cells (Dor *et al.* 2004).

All of these studies utilized surgical injury models (partial pancreatectomy or duct ligation) to induce beta cell proliferation (Pearson *et al.* 1977; Wang *et al.* 1995) and beta cell dysfunction (Leahy *et al.* 1988). The degree of injury appears to impact the potential contribution of neogenesis versus hyperplasia (reviewed in (Bouwens and Rooman 2005), which further confounds the role of neogenesis in beta cell proliferation.

Mature beta cell hypertrophy and hyperplasia has been observed in animal models of obesity, type 2 diabetes, and in pregnancy. For example, in two mouse models of diabetes, the ob/ob and db/db mouse, islet hyperplasia accompanies the obese diabetic phenotype (Gapp *et al.* 1983; Bock *et al.* 2003). The large increase in islet mass seen in the Zucker diabetic fatty rat has also been attributed to beta cell hyperplasia (Milburn *et al.* 1995; Hirose *et al.* 1996). In some instances, an increase in beta cell mass is a combination of both hyperplasia and hypertrophy. Such is the case in pregnancy, where beta cells adapt to an increase in body weight (Van Assche *et al.* 1980). It should be noted that contributions of neogenesis were not ruled out in the animal studies described here; however, the conclusions made in these studies attribute the increase in beta cell numbers to hyperplasia. Hence, beta cell hypertrophy and hyperplasia are commonly described as the most relevant mechanisms of increasing beta cell mass, but analysis has been incomplete in many instances.

A common characteristic of type 1 and type 2 diabetes is a decrease in beta cell mass (reviewed in (Donath and Halban 2004)) emphasizing the importance of balancing beta cell proliferation, apoptosis, and function. For example, in type 1 diabetes, immune-mediated apoptosis results in a near total loss in functional beta cell mass. The interplay between beta cell proliferation (whether through hyperplasia or neogenesis) and apoptosis in exemplified

in the study of the sand rat, a model of type 2 diabetes. This animal is naturally insulin resistant due to its calorie-restricted diet. When maintained on a high calorie diet, beta cell hyperplasia is insufficient to compensate for the increased demand placed on the beta cells. In addition, beta cell apoptosis increases, thus resulting in a functional decrease in beta cell mass leading to diabetes.

In sum, the various animal models discussed in this section demonstrate the dynamics of beta cell mass and how perturbations in any of the 4 mechanisms that influence beta cell mass can have pathological results. Understanding the factors that influence these changes may identify new points of intervention in both type 1 and type 2 diabetes.

Cell cycle control and beta cell proliferation

As in all eukaryotic cells, the cell cycle in adult beta cells consists of 4 distinct phases (G_1, S, G_2, and M). There is also a G_0 phase, which describes non-dividing quiescent cells. This can occurs at a point in G_1 before a cell commits to replication, a point known as the restriction point. A quiescent cell has one of three fates: 1) upon proper stimulation, quiescence can be reversed and the cell can enter the cell cycle; 2) the cell commits to an irreversible post-mitotic state; or 3) the cell undergoes apoptosis. Growth factor stimulation is only required for cells up to the restriction point, at which point cells are committed to replication (Rane and Reddy 2000). Beta cells have been shown to be responsive to a range of mitogenic stimuli, such as the gut hormones GLP-1, GIP and FSH.

Progression through the cell cycle is regulated by a family of proto-oncoproteins know as the cyclins and cyclin-dependent kinases (cdk's). The activation of various cyclin complexes and their transport into the nucleus results in the phosphorylation of various substrates, the result of which initiates a cascade of downstream events necessary for progression though the cell cycle.

Initial studies of mature beta cell proliferation using thymidine incorporation revealed that the majority of beta cells (97%) are predominantly in G_0 and are not actively proliferating. Thus only 3% of beta cells are actively progressing through the cell cycle, a process that takes approximately 15 h (Swenne 1983). More recent analysis of beta cell proliferation using the immunohistological detection of BrdU incorporation, found that only a small percentage of beta cells were proliferating and that this percentage decreased with age, from 0.99% in young rats, to 0.14% in older adult rats (Montanya *et al.* 2000). Therefore, the mitotic capacity of beta cells in primary islets is remarkably low. Factors that regulate cell cycle-related genes in primary beta cells are not well defined. The reduced proliferative capacity of beta cells cannot be attributed to a single gene, and due to the complexity of cell cycle progression, multiple factors are most likely involved in initiating and maintaining beta cell replication.

In a comparative analysis of mRNA levels in multiple tissues, it was found that beta cells expressed relatively low levels of the mitotic cyclin B1 and p34Cdc2, whereas there was little difference in other cell cycle factors, such as pRB, p53, and cyclin D1 (Mares and Welsh 1993). Interestingly, cyclin B1 and p34Cdc2 form a complex known as the M phase or maturation promoting factor (MPF) which triggers mitosis and is required for normal cell

cycle progression (Mares and Welsh 1993). The authors conclude that low levels of these two factors could explain the low proliferative capacity of mature beta cells, but no follow up studies were ever performed.

Increasing beta cell proliferation

An increase in beta cell proliferation can occur either via an increase in the rate of progression through the cell cycle or an increase in the number of proliferating beta cells. In studies of glucose-induced proliferation in mature beta cells it was found that the rate of cell cycle progression was not changed with an increase in glucose concentration; rather, the increase in proliferation was due to an increase in the number of replicating cells (Swenne 1982). It appears that mature islets have multiple pools of beta cells with a varying capacity to proliferate and respond to growth stimuli.

Further studies of the mechanisms involved in regulation of beta cell replication are important for a variety of reasons: 1) to understand the mechanisms behind normal beta cell proliferation and how these mechanisms change in response to different stimuli such as diet and pharmacological interventions; 2) to potentially curb the auto-immune destruction seen in type 1 diabetes or promote expansion of the remaining beta cell mass; 3) preventing the loss of functional beta cell mass seen in type 2 diabetes; 4) expansion of beta cells *ex vivo* for use in islet transplantation; and 5) procurement of primary beta cell lines, especially human lines, that would serve as an invaluable tool in researching beta cell biology.

Transfection of various oncogenes, such as the tyrosine kinase v-src, ras, and myc into primary beta cells has been shown to increase beta cell proliferation up to 4-fold (Welsh *et al.* 1988; de la Tour *et al.* 2001). Additionally, stimulation of beta cell growth has been reported in response to cell matrix proteins and hepatocyte growth factor (HGF) (Hayek *et al.* 1989; Hayek *et al.* 1995; Beattie *et al.* 2002). However, stimulating beta cell proliferation through tyrosine kinases such as FRK/RAK (also known as GTK) results in an increase in beta cell sensitivity to cytokine-induced killing and other toxins that are relevant to autoimmune destruction of beta cells in type 1 diabetes (Anneren and Welsh 2001; Anneren 2002; Welsh *et al.* 2004).

Transgeneic mice overexpressing c-myc specifically in beta cells exhibited increased beta cell proliferation, but this was accompanied by an even greater increase in apoptosis and a decrease in insulin gene transcription, resulting in the development of overt diabetes (Laybutt *et al.* 2002). Islets overexpressing c-myc also had significantly larger cell volumes. Interestingly, mRNA levels of the transcription factors Pdx1 and Nkx6.1 in islets from c-myc transgenic animals were similar to levels in control mice, but Nkx6.1 protein had abnormal cytoplasmic localization versus the strong nuclear localization seen in control mice (Laybutt *et al.* 2002).

Decreases in insulin content are observed when primary beta cells are grown in monolayers (Halvorsen *et al.* 2000) reemphasizing the importance of islet structure and cell-cell contact in maintaining the primary beta cell phenotype. Subsequently, islet hormone expression was restored when expanded monolayers were removed from extracellular matrix and re-aggregated (Beattie *et al.* 1996). The removal from extracellular matrix, however,

increased apoptosis and necrosis (Beattie *et al.* 2000). Other attempts at stimulating beta cell proliferation have resulted in insulinoma formation (tumorgenic beta cells), as seen with the overexpression of the SV40 large T antigen (Hanahan 1985).

The foregoing summary serves to highlight that the general experience has been that stimulation of beta cell proliferation often results in a loss of differentiated function, a net decrease in beta cell mass, or tumorgenesis. There have been two cases where stimulation of beta cell proliferation has not had a detrimental effect on beta cell function. The laboratory of Andrew Stewart has published a series of reports on the effect of overexpressing hepatocyte growth factor (HGF) in islet beta cells. Analysis of islets from transgenic mice overexpressing hepatocyte growth factor (HGF) under control of the insulin promoter showed an increase in beta cell proliferation, beta cell mass, insulin content and glucose responsiveness (Garcia-Ocana *et al.* 2000; Garcia-Ocana *et al.* 2001).

Further study of HGF-induced effects in primary beta cells implicated the phosphatidylinositol 3-kinase/Akt (PI-3/Akt) intracellular-signaling pathway in mediating the proliferative effects (Garcia-Ocana *et al.* 2003). Furthermore, *ex vivo* manipulation of islets by adenovirus-mediated overexpression of HGF increased the function of islets after transplantation into diabetic mice (Garcia-Ocana *et al.* 2003). PI-3/Akt signaling inactivates forkhead transcription factors resulting in an increase in cyclin D1 and D2 activity, triggering the exit of G_0 (quiescence) and entry into the cell cycle, as demonstrated in a variety of cell types (Medema *et al.* 2000; Kops *et al.* 2002; Alvarez *et al.* 2003). Therefore the increase in PI-3/Akt signaling through HGF-overexpression mediates beta cell proliferation in part through cyclin D activation. A subsequently study that demonstrated overexpression of

cyclin D1 and its molecular partner, Cdk4, in rat and human primary islets also stimulated proliferation while maintaining insulin secretion, similar to the effects of HGF-overexpression (Cozar-Castellano *et al.* 2004).

An important role for Cdk4 in islet mass is evident in the analysis of Cdk4 knockout mice, which resulted in a large reduction in islet mass, shown to be beta cell-specific effect, and these animals exhibited hyperglycemia, in part due to a 90% reduction in insulin levels (Rane *et al.* 1999). Conversely, transgenic overexpression of a dominant positive Cdk4 ($Cdk4^{R24C/R24C}$) resulted in beta cell hyperplasia (Rane *et al.* 1999). This prompted several follow up studies including functional analysis of islets from $Cdk4^{R24C/R24C}$ mice which were shown to maintain glucose-stimulated insulin secretion in the face of hyperplasia (Marzo *et al.* 2004). Lentiviral-mediated gene transfer of Cdk4 into human islets also resulted in an increase in beta cell proliferation (Marzo *et al.* 2004). Moreover, it has been shown that transgenic overexpression of a dominant positive form of Cdk4 under control of the insulin promoter results in a large increase in functional beta cell mass, with no indications of insulinoma formation or a loss in beta cell function (Hino *et al.* 2004). The primary drawback to Cdk4 modulation is its lack of tissue-specific expression. Other defects such as a lack of growth and infertility seen in $Cdk4^{-/-}$ mice as well as increased proliferation of other cell types such as Leydig cells in the $Cdk4^{R24C/R24C}$ mice (Rane *et al.* 1999) demonstrate that targeting Cdk4 may have deleterious non-beta cell effects. In addition, the analysis of the cyclin D1 and cyclin D2 knockout mice indicate a critical role for these factors in post-natal beta cell proliferation (Georgia and Bhushan 2004; Kushner *et al.* 2005). Taken together, increasing the expression or activation of cyclin D/Cdk4 is a key molecular event the

stimulation of beta cell proliferation, although this does not exclude other cell cycle factors playing an important role.

In sum, these studies demonstrate that beta cell proliferation can be influenced both in primary cell cultures and in whole animal studies through overexpression of oncogenes, growth factors, or direct manipulation of cell cycle gene expression, but resulting in most instances in a dramatic loss of differentiated function. Understanding the similarities and differences that the various manipulations described above have on beta cell proliferation and function on a molecular level will provide much needed insight into the mechanisms of mature beta cell proliferation and potentially provide new targets for diabetic therapies.

Specific aims

Due to the central role of beta cell function and mass in control of metabolic fuel homeostasis, this dissertation focused on identification and characterization of genes and factors that regulate these aspects of beta cell biology. Beta cell lines with varying capacities for glucose-stimulated insulin secretion (Hohmeier *et al.* 2000) were used as an initial model system for the identification of such factors. By directly comparing the gene expression profiles of robustly versus poorly glucose responsive beta cell lines, Nkx6.1 was identified as a gene with consistently higher expression in the robustly glucose responsive cells. This led to an in depth analysis of the effects of manipulation of Nkx6.1 expression in cell lines and primary rat islets. The results of these studies are summarized in the following two chapters of this dissertation, and in aggregate, provide new information about the role of Nkx6.1 in control of mature beta cell function and proliferation.

CHAPTER TWO

THE NKX6.1 HOMEODOMAIN TRANSCRIPTION FACTOR SUPPRESSES GLUCAGON EXPRESSION AND REGULATES GLUCOSE-STIMULATED INSULIN SECRETION IN ISLET BETA CELLS

Introduction

Type 1 and type 2 diabetes are both diseases of insulin deficiency, but with different etiologies. Type 1 diabetes occurs when pancreatic islet beta cells are destroyed by the host immune system. Type 2 diabetes involves loss of key beta cell functions such as glucose-stimulated insulin secretion (GSIS), and a gradual loss of beta cell mass by non-autoimmune mechanisms. The recent success of a human islet transplant trial in patients with type 1 diabetes (Shapiro *et al.* 2000) has focused fresh attention on cell-based insulin replacement as a method for treating the disease. However, a major obstacle to broad application of this therapeutic approach is the inadequate supply of fully differentiated human islets. In the case of type 2 diabetes, several classes of drugs have been developed for stimulation of insulin secretion, but complications in their use including loss of efficacy over time and episodic hypoglycemia have been noted (Lebovitz 2004). Thus, a clearer understanding of the factors that control the development of beta cells is critical for ultimate success in creation of "surrogate" cells for insulin replacement therapy in type 1 diabetes, and for better understanding of potential drug targets for enhancing beta cell function and mass in type 2 diabetes.

Recently, our laboratory described a set of robustly and poorly glucose responsive subclones of the rat insulinoma cell line INS-1 (Hohmeier *et al.* 2000). These lines were procured by stable transfection of the parental INS-1 cell line (Asfari *et al.* 1992) with a plasmid containing a neomycin resistance gene. From among 60 clones isolated by this procedure, 67% were found to be poorly responsive to glucose in terms of insulin secretion (≤ 2-fold stimulation by 15 mM compared to 3 mM glucose), 17% of the clones were moderately responsive (2-5-fold stimulation), and 16% were robustly responsive (5-13-fold stimulation).

In the current study, I have begun to exploit the differences between robustly and poorly glucose responsive INS-1-derived cell lines for the purpose of defining the genes that control key functions of the differentiated beta cell. As a first step in this process, a "candidate genes" approach was employed, involving assay of the expression level of a set of known genes in several independent robustly versus poorly glucose responsive lines, resulting in identification of three differentially expressed genes – glucagon, Nkx2.2, and Nkx6.1. Experimental manipulation of Nkx6.1 expression in these cell lines demonstrates novel roles for this transcription factor in regulation of glucagon expression and control of GSIS.

Materials & methods

Cell culture conditions

INS-1-derived cell lines were prepared and cultured as previously described (Chen *et al.* 2000; Hohmeier *et al.* 2000). The culture medium was RPMI-1640 with 11.1 mM D-glucose supplemented with 10% fetal bovine serum, 10 mM HEPES, 2 mM L-glutamine, 1 mM sodium pyruvate, and 50 μM β-mercaptoethanol.

Preparation and use of recombinant adenoviruses

The cDNA encoding hamster Nkx6.1 was cloned from plasmid pBAT12-Nkx6.1 (Mirmira *et al.* 2000) into the Xho1/Xba1 site in the adenovirus vector pACCMV.pLpA (Gomez-Foix *et al.* 1992) generating a new plasmid, pACCMV-Nkx6.1. The human embryonic kindney cell line (HEK 293) was used for transient co-transfection of pACCMV-Nkx6.1 and a plasmid containing the complete adenovirus genome plus an additional prokaryotic DNA fragment inserted into the E1a gene, pJM17 (Becker *et al.* 1994). 1 x 10^6 HEK 293 cells were seeded in 6-well plates cultured in DMEM (Sigma D5671) with high glucose (4500 mg/l) and 10% fetal bovine serum. When cells reached 50% confluence they were co-transfected with 0.5 μg both of pACCMV-Nkx6.1 and pJM17 using FuGene transfection reagent (Roche). Cells were monitored for lysis, an indication of viral production. Lysates were collected and used for a subsequent viral amplification. Crude lysates were screened for overexpression of the Nkx6.1 transgene via RT-PCR and immunobloting, confirming the production of the recombinant adenovirus, AdCMV-Nkx6.1. A virus containing the bacterial β-galactosidase gene (AdCMV-βGAL) was used as a control

(Herz and Gerard 1993). Viruses were purified using the Adeno-X purification kit (BD Biosciences), and used to treat cell lines at multiplicities of infection (MOI) ranging from 10-2500 for 2 h. Assays and analyses were undertaken 24-48 h later.

Construction of adenoviruses expressing small interfering RNA sequences (siRNAs) was performed according to recently described methods (Bain *et al.* 2004). Oligos containing siRNAs corresponding to rat Nkx6.1 (accession number AF004431; GAGCACGCTTGGCCTATTC) and rat Pdx1 (accession number NM_022852; GAAAGAGGAAGATAAGAAA) were annealed into a lineralized expression plasmid (pSUPER, OligoEngine) between the human H1 RNA pol III promoter and T5 transcriptional terminator. This expression cassette was subsequently subcloned into a modified vector of pACCMV.pLpA that does not contain a CMV-promoter (EH006) and used for construction of recombinant adenoviruses Ad-siNkx6.1 and Ad-siPdx1 as described above. siRNA sequences corresponding to the *Photinus pyralis* luciferase gene, GL2 (Elbashir *et al.* 2001), or a random siRNA sequence (GAGACCCTATCCGTGATTA) were cloned into adenoviruses (Ad-siLuc and Ad-siRNAcontrol, respectively) for use as controls. Titers of tertiary viral lysates were measured by immunoassay (Adeno-X Rapid Titer Kit, BD Biosciences) in HEK293 cells (Bewig and Schmidt 2000), and these viral stocks were used to treat cell lines at an MOI of 20 for 18 h. Assays and analyses were undertaken 96 h later.

Glucose-stimulated insulin secretion; insulin and glucagon content

Cells were aliquoted in 12-well plates, treated with the various recombinant adenoviruses, and grown in culture medium (see above) containing 11 mM glucose. Insulin secretion was measured by static incubation as described (Hohmeier *et al.* 2000) following a switch to culture medium containing 5 mM glucose for 12 h, using basal and stimulatory glucose concentrations as indicated in the figures and legends. For measurements of insulin and glucagon content, cells were extracted with 1 M acetic acid, 0.1% BSA. Media and extract samples were analyzed for insulin or glucagon concentrations by radioimmunoassay (RIA) with the insulin Coat-a-Count kit (Diagnostic Products, Los Angeles, CA) (Clark *et al.* 1997; Hohmeier *et al.* 1997), or a glucagon-specific RIA (Linco Research Inc.).

Semiquantitative multiplex-PCR and real-time PCR measurements of RNA levels

Total RNA was isolated and purified using TRizol reagent (Invitrogen) or RNeasy (Qiagen) and treated with DNase to avoid genomic contamination. Superscript II (Invitrogen) or iScript (BioRad) was used for first strand synthesis of cDNA using 0.5-1.0 μg of RNA.

Semi-quantitative multiplex-PCR was performed as described previously (Jensen *et al.* 1996). Briefly, primers were optimized to yield products between 160-280 bp. Reactions were carried out using Platinum Taq polymerase (Invitrogen) in 25 μl volumes containing 20 ng of various cDNA samples, 25 μM dATP, 25 μM dGTP, 25 μM dTTP, 12.5 μM dCTP, and 0.125 μl of 3000 Ci/mmol [α-^{33}P]dCTP (NEN Life Science Products). Reactions were carried out at cycles in the exponential range of product formation (between 16-24 cycles,

dependent on target) and separated on 0.4 mm, 7 M urea, 6% polyacrylamide gels. Images were acquired using a Storm phosphor-imager (Amersham Biosciences). Standard band/volume analysis using local background correction was used to quantify PCR products. Appropriate internal standards amplified at similar cycle numbers as the target gene product(s) were included. cDNA reactions conducted in the absence of reverse transcriptase served as negative controls. Candidate gene expression profiling in the various cell lines was performed with primers specific for islet hormones (insulin, glucagon, somatostatin), enzymes and transporters (glucose-6-phosphatase, glucokinase, GLUT2, hexokinases 1 and 2, and glutamate dehydrogenase), transcription factors (Brn4, E47, HNF-1α, IB1, NeuroD, Nkx2.2, Nkx6.1, Pax4, Pdx1) and glycogen targeting subunits (PTG, G_L). Other studies of the effects of modulation of Nkx6.1 levels involved measurements of additional target genes and loading controls, including α-tubulin, G6PDH, Pax6, HNF3α (FOXO1), rat Nkx6.1, hamster Nkx6.1, and total Nkx6.1 (rat + hamster). Primer sequences for each of these targets are listed in Appendix A.

Real-time PCR reactions were performed with using the ABI PRISM® 7000 sequence detection system, software and reagents (An *et al.* 2004). Primers and probes used in real-time PCR reactions are listed in Appexdix A. Primers used with SYBR-Green PCR chemistry were used at final concentrations of 100 nM. Pre-validated primer and probe sets based on Taqman® chemistry (Applied Biosystems) were used as indicated in Appendix A. Triplicate reactions from independent RNA samples were carried out in a final volume of 25 μl containing 20 ng of cDNA template. RNA input was calibrated with 18S or cyclophilin B

expression levels and relative mRNA levels were normalized to either AdCMV-βGAL or Ad-siRNAcontrol treated samples where appropriate.

Isolation of nuclear extracts

INS-1-derived cells were cultured in 10-cm plates and treated with various adenoviruses as indicated in the figure legends. Plates were washed with twice with 10 ml of cold PBS followed by the addition of 0.5 ml of cold Buffer A (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 1 mM DDT, 4% IGEPAL, and protease inhibitor cocktail (Sigma P8340)) and incubated for 10 min at room temperature. Cells were scraped and mixed by pipeting, collected in 1.5 ml microcentrifuge tubes, and centrifuged at 15000 x G for 3 min at 4 °C. The supernatant (cytosolic fraction) was removed and 150 µl of Buffer B (20 mM HEPES, pH 7.9, 0.4 M NaCl, 1 mM EDTA, 10% glycerol, 1 mM DTT, and protease inhibitor cocktail) was used to resuspend the pellet, mixed well by pipeting. Samples were vortexed at high speed for 2 h at 4 °C. Subsequently, samples were centrifuged at >15,000 x G for 5 min at 4 °C. The supernatant (nuclear fraction) was aliquoted to pre-chilled tubes and flash frozen in liquid nitrogen. Protein concentration was determined via Bradford assay (Bradford 1976).

Electrophoretic mobility shift (EMSA) and chromatin immunoprecipitation (ChIP) assays

EMSA buffers and procedures were used described previously (German *et al.* 1992). 5 µg of nuclear extract protein was added to each EMSA reaction containing EMSA reaction

buffer (10 mM HEPES, pH 7.9, 75 mM KCl, 2.5 mM MgCl2, 0.1 mM EDTA, 1 mM DTT and 3% Ficoll), 0.5 mg/ml BSA, 0.05 mg/ml polydI-dC, ^{32}P-labeled probe (~10K CPM) in a final volume of 20 µl. Reactions were performed for 15 min at room temperature. Where supershift assays were performed, 1 µl of undiluted anti-Nkx6.1 or anti-Pdx1 antisera were also added. 18 µl of the reaction were loaded of a 5% polyacrylamide gel, run at 150V for 2 h, dried and exposed to film for 16 – 48 h at -80 °C. EMSA was performed with an oligonucleotide corresponding to the G1 element of the rat glucagon promoter: 5'-GAACAAAACCCCATTATTTACAGATGAGAA-3' (top strand shown).

ChIP assays were performed as detailed previously (Chakrabarti *et al.* 2002; Chakrabarti *et al.* 2003). 10-cm plates of 832/3 cells were washed once with 10 ml of PBS and then treated with 1% formaldehyde by adding 0.27 ml of 37% formaldehyde directly to 10 ml of PBS. After incubating in formaldehyde for 10 min at room temperature, glycine was added to a final concentration of 0.125 M. Cells were washed twice with 10 ml of cold PBS. 0.5 ml of cold PBS with containing protease inhibitors (Sigma P8340) was added to the plate, and cells were scraped and collected in 2 ml microcentrifuge tubes. Cells were spun down at 2000 x G for 2 min at 4 °C and resuspended in 0.6 ml of lysis buffer (50 mM Tris-Cl, pH 8.1, containing 1% Triton X-100, 0.1% deoxycholate, 150 mM NaCl, and 5 mM EDTA) plus protease inhibitors (leupeptin, phenylmethylsulfonyl fluoride, and aprotinin) and subjected to sonication (using a Fisher Scientific model 60 sonic dismembrator with a microtip at a setting of 10). 15 x 5 sec sonication pulses were required to shear chromatin to 500-1500 bp fragments. The effectiveness of shearing was confirmed by incubating a 10 µl aliquot of the extract at 65 °C for 3 h (to reverse cross-links) and subsequently subjecting it to

electrophoresis on a 1% agarose gel. 0.25 ml aliquots of the clarified extracts were diluted to 1 ml in lysis buffer containing protease inhibitors and then incubated with either 5 µl of anti-Nkx6.1 antiserum or normal rabbit serum (25 µl was also divided into separate aliquots and stored for later PCR analysis as 10% of the input extract). Incubations occurred overnight at 4 °C on a rocking platform, after which 40 µl of protein A-agarose slurry (Santa Cruz Biotechnology) and 2 µl of a 10 mg/ml herring sperm DNA solution (Sigma) were added, and incubation was continued an additional 1 hour.

The agarose was pelleted by centrifugation, and the pellets were washed consecutively with 1 ml of lysis buffer, lysis buffer plus 500 mM NaCl, lysis buffer plus 0.25 M LiCl, and Tris/EDTA. DNA and protein were eluted from the pellets by incubating the pellets 2 times in 0.25 ml of elution buffer (0.1 M $NaHCO_3$ with 1% SDS and 20 µg/ml herring sperm DNA, and protein-DNA cross-links were reversed by incubating at 65 °C for 3 h. DNA and protein were ethanol-precipitated overnight at −20 °C. The precipitated samples were pelleted and dissolved in proteinase K buffer (10 mM Tris-Cl, pH 7.5 with 1% SDS) and incubated with 1 µg of proteinase K (Roche Molecular Biochemicals) for 1 h at 55 °C. The samples were extracted once with phenol/chloroform and ethanol-precipitated overnight at −20 °C. Samples were pelleted, washed with 70% ethanol, and dissolved in 100 µl of Tris/EDTA. 3-µl aliquots were used for each real time PCR reaction to quantitate co-immunoprecipitated promoter fragments.

Each ChIP assay was quantitated in triplicate by real-time PCR for recovery of either the rat glucagon or myoD1 promoters. Forward and reverse primers used to amplify the glucagon gene (-1 bp to –279 bp relative to the transcriptional start site) were, respectively:

5’- GGATCCTTCAGAGAGCTGAATAG and 5’- GGCAAGCTTCACCAGGGTGCTGTG. Forward and reverse primers used to amplify the myoD1 gene were, respectively: 5’-CCACTTCGTCCTTGGCTCAAC and 5’- GGGATACCAGGCACAGCATAGG.

Immunoblot analysis

Aliquots of 5 μg of nuclear extract protein were resolved on 4-12% Bis-Tris-HCl buffered (pH = 6.4) polyacrylamide gels (Invitrogen) and transferred to polyvinylidene difluoride (PVDF). Membranes were incubated with anti-Nkx6.1 (1:4000) or anti-Pdx1 (1:10,000) antibodies overnight at four degrees, or anti-γ-tubulin (1:10,000 Sigma) antibody for 3 h at room temperature. Antibodies were detected using appropriate HRP-linked secondary antibodies and visualized using ECL Advance (Amersham) on a Versadoc 5000 (Biorad).

Transient transfection assays

αTC1.6 cells were aliquoted into 6-well plates at a density of 7.5×10^5 cells per well one day before transfection. A total of 2 μg of plasmid DNA (consisting of 1.0 μg of a luciferase reporter plasmid under control of the proximal 450 bp of the rat glucagon promoter (pFoxLucGlucagon), 0.25 μg of pBAT12.Nkx6.1 or pBAT12 lacking a cDNA insert, and the balance consisting of a CMV promoter-driven β-galactosidase reporter plasmid) was mixed with 6 ml of Transfast® reagent (Promega), and transfections performed according to the manufacturer’s protocol. Additional control experiments utilized plasmids with luciferase under control of thymidine kinase, CMV, or no promoter in place of 1 μg pFoxLucGlucagon.

Cells were harvested 48 h after transfection, and luciferase activities were measured using a commercially available assay kit (Promega) and an FB15 luminometer (Zylux).

Islet isolation

Islets from male Wistar rats weighing approximately 250 g were isolated via pancreatic perfusion as described (Johnson *et al.* 1990) using the Liberase R1 enzyme (Roche), and GSIS assays were performed as described (Antinozzi *et al.* 1998).

Statistical methods

Statistical significance was determined using a two-tailed Student's t-Test. P values less than 0.05 were considered significant.

Results

Glucose-stimulated insulin secretion in INS-1-derived cell lines

GSIS was measured in six independent INS-1-derived cell lines (Hohmeier *et al.* 2000). Four of the cell lines were poorly glucose responsive (lines 834/105, 834/112, 832/1, 832/2; average responses of 2.4-fold, 2.1-fold, 2.2-fold, and 3.8-fold as glucose was raised from 1 to 20 mM, respectively) whereas two cell lines were robustly glucose responsive (lines 833/15, 832/13; average responses of 21-fold and 30-fold, respectively) (Figure 2-1). These six lines were used for subsequent experiments.

Candidate gene screen

The levels of mRNA encoding a panel of twenty transcription factors, metabolic enzymes, or hormones (Table 2-1) thought to serve either as markers or mediators of beta cell differentiation were measured by semi-quantitative multiplex-PCR. Among these, three were differentially expressed in the 6 INS-1-derived cell lines. The expression pattern for the gene encoding glucagon identified new subclasses of INS-1-derived cell lines (Figure 2-2A). Thus, a subset of the poorly glucose responsive cell lines (834/105 and 834/112) were found to contain substantial quantities of the mRNA encoding glucagon, and are hereafter referred to as Class 1 cells. Two other poorly glucose responsive cell lines (832/1 and 832/2) contained little glucagon transcript, and are hereafter referred to as Class 2 cells. Finally, the two robustly glucose responsive cell lines (832/13 and 832/15) also contained very low levels

of glucagon mRNA, in keeping with their well-differentiated phenotype, and are hereafter referred to as Class 3 cells.

The other two differentially expressed candidate genes emerging from this study are members of the Nkx transcription factor family (Figure 2-2B and 2-2C). Nkx2.2 was most highly expressed in the glucagon positive, poorly glucose responsive Class 1 cells, and was less abundant in Class 2 and Class 3 cells (relative RNA levels of 3.3: 1.0: 1.0 in Class 1: Class 2 and Class 3) (Figure 2-2B). Interestingly, the inverse pattern was seen for another member of the gene family, Nkx6.1, which was most abundant in Class 3 cells and least abundant in Class 1 cells (relative RNA levels of 1.0: 2.6: 6.4 in Class 1: Class 2: Class 3) (Figure2-2C).

Expression of Nkx6.1 in Class 1 cells suppresses glucagon gene expression and decreases glucagon content and secretion

The expression pattern of Nkx6.1 shown in Figure 2-2C suggests a potential role for this transcription factor in regulation of glucagon expression and/or other differentiated functions of the beta cell such as GSIS. To evaluate these possibilities, I first constructed a recombinant adenovirus containing the cDNA encoding hamster Nkx6.1. Treatment of 834/105 cells with the AdCMV-Nkx6.1 adenovirus resulted in increases in Nkx6.1 mRNA (Figure 2-3A) and protein (Figure 2-3B). Moreover, overexpression of Nkx6.1 in these Class 1 cells suppressed glucagon mRNA levels in a dose-dependent manner (Figure 2-3A and 2-3C).

Importantly, at a dose of AdCMV-Nkx6.1 adenovirus that caused a 62% suppression of glucagon mRNA levels, no effect on expression of prominent beta cell transcription factors such as Pdx1, IB1, E47, HNF-1α, Nkx2.2, and endogenous rat Nkx6.1 (Figure 2-3D and 2-3E), or PAX4, PAX6 or HNF3α (FOXO1) (data not shown) were observed. Overexpression of Nkx6.1 also did not affect insulin mRNA levels (Figure 2-3D and 2-3E). Overexpression of Nkx6.1 did cause a small (10%), but statistically significant decrease in expression of NeuroD ($p = 0.049$). These data suggest that Nkx6.1 overexpression is largely sufficient to suppress glucagon gene expression in INS-1-derived cell lines.

To determine if the decrease in glucagon mRNA seen with the overexpression of Nkx6.1 in class 1 cells correlates to changes in glucagon peptide levels, the cellular content of glucagon peptide as well as the amount of glucagon peptide secreted was measured in 24 h intervals over 3 days of Nkx6.1 overexpression. At a dose of AdCMV-Nkx6.1 virus that caused a 50% decrease in glucagon mRNA over 24 h, a 71% decrease in glucagon peptide content was observed 48 h after viral treatment (134 ± 24 versus 39 ± 3 ng glucagon/mg protein in AdCMV-βGAL versus AdCMV-Nkx6.1-treated cells, respectively; $p = 0.0014$) (Figure 2-4A). Additionally, both glucagon content and secretion was significantly lower at all three time points in cells treated with AdCMV-Nkx6.1 compared to cells treated with AdCMV-βGAL (Figure 2-4A and Figure 2-4B). Overexpression of Nkx6.1 over the 72 h time course was confirmed via immunoblot analysis (Figure 2-4C). These data demonstrate that a change in glucagon mRNA is reflected in changes in glucagon peptide.

Nkx6.1 interacts with the glucagon promoter and suppresses its function

In collaboration with Raghu Mirimira and David Thomas (University of Virginia) we further investigated the mechanism of Nkx6.1-mediated suppression of glucagon expression, by the ability of the transcription factor to interact with the G1 element of the rat glucagon promoter in electromobility shift assays (EMSAs). Consistent with this approach, the G1 element contains a potential Nkx6.1 binding site (5'-TAAT-3') (Jorgensen *et al.* 1999; Mirmira *et al.* 2000). I prepared nuclear extracts from Class 3 cell cells in a variety of conditions for subsequent EMSA analysis performed by R.M and D.T. Class 3 cells contain significant Nkx6.1 protein, as demonstrated by supershift of a specific protein from the G1/nuclear extract complex in response to treatment with an Nkx6.1-specific antibody (Figure 2-5A, lane 4). Adenovirus-mediated overexpression of Nkx6.1 caused a large increase in the amount of super-shifted protein (Figure 2-5A, lane 2), demonstrating that the overexpressed hamster Nkx6.1 protein can bind to elements found within the rat glucagon promoter.

To assess the functional consequence of interaction of Nkx6.1 with the glucagon promoter, R.M. and D.T. performed co-transfection studies in the alpha cell line αTC1.6. Overexpression of Nkx6.1 caused a 9.4 ± 1.1-fold repression of the activity of a glucagon promoter-driven luciferase reporter relative to control cells transfected with empty vector (Figure 2-6A). By contrast, repression of control reporters (promoterless, thymidine kinase promoter, and CMV promoter) by overexpressed Nkx6.1 was in the 1.5- to 2.5-fold range, suggesting that repression of the glucagon promoter-driven luciferase reporter by Nkx6.1 is specific. We attempted to repeat these reporter gene co-transfection studies in 834/105 cells

(one of the Class 1 cell lines with endogenous glucagon expression), but found that the glucagon promoter fragment used in our studies had no detectable activity in these cells. Importantly, however, Nkx6.1 also suppresses activity of the endogenous glucagon promoter in αTC1.6 cells, as adenovirus-mediated expression of Nkx6.1 caused a 58% suppression of glucagon mRNA relative to AdCMV-βGAL-treated cells, as measured by RT-PCR (Figure 2-6B). Our findings clearly demonstrate that overexpression of Nkx6.1 is sufficient to suppress both exogenously introduced and endogenous glucagon promoter function.

Suppression of Nkx6.1 or Pdx1 expression activates glucagon expression

A previous study has demonstrated that overexpression of Pdx1 in INS-1 cells causes increased expression of Nkx6.1 and suppression of glucagon expression (Wang *et al.* 2001). However, the specific roles played by Nkx6.1 versus Pdx1 in suppression of glucagon expression were not elucidated. To gain more insight into this issue, we prepared recombinant adenoviruses containing small interfering RNAs specific for Nkx6.1 or Pdx1 (Ad-siNkx6.1 and Ad-siPdx1), and used these reagents to suppress the expression of the individual transcription factors in Class 3 cells (832/13). Treatment of these cells with Ad-siNkx6.1 resulted in suppression of Nkx6.1 mRNA levels by 65%, whereas treatment with Ad-siPdx1 caused an 85% decrease in Pdx1 mRNA content (Figure 2-7). Moreover, treatment of Class 3 cells with Ad-siNkx6.1 caused near-complete extinction of the supershifted Nkx6.1 band detected by EMSA analysis (performed by R.M. and D.T.) with the glucagon promoter G1 element probe (compare lanes 4 and 2 in Figure 2-5B). Class 3 (832/13) cells contain large amounts of endogenous Pdx1 that also binds to the glucagon

promoter G1 element and is supershifted by Pdx1-specific antibody in EMSA analysis (lane 6, Figure 2-5B). This protein band was suppressed by more than 90% in cells treated with the Ad-siPdx1 adenovirus (compare lanes 8 and 6 in Figure 2-5B).

The level of Nkx6.1 suppression demonstrated in Figure 2-5B and Figure 2-7 resulted in a 2-fold increase in glucagon mRNA levels, further supporting a direct role for Nkx6.1 in control of glucagon expression in beta cells (Figure 2-7). Interestingly, Pdx1 suppression caused a much larger increase (12-fold) in glucagon expression than achieved by Nkx6.1 suppression. Pdx1 suppression resulted in modest decreases in Nkx6.1 mRNA levels (Figure 2-7), and in Nkx6.1 protein levels as judged by EMSA analyses (Figure 2-5C). Conversely, suppression of Nkx6.1 by RNAi treatment had no effect on Pdx1 mRNA (Figure 2-7) or protein levels (Figure 2-5C). Thus, we conclude that Nkx6.1 suppression is sufficient to enhance glucagon expression, but suppression of Pdx1 causes a larger enhancement. Moreover, our data suggest that the two transcription factors have independent effects on glucagon expression in beta cells.

The effects of Nkx6.1 and Pdx1 silencing on expression of the large Maf transcription factors.

The expression of several members of large Maf transcription factor family has been characterized in islet cell types and implicated in the regulation of islet hormones (Matsuoka *et al.* 2003; Kataoka *et al.* 2004). Therefore, the mRNA level of three Maf transcription factors (MafA, MafB, and cMaf) was measured in Class 3 cells that were treated with either

Ad-siNkx6.1 or Ad-siPdx1 and compared to levels found in Ad-siRNAcontrol-treated cells. As shown in

Figure 2-8, silencing of Pdx1 expression by 78% ± 1% resulted in an 86 ± 3% decrease in MafA expression and a 492% ± 64% increase in MafB expression compared to Ad-siRNAcontrol-treated cells. The changes in MafA and MafB were specific to Pdx1 silencing, since a 66% ± 2% reduction in Nkx6.1 expression did not have any effect on MafA or MafB levels. Interestingly, significant changes in cMaf mRNA levels were only seen in Ad-siNkx6.1-treated cells, (229% ± 40% compared to Ad-siRNAcontrol). Therefore, Nkx6.1 and Pdx1 have divergent functions in control of Maf gene expression in beta cell lines.

Nkx6.1 interacts with the endogenous glucagon promoter in Class 3 cells

Data in Figure 2-5 and Figure 2-7 provide evidence that Nkx6.1 levels regulate glucagon promoter activity in alpha and beta cell lines, but do not prove that Nkx6.1 binds directly to the endogenous glucagon promoter in these cells. To address this issue, in collaboration with R.M. and D.T., we performed chromatin immunoprecipitation (ChIP) assays. Nkx6.1 is present at relatively low abundance in beta cells, so as a prelude to these studies, we screened multiple cell lines with robust GSIS for their endogenous levels of Nkx6.1. Line 832/3, previously identified as a line with robust GSIS (Hohmeier *et al.* 2000) contained 4.8 times more Nkx6.1 mRNA than lines 832/13 or 832/15 characterized earlier, and glucagon expression was virtually undetectable in these cells. I prepared cross-linked cell samples from the 832/3 cell line for subsequent ChIP analysis by R.M. and D.T. As shown in Figure 2-6C, a significantly larger fraction of input glucagon promoter was

recovered (2.5 times more, $p = 0.001$) in nuclear extract samples from 832/3 cells immuno-precipitated with anti-Nkx6.1 antibody compared to samples treated with non-specific rabbit antiserum. Moreover, the anti-Nkx6.1 antibody did not preferentially immunoprecipitate a control promoter (myoD). These data demonstrate direct interaction of Nkx6.1 with the glucagon promoter in living beta cells.

Nkx6.1 suppression causes impairment of GSIS in Class 3 cells

The expression pattern of Nkx6.1 in the three classes of INS-1-derived cell lines suggests a possible role for this transcription factor in the robust GSIS exhibited by Class 3 cells. To test this possibility directly, we used Ad-siNkx6.1 to suppress Nkx6.1 mRNA levels by 65% in a representative Class 3 cell line, 832/13. As shown in Figure 2-9, suppression of Nkx6.1 caused a significant impairment in GSIS, with a decline from a 13.9-fold response in cells treated with the Ad-siRNAcontrol virus to a 3.7-fold response in cells treated with Ad-siNkx6.1, as glucose concentrations were raised from 3 to 15 mM. Similar effects of Nkx6.1 suppression were observed in a second Class 3 cell line, 832/3 (data not shown). In contrast, suppression of Pdx1 expression caused a decrease in both basal and stimulated insulin secretion, but did not affect the fold response. The loss of fold response in cells with suppressed Nkx6.1 levels versus the decrease in total insulin output in cells with suppressed Pdx1 suggests that these transcription factors control distinct sets of genes involved in insulin secretion. Consistent with this, suppression of Pdx1 caused a 63% decrease, whereas Nkx6.1 suppression caused a 2.3-fold increase, in GLUT-2 mRNA levels (Figure 2-7). Further investigation of Nkx6.1-regulated gene expression by microarray

analysis will be required in order to fully explain our findings. Importantly, neither Pdx1 suppression nor Nkx6.1 suppression affected insulin content (16.8 ± 3.5, 14.3 ± 1.9, 15.5 ± 3.5 μU insulin/μg protein in Ad-siRNAcontrol-, Ad-siNkx6.1-, and Ad-siPdx1-treated cells, respectively).

We also investigated whether increasing the levels of Nkx6.1 in Class 1 or Class 2 cells would result in improved GSIS in these lines. AdCMV-Nkx6.1 was used to raise Nkx6.1 mRNA levels in Class 1 or Class 2 cells to a level approximating the endogenous levels of Class 3 cells as measured by semiquantitative multiplex-PCR (Figure 2-10B) and subsequently validated via real-time PCR (Figure 2-10C). This maneuver did not improve GSIS relative to AdCMV-βGAL-treated Class 1 or Class 2 cells (Figure 2-10A). Overexpression of Nkx6.1 in Class 1 or Class 2 cells to levels higher than endogenous levels in Class 3 cells also had no effect on GSIS (data not shown). These data and the data of Figure 2-9 suggest that Nkx6.1 is a necessary but not sufficient factor for conferring GSIS in INS-1-derived cell lines.

Suppression of Nkx6.1 expression impairs GSIS in rat islets

To determine whether our findings of a role of Nkx6.1 in control of GSIS in INS-1-derived cell lines also applies to normal rat islets, we used the Ad-siNkx6.1 virus to suppress Nkx6.1 mRNA levels by 46% in primary cells. This maneuver resulted in a decrease in GSIS from 7.1 ± 0.2- fold in Ad-siRNAcontrol-treated islets to 5.2 ± 0.5-fold in Ad-siNkx6.1-treated islets ($p = 0.012$), and a 56% decrease in insulin secretion at stimulatory

glucose (16.7 mM) (Figure 2-11). Consistent with our findings in the INS-1-derived cell lines, suppression of Nkx6.1 did not affect insulin content in the primary islet experiments.

Expression of Nkx6.1 is reduced in islets from ZDF rats.

To investigate the potential significance of Nkx6.1 in functional impairment of β-cells in type 2 diabetes, we used real-time PCR to measure the levels of mRNA encoding the transcription factor in islets isolated from 12 week old Zucker diabetic fatty (ZDF; fa/fa) rats, or islets from lean control Zucker rats (+/+) of the same age. We also measured the levels of PGC-1α, a transcriptional co-activator recently shown to be increased in islets from murine models of type 2 diabetes (Yoon *et al.* 2003). As shown in Figure 2-12, PGC-1α mRNA levels were increased by 60% in islets from fa/fa rats compared to controls, whereas Nkx6.1 mRNA levels were reduced by 63% in the fa/fa islets. Thus, expression of Nkx6.1 is reduced in concert with loss of beta cell function in the ZDF rat model. Given our demonstration of a direct effect of Nkx6.1 levels on GSIS (Figure 2-9), these findings suggest that the decrease in Nkx6.1 expression in ZDF islets could contribute to the development of beta cell dysfunction during the progression to diabetes.

Discussion

INS-1 cells (Asfari *et al.* 1992) have been used to study many aspects of beta cell biology including glucose and lipid metabolism(Antinozzi *et al.* 1998; Antinozzi *et al.* 2002; Wang *et al.* 2003a), beta cell survival mechanisms (Chen *et al.* 2000; Chen *et al.* 2001; Tran *et al.* 2003), and glucose-stimulated insulin secretion (GSIS) (Zhang and Kim 1995; Khoo and Cobb 1997; Noel *et al.* 1997; Antinozzi *et al.* 1998; Ahren and Havel 1999; Smukler *et al.* 2002; Khoo *et al.* 2004). The parental INS-1 cell line is now recognized as a mixed population of cells with different phenotypic features, as subclones derived from these cells have varying degrees of glucose responsiveness. This chapter summarizes our first attempt at defining differentially expressed genes that contribute to beta cell function in INS-1-derived cell lines. Microarray analyses of rodent insulinoma cell lines with differing capacities for GSIS (Zimmer *et al.* 1999; Lilla *et al.* 2003; Wang *et al.* 2003b) or as a function of exposure to inflammatory cytokines (Eizirik *et al.* 2003; Kutlu *et al.* 2003; Rasschaert *et al.* 2003) have been reported, with hundreds of differentially expressed genes appearing in each of these studies. However, follow-up functional analysis aimed at determining which of the differentially expressed genes actually contribute to the phenotype under study has been rare. Therefore, a key goal of the current work was to combine analysis of differentially expressed genes with direct testing of their potential roles in controlling beta cell phenotype.

The candidate gene strategy reported herein has led to the identification of three classes of INS-1-derived subclones. Class 1 cells were found to co-express insulin and glucagon and were poorly glucose responsive, Class 2 cells had very low glucagon expression and were also poorly glucose responsive, and Class 3 cells had low glucagon expression and robust GSIS. These phenotypes were negatively and positively correlated with levels of expression of Nkx2.2 and Nkx6.1, respectively, such that Nkx2.2 expression was highest in Class 1 cells and lowest in Class 3 cells, whereas the opposite was true for Nkx6.1.

Co-expression of insulin and glucagon has been reported previously in the parental INS-1 cell line (Poitout *et al.* 1996). In addition Wang, *et al.* described two INS-1 derived clones, INSrαβ and INSrβ; INSrαβ co-expressed glucagon and insulin, whereas glucagon expression was absent in INSrβ cells (Wang *et al.* 2001). GSIS was modest in both of these clones (3-4-fold), but clear understanding of this aspect of their phenotype was complicated by the fact that the cells were transfected with either a Pdx1 transgene (INSrβ) or a dominant negative Pdx1 construct (INSrαβ). Wang and colleagues demonstrated that overexpression of Pdx1 in INSrαβ cells resulted in stimulation of Nkx6.1 expression in concert with suppression of glucagon expression. However, their studies did not determine whether the suppression of glucagon expression was mediated directly by Nkx6.1 or via another mechanism.

The work summarized in this chapter shows that Nkx6.1 maintains the mature beta cell phenotype in part via participation in suppression of glucagon expression. Several lines of evidence support this conclusion: 1) Overexpression of Nkx6.1 decreases glucagon mRNA

and peptide levels in a dose-dependent fashion in Class 1 cells; 2) Lowering of Nkx6.1 levels in Class 3 cells results in an increase in glucagon mRNA; 3) EMSA analysis demonstrates interaction of Nkx6.1 with a sequence from the endogenous rat glucagon promoter; 4) Nkx6.1 suppresses activity of a glucagon promoter/reporter construct in co-transfection experiments in αTC-1 cells, and adenovirus-mediated overexpression of Nkx6.1 also suppresses endogenous glucagon expression in these cells; 5) ChIP analysis demonstrates direct interaction of Nkx6.1 with the glucagon promoter in Class 3 cells; 6) Nkx6.1 expression does not affect the expression of other transcription factors that have been prominently implicated in beta cell development such as Pdx1, IB1, PAX6, or E47, although a small suppression of NeuroD expression was noted. Our findings contrast with a previous study in a different alpha cell line (MSL-G-AN) in which no suppression of endogenous glucagon expression was reported in response to stable expression of Nkx6.1 (Jorgensen *et al.* 1999). However, in that study, changes in glucagon expression were only compared in Nkx6.1 transfected and control cells after prolonged *in vivo* passage to allow tumor formation, whereas the use of adenovirus vectors in the current study allowed us to examine the acute effects of up- and down-regulation of Nkx6.1 levels directly in cultured cells.

RNAi-mediated suppression of Pdx1 caused a larger increase in glucagon expression than suppression of Nkx6.1, and Pdx1 protein clearly interacts directly with the G1 element of the glucagon promoter, as demonstrated by EMSA analysis. These findings suggest that suppression of Pdx1 expression affects glucagon expression in part by causing a decrease in Nkx6.1 protein levels and promoter binding, but that Pdx1 has additional and apparently additive suppressive effects on glucagon expression in beta cell lines.

The transcription factors MafA, MafB, and cMaf are members of the large Maf family of transcription factors and the detection of their expression in islet cell types have focused attention on the role they may play in islet biology. In particular, these factors have been found to participate in the transcriptional regulation of both glucagon and insulin gene expression (Matsuoka *et al.* 2003; Kataoka *et al.* 2004). MafA has been the best studied of the Maf factors and has been shown to interact directly with the insulin promoter and activate its transcription (Kataoka *et al.* 2002; Olbrot *et al.* 2002; Matsuoka *et al.* 2003; Kataoka *et al.* 2004; Matsuoka *et al.* 2004). It has also been reported to be a necessary factor for GSIS, as demonstrated in the MafA knockout mouse (Zhang *et al.* 2005). In contrast, MafB is a positive regulator of glucagon gene expression (Artner *et al.* 2006). Consistent with the functions described above, MafA and MafB expression are restricted to beta or alpha cells, respectively, whereas cMaf as not been detected in mature rodent islets, and its role in islet biology, if any, remains obscure (Artner *et al.* 2006). We hypothesized that some of the effects of siRNA-mediated suppression of Nkx6.1 and Pdx1 on glucagon expression or GSIS may be due to effects on the Maf transcription factors. The silencing of Pdx1 resulted in an 86% drop in MafA and 492% increase in MafB mRNA levels, whereas the levels of these two factors were unchanged in cells with reduced Nkx6.1 expression. Therefore, Pdx1, in addition to its direct role in insulin (Iype *et al.* 2005) and glucagon transcription (Schisler *et al.* 2005), may also affect these processes indirectly through changes in MafA and MafB levels, respectively. The ability of Pdx1, but not Nkx6.1 to suppress MafB may explain the more potent stimulation of glucagon gene expression that we observed in our studies

comparing the effects of Pdx1 and Nkx6.1 suppression in INS-1-derived Class 3 cells (Schisler *et al.* 2005).

We also report that siRNA-mediated silencing of Nkx6.1 in Class 3 cells causes a dramatic decrease in GSIS. siRNA-mediated suppression of Pdx1 expression, in contrast, caused a decrease in the amount of insulin secreted at both basal and stimulatory glucose concentrations, but did not affect fold-response. These effects on insulin secretion were not attributable to changes in insulin content, as this variable was not affected by Ad-siNkx6.1 or Ad-siPdx1 treatment. Importantly, suppression of Nkx6.1 expression also impairs GSIS in primary rat islets.

Both glucagon-expressing alpha cells and insulin-expressing beta cells arise from a common neurogenin3-expressing precursor cell (Schwitzgebel *et al.* 2000). Differentiation from these common precursors into either alpha or beta cells is directed by an array of transcription factors that are expressed in a specific temporal and spatial context (Wilson *et al.* 2003). Nkx2.2 is expressed early in pancreatic differentiation (e9.5), later restricted to endocrine cells (e15.5), and expressed in alpha, beta, and PP cells in mature islets. Disruption of the Nkx2.2 gene results in a decrease in islet mass with an absence of insulin-expressing cells and a reduction in both glucagon- and PP-expressing cells (Sussel *et al.* 1998). Nkx6.1 can first be detected at E10.5, is dependent upon Nkx2.2 for its expression, and is tightly restricted to the beta cell lineage (Sander *et al.* 2000), consistent with the idea that Nkx6.1 could function as a glucagon suppressor. Islets from Nkx6.1 knockout animals are slightly smaller than littermate controls, with 94% reduction in the number of beta cells, but with no change in the number of glucagon-positive cells (Sander *et al.* 2000). The surfeit

of beta cell mass in Nkx6.1-/- animals is due in part to an absence of mature beta cells produced from the so-called "secondary transition". These features of the model prevent a full assessment of the role of Nkx6.1 in maintenance of mature beta cell function due to an insufficient number of beta cells for study. Thus, our work with overexpression and RNAi-mediated suppression of Nkx6.1 in well differentiated insulinoma cells and primary rat islets has allowed us to uncover new roles for the transcription factor that were not discernable earlier.

Our findings may have implications for development of beta cell dysfunction in diabetes. Of interest in this context is the finding of impaired GSIS induced by partial pancreatecomy is accompanied by reduced Nkx6.1 expression (Jonas *et al.* 1999). Similarly, we have observed a 60% decrease in Nkx6.1 mRNA levels in islets of Zucker diabetic fatty (ZDF) rats relative to islets from lean control animals. Nkx6.1 usually functions as a transcriptional repressor; therefore, a decrease in its expression in islets during development of diabetes might lead to an increase in expression of other genes that cause impairment of beta cell function. Further study, including microarray analysis of Class 3 cells and normal islets with and without Nkx6.1 suppression as described in the next chapter of this dissertation, will be required to identify Nkx6.1 target genes that mediate GSIS, and to determine if such genes play a significant role in beta cell failure of diabetes.

Figures

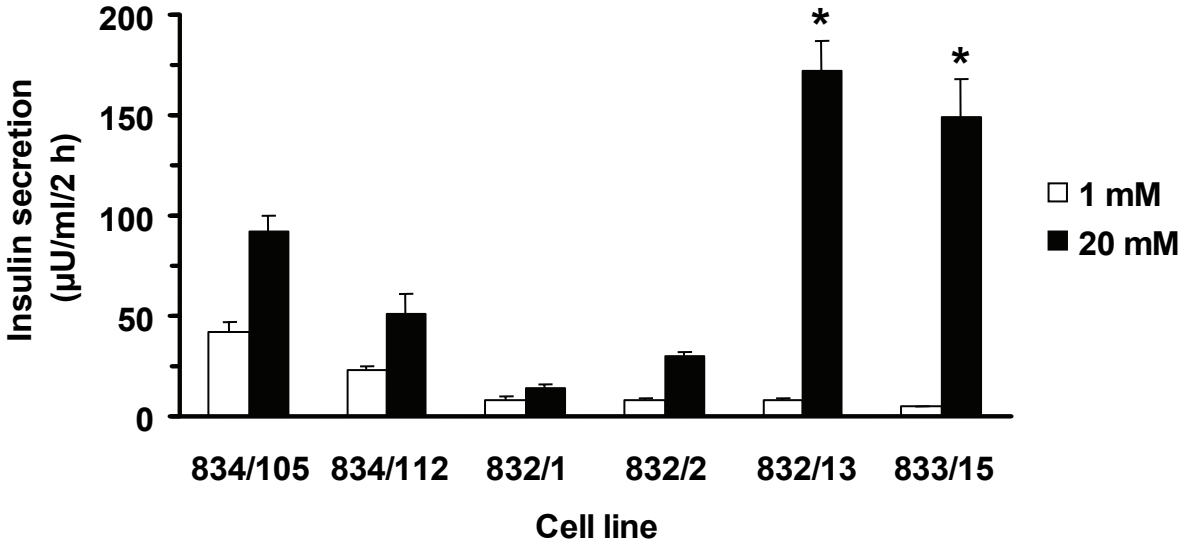
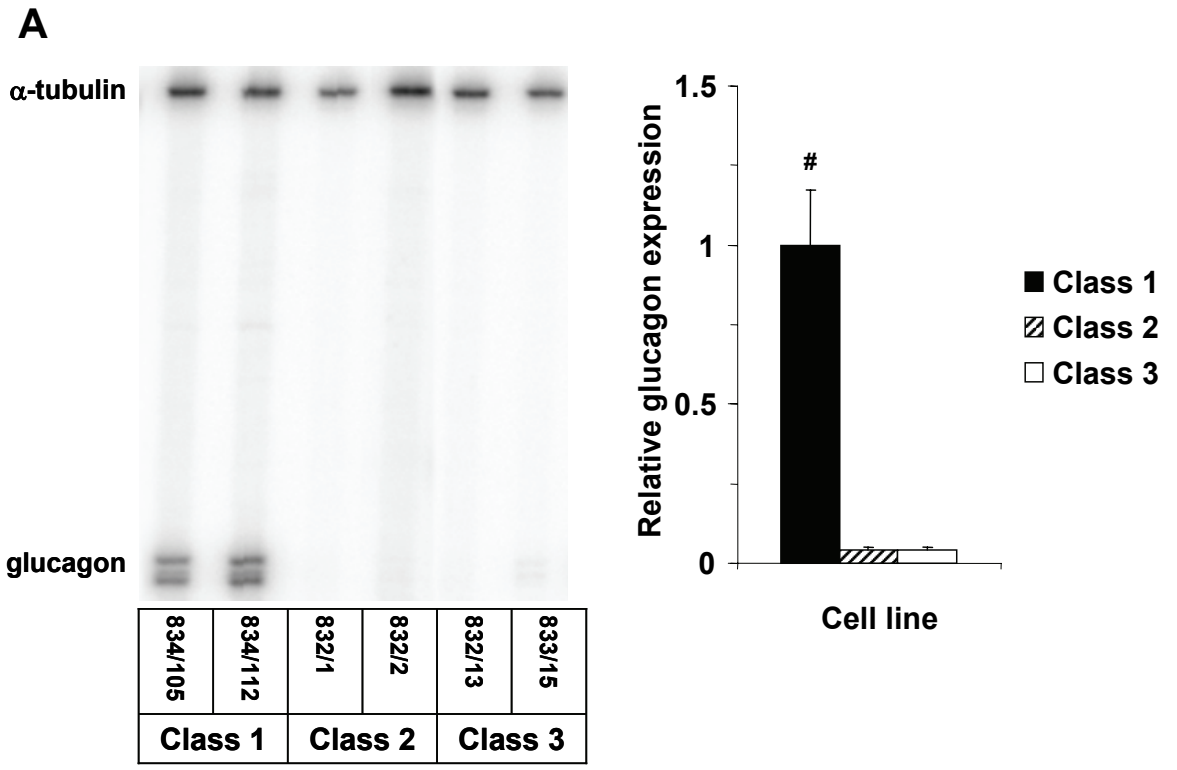
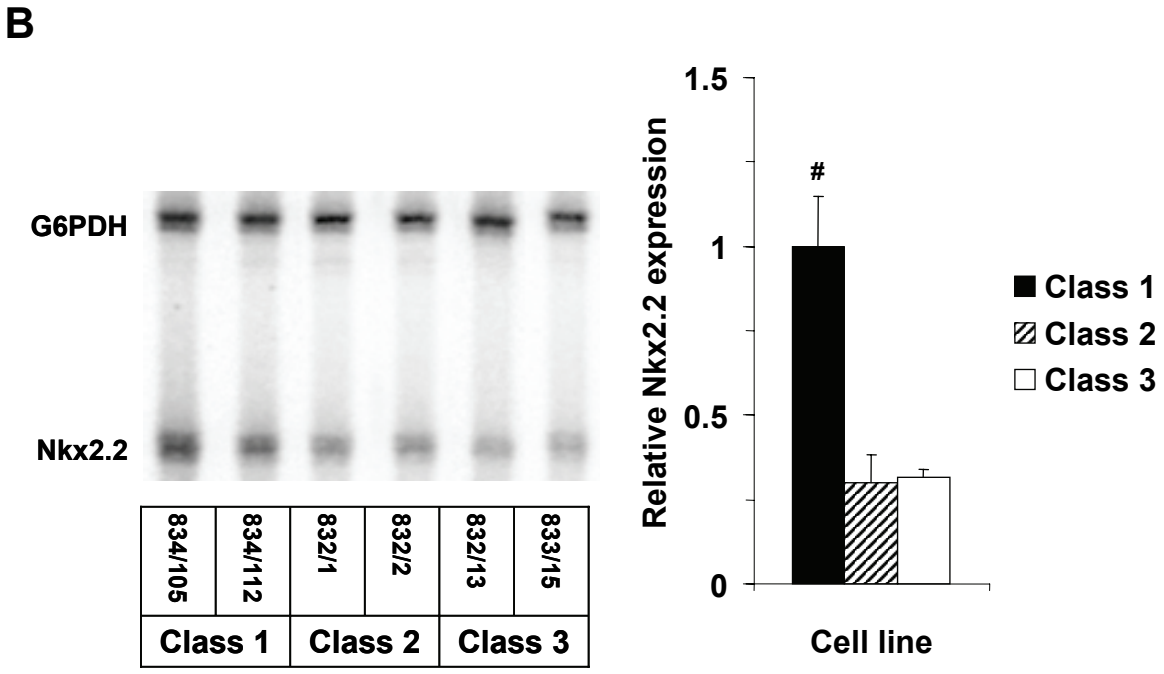


Figure 2-1. Glucose-stimulated insulin secretion from INS-1-derived cell lines
Insulin secretion was measured at 1 or 20 mM glucose in a 2 h static incubation assay in the indicated INS-1-derived cell lines. Data represent the mean ± SEM for 3-4 independent assays, each performed in triplicate. The symbol * indicates that cell lines 832/13 and 833/15 had larger fold responses to stimulatory glucose (insulin secretion at 20 mM glucose divided by insulin secretion at 1 mM glucose) than the other four cell lines, with $p < 0.0025$.

Hormones	Transcription Factors
Insulin	Brn4
Glucagon	E47
Somatostatin	HNF-1a
	IB1
Metabolic Enzymes	NeuroD
GL	***Nkx2.2***
PTG	***Nkx6.1***
G6Pase	Pax4
Glucokinase	Pdx1
GLUT2	
HK1	
HK2	
GDH	

Table 2-1. Candidate genes chosen for expression analysis
A panel of 20 genes, including the various islet hormones, various metabolic enzymes, and transcription factors implicated in beta cell function or differentiation were chosen for expression analysis. The glucagon gene and the two NK-family transcription factors, Nkx6.1 and Nkx2.2, were found to be differentially expressed.


A
α-tubulin
glucagon
834/105
834/112
832/1
832/2
832/13
833/15
Class 1
Class 2
Class 3
Relative glucagon expression
1.5
1
0.5
0
#
Cell line
Class 1
Class 2
Class 3


B
G6PDH
Nkx2.2
834/105
834/112
832/1
832/2
832/13
833/15
Class 1
Class 2
Class 3
Relative Nkx2.2 expression
1.5
1
0.5
0
#
Cell line
Class 1
Class 2
Class 3

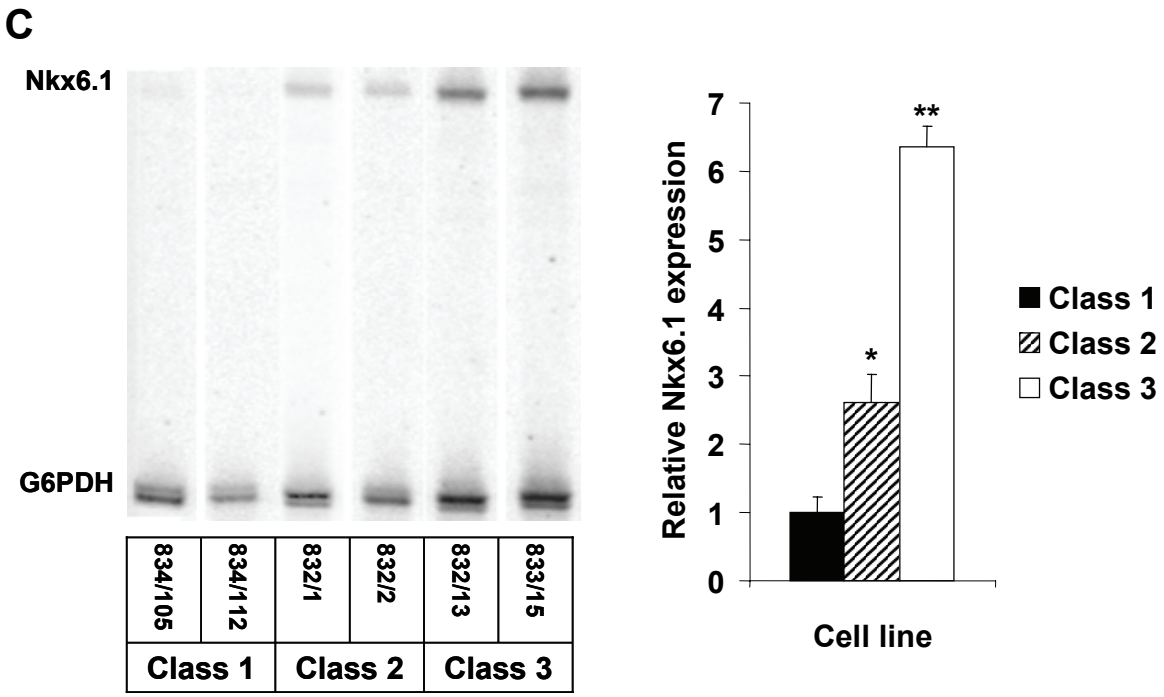
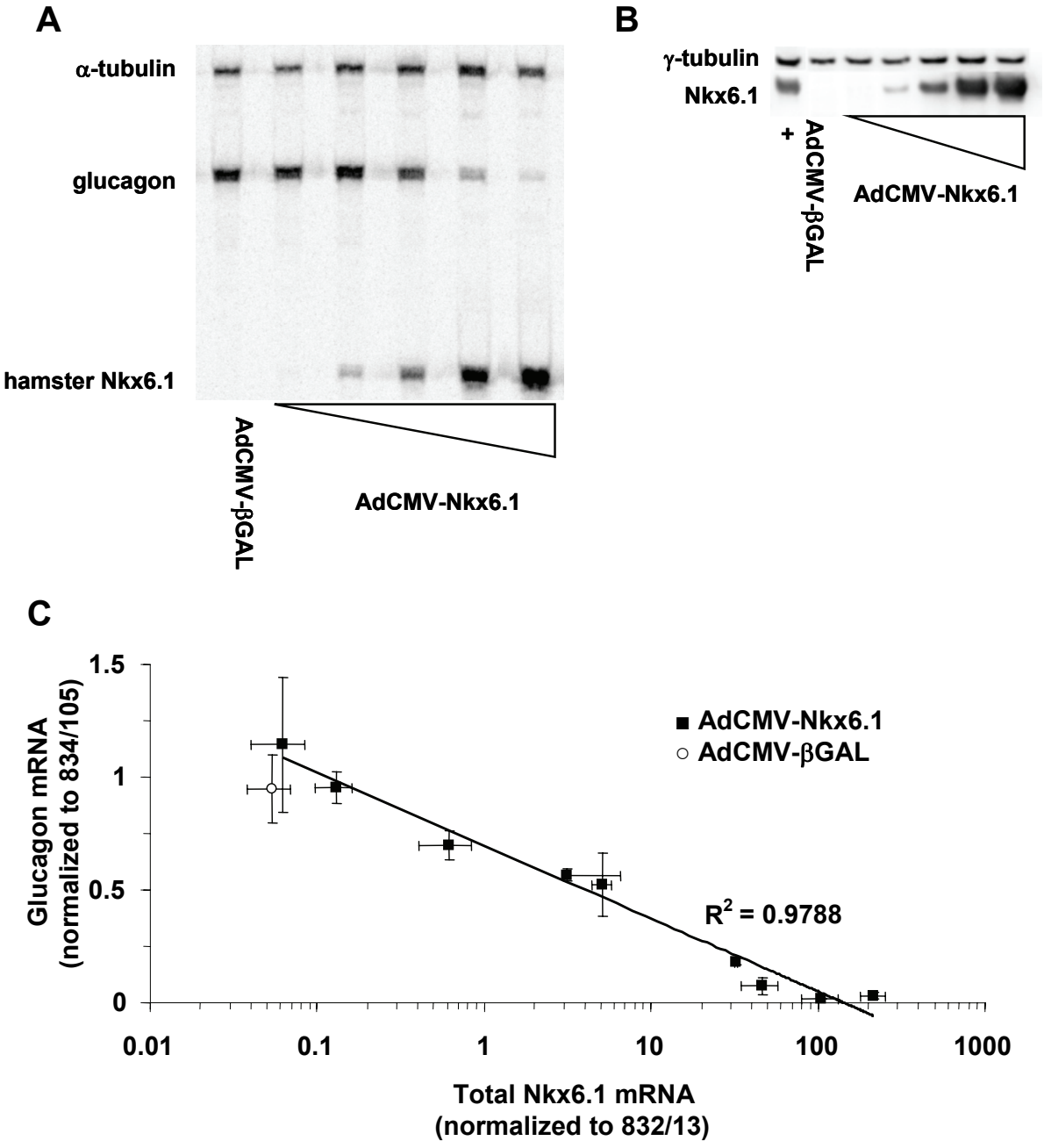
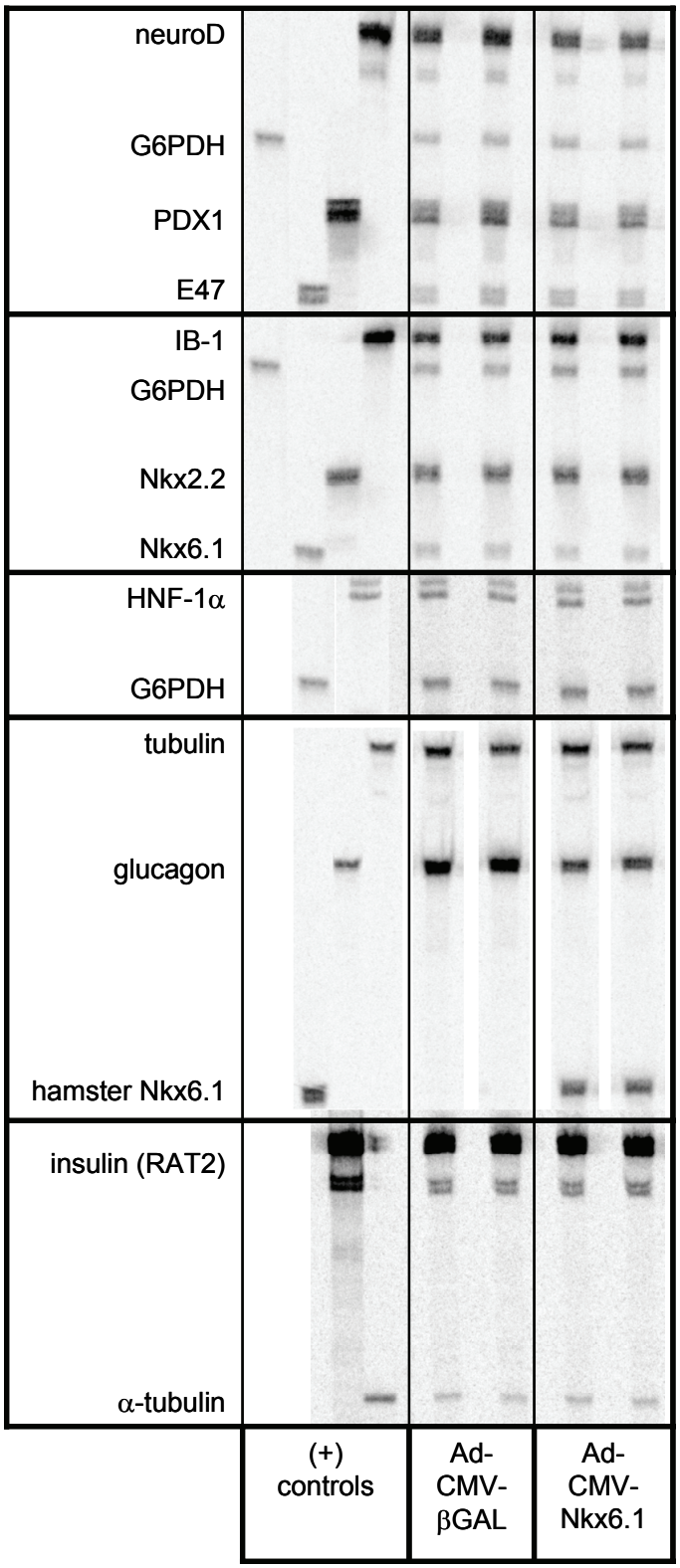


Figure 2-2. Differentially expressed genes in INS-1-derived cell lines.
Among 20 genes surveyed in INS-1-derived cell lines (Table 1), 3 were found to be differentially expressed: glucagon (**Panel A**), Nkx2.2 (**Panel B**), and Nkx6.1 (**Panel C**). Each panel contains a representative gel and a bar graph summary, with data in the bar graphs quantified by Phosphor-Imaging and representing the mean ± SEM for 3 independent experiments/cell line, each performed in duplicate. The symbol # in panels A and B indicate that Class 1 cells have more glucagon and Nkx2.2 mRNA, respectively than the other two classes of cells, with $p < 0.008$. In panel C, the symbol ** indicates that Class 3 cells have more Nkx6.1 mRNA than the other two classes, with $p < 0.001$, and the symbol * indicates that Class 2 cells have more Nkx6.1 mRNA than Class 1 cells, with $p < 0.05$.


A
α-tubulin
glucagon
hamster Nkx6.1
AdCMV-βGAL
AdCMV-Nkx6.1
B
γ-tubulin
Nkx6.1
+
AdCMV-βGAL
AdCMV-Nkx6.1
C
Glucagon mRNA
(normalized to 834/105)
1.5
1
0.5
0
AdCMV-Nkx6.1
AdCMV-βGAL
R2 = 0.9788
0.01
0.1
1
10
100
1000
Total Nkx6.1 mRNA
(normalized to 832/13)

D


neuroD
G6PDH
PDX1
E47
IB-1
G6PDH
Nkx2.2
Nkx6.1
HNF-1α
G6PDH
tubulin
glucagon
hamster Nkx6.1
insulin (RAT2)
α-tubulin
(+) controls
Ad-CMV-βGAL
Ad-CMV-Nkx6.1

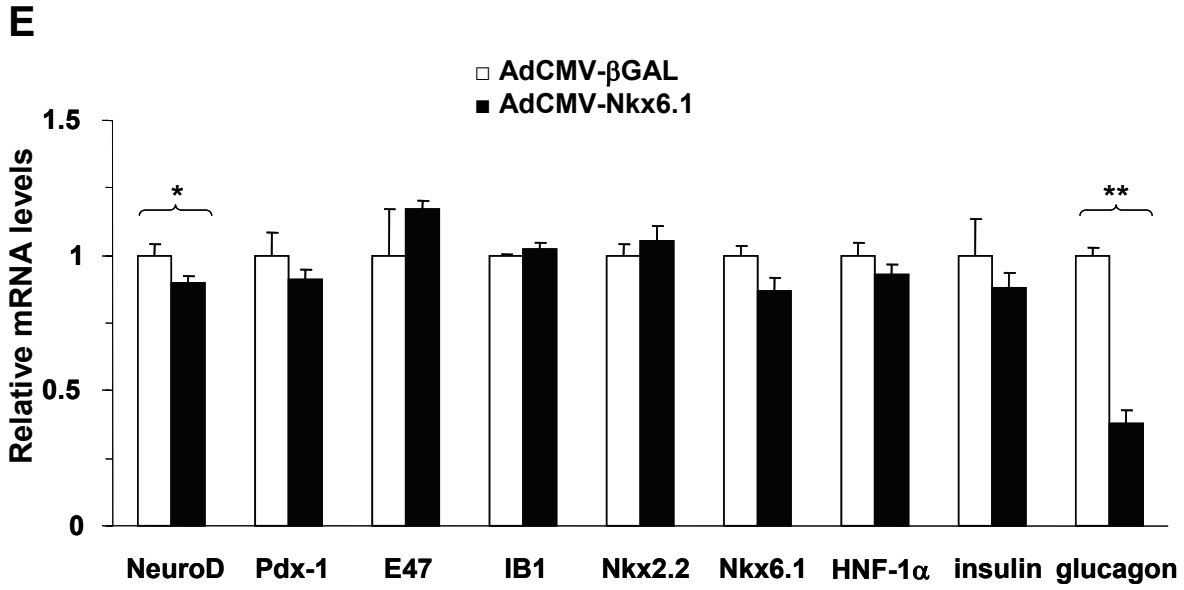


Figure 2-3. Overexpression of Nkx6.1 in Class 1 cells suppresses glucagon expression. Class 1 cells were treated with recombinant adenovirus containing the cDNA encoding hamster Nkx6.1 (AdCMV-Nkx6.1), or the β-galactosidase gene (AdCMV-βGAL). **Panel A** shows seminquantitative-multiplex PCR analysis of hamster Nkx6.1 and glucagon mRNAs, with α-tubulin as an internal control. **Panel B** shows immunoblot analysis of Nkx6.1 protein levels for the same range of viral titers as used in panel A. **Panel C** shows the dose-dependent effects of Nkx6.1 overexpression on glucagon mRNA levels quantified by real-time PCR. Data represent the mean ± SEM for 4 independent experiments per viral dose, with each measurement in duplicate. **Panel D** shows a representative semiquantitative-multiplex PCR analysis of a host of important beta cell transcription factors. **Panel E** summarizes the data shown in Panel D and shows that Nkx6.1 overexpression had little effect on expression of other beta cell factors except for glucagon gene expression. Data represent the mean ± SEM of 2 independent experiments, with each measurement in duplicate. The symbols * and ** indicate that Nkx6.1 overexpressing cells had less NeuroD or glucagon mRNA, with $p < 0.05$ and $p < 0.01$, respectively.

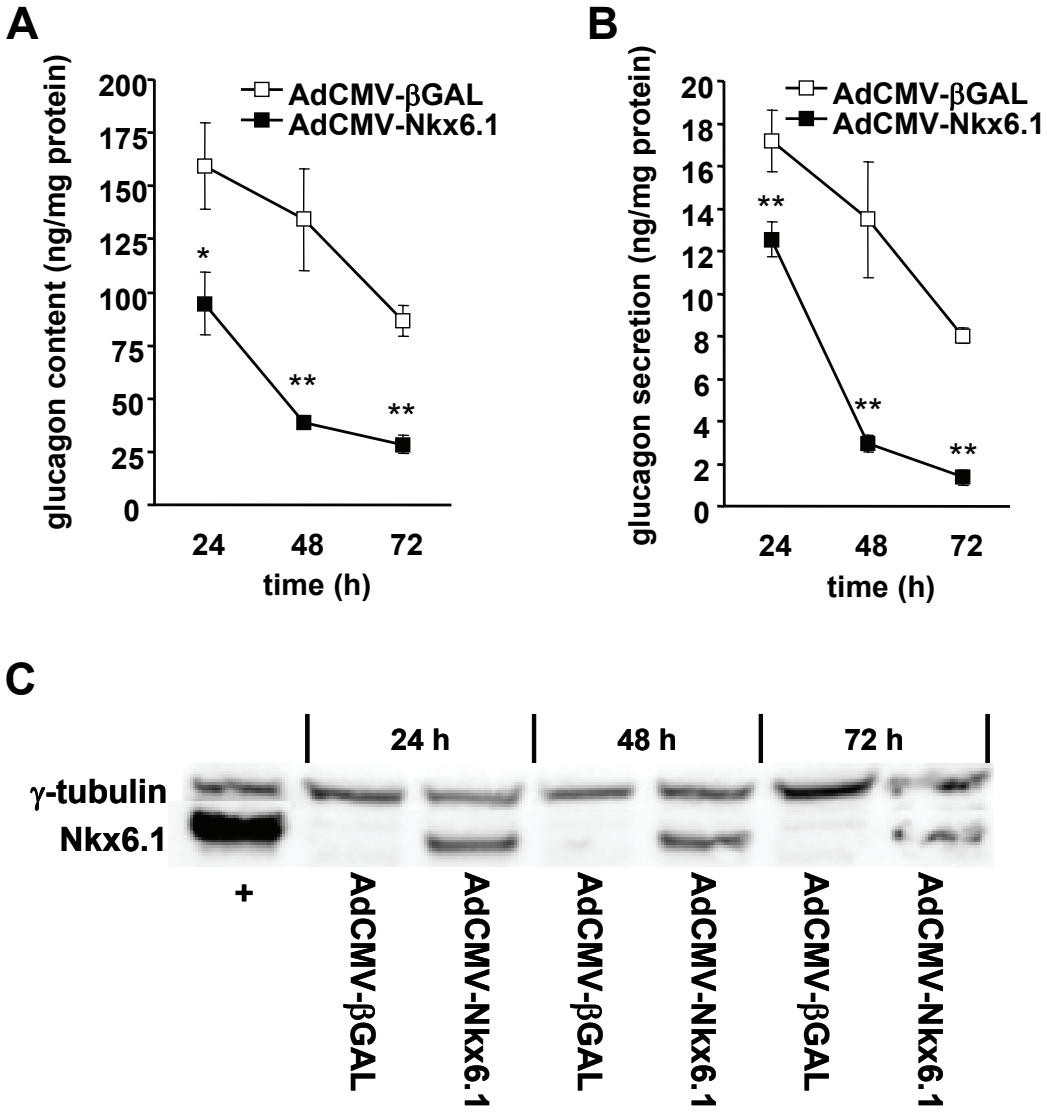


Figure 2-4. Effect of Nkx6.1 overexpression on glucagon content and secretion. **Panel A** shows the change in glucagon content in Class 1 cells measured every 24 h after treatment with either AdCMV-βgal or AdCMV-Nkx6.1. Data represent the mean ± SEM from 3 independent experiments with each time point measured in triplicate. **Panel B** shows the change in secreted glucagon in 24 hour intervals after treatment with either AdCMV-βgal or AdCMV-Nkx6.1. Data represent the mean ± SEM from 3 independent experiments with each time point measured from 6 replicates. The symbols * and ** indicate that Nkx6.1 overexpressing cells had lower glucagon content or secreted glucagon, with $p < 0.05$ and $p < 0.005$, respectively. **Panel C** shows a representative immunoblot confirming overexpression of the Nkx6.1 transgene throughout the time course of the experiments represented in Panels A and B.

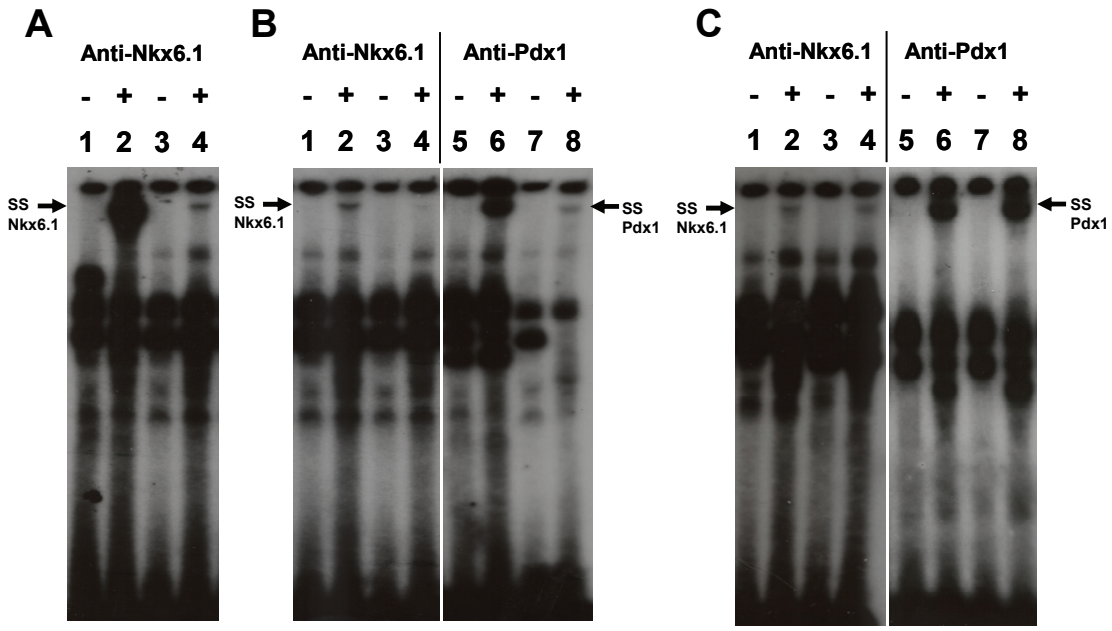


Figure 2-5. EMSA analysis of Nkx6.1 binding to the G1 element of the glucagon promoter.

Panel A. Nuclear extracts were prepared from 832/13 cells treated with AdCMV-Nkx6.1 (lanes 1 and 2) or no virus (lanes 3 and 4) and mixed with a radiolabeled oligonucleotide corresponding to the G1 element of the glucagon promoter. **Panel B.** Effects of Ad-siNkx6.1 (left) and Ad-siPdx1 (right) viruses on their target proteins in 832/13 cells. **Panel C.** Effects of Ad-siPdx1 on Nkx6.1 protein level (left) and of Ad-siNkx6.1 on Pdx1 protein level (right) in 832/13 cells. Nuclear extracts were prepared and mixed with a radiolabeled oligonucleotide corresponding to the G1 element of the glucagon promoter. Prior to gel electrophoresis, samples were left untreated or were treated with anti-Nkx6.1 or anti-Pdx1 antibodies as indicated above each panel. In panel B, lanes 1, 2, 5, and 6 are Ad-siLuc-treated, lanes 3 and 4 are Ad-siNkx6.1-treated, and lanes 7 and 8 are Ad-siPdx1-treated samples. In panel C, lanes 1, 2, 5, and 6 are Ad-siRNAcontrol-treated, lanes 3 and 4 are Ad-siPdx1-treated, and lanes 7 and 8 are Ad-siNkx6.1-treated samples. In all panels, samples were untreated (-) or treated (+) with anti-Nkx6.1 or anti-Pdx1 antibodies as indicated to cause "supershift" of the specific Nkx6.1/G1 (SS Nkx6.1) or Pdx1/G1 (SS Pdx1) complexes. Data are representative of 2-3 independent experiments.

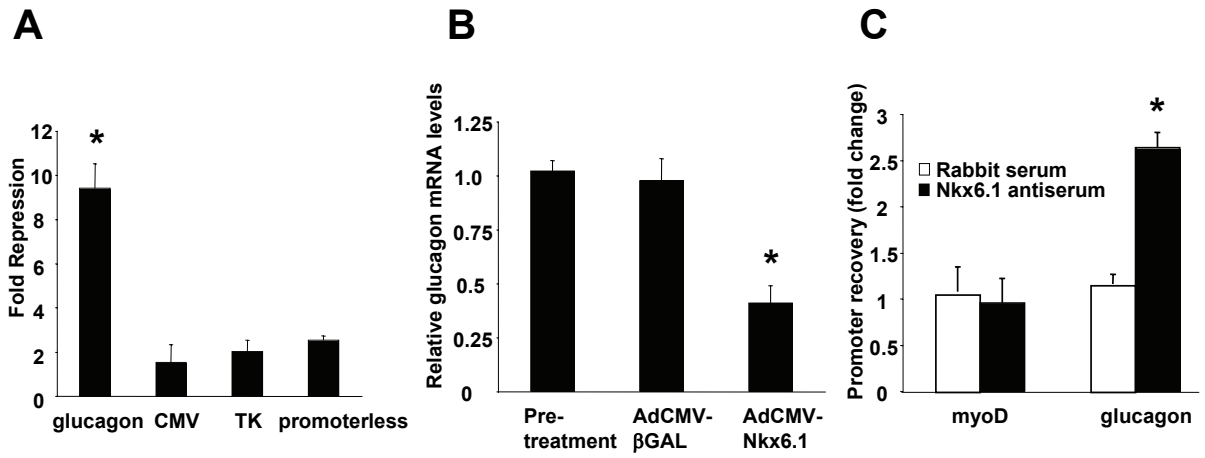
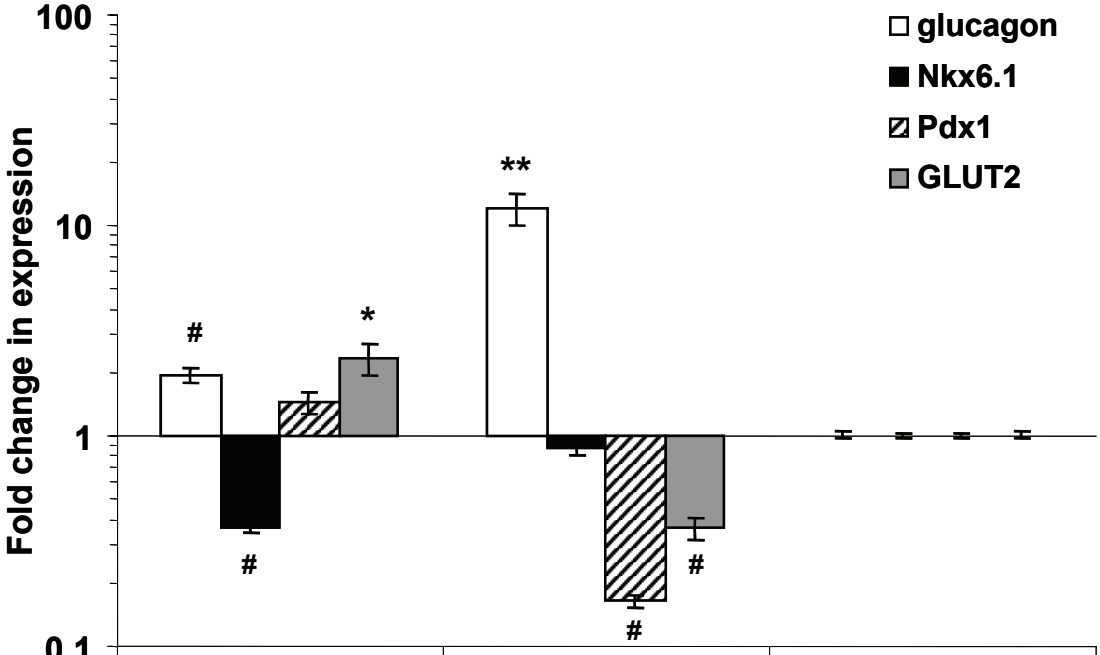


Figure 2-6. Suppression of exogenous and endogenous glucagon promoter function by Nkx6.1 and ChIP analysis.

Panel A. αTC1.6 cells were co-transfected with the pBAT12.Nkx6.1 plasmid expressing Nkx6.1 and plasmids containing the luciferase reporter under control of the glucagon promoter, CMV promoter, TK promoter, or no promoter (promoterless). Luciferase activity is expressed as fold repression relative to activity in cells co-transfected with the glucagon/luciferase reporter construct and the pBAT12 plasmid lacking a cDNA insert (empty vector). Data represent the mean ± SEM for 3 independent co-transfections. **Panel B.** Suppression of endogenous glucagon expression in αTC1.6 cells in response to adenovirus-mediated overexpression of Nkx6.1. Data represent the mean ± SEM for 2 independent experiments, each performed in triplicate. **Panel C.** Chromatin immunoprecipitation (ChIP) assay of Nkx6.1 binding to the endogenous glucagon promoter in a Class 3 cell line (832/3). Immunoprecipitation reactions were performed with non-specific rabbit serum or anti-Nkx6.1 antibody, followed by RT-PCR analysis of associated DNA with primers specific for the glucagon or myoD genes. Data are expressed as fold increase in recovery of input glucagon or myoD DNA by immunoprecipitation with the respective antibodies, and represent the mean ± S.E.M. for 5 independent experiments, each performed in triplicate. Primary data for these experiments (RT-PCR amplification curves) are available upon request from the authors. For all panels, the symbol * indicates significant differences, with $p < 0.01$.



	Ad-siNkx6.1	Ad-siPdx1	Ad-siRNAcontrol
glucagon	1.94	12.00	1.01
Nkx6.1	0.37	0.87	1.00
Pdx1	1.43	0.16	1.00
GLUT2	2.34	0.37	1.01

Figure 2-7. Effects of RNAi-mediated suppression of Nkx6.1 or Pdx1 expression in 832/13 cells.

832/13 cells were treated with Ad-siNkx6.1 (left group of bars), Ad-siPdx1 (center group of bars), or Ad-siRNAcontrol (right group of bars). The effect of these manipulations on glucagon, Nkx6.1, Pdx1, and GLUT2 mRNA levels was analyzed by real-time PCR. Data are normalized to the levels in Ad-siRNAcontrol-treated cells, and represent the mean ± SEM for 3-5 independent experiments, each performed in triplicate. Symbols denote statistically significant increases or decreases in mRNA levels, with $p < 0.05$ (*), $p < 0.01$ (**), or $p < 0.002$ (#). Numbers below the graph indicate the average fold-changes in expression of the indicated mRNAs in response to Nkx6.1 or Pdx1 suppression.

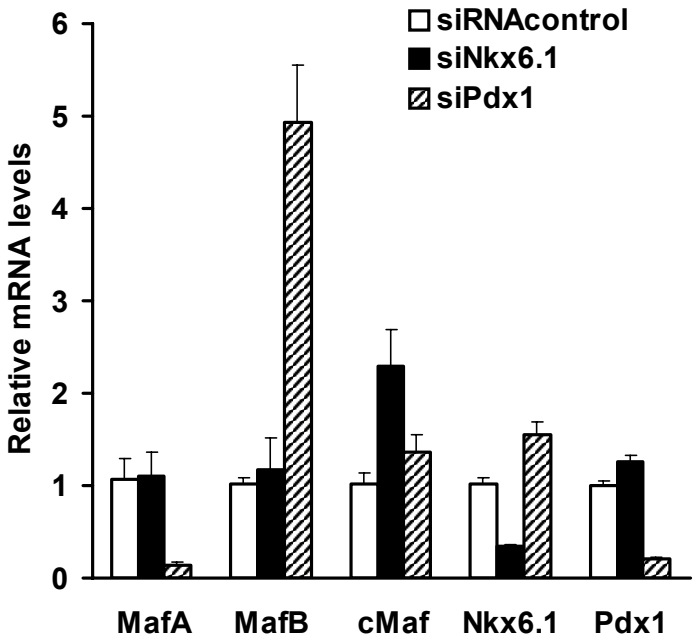


Figure 2-8. The effect of Nkx6.1 and Pdx1 silencing on Maf transcription factor expression.
Class 3 832/13 cells were treated with siRNA adenoviruses targeting either Nkx6.1 or Pdx1 mRNA or a random sequence control siRNA. Real-time PCR analysis compared the relative mRNA levels of MafA, MafB, and cMaf in cells with reduced Nkx6.1 and Pdx1 expression normalized to levels found in siRNAcontrol-treated cells from 3 independent experiments each performed in duplicate. The effectiveness of the siRNA-mediated silencing of the target genes is also shown. The symbols # and * indicates statistically significant changes with $p < 0.01$ and $p < 0.005$ respectively.

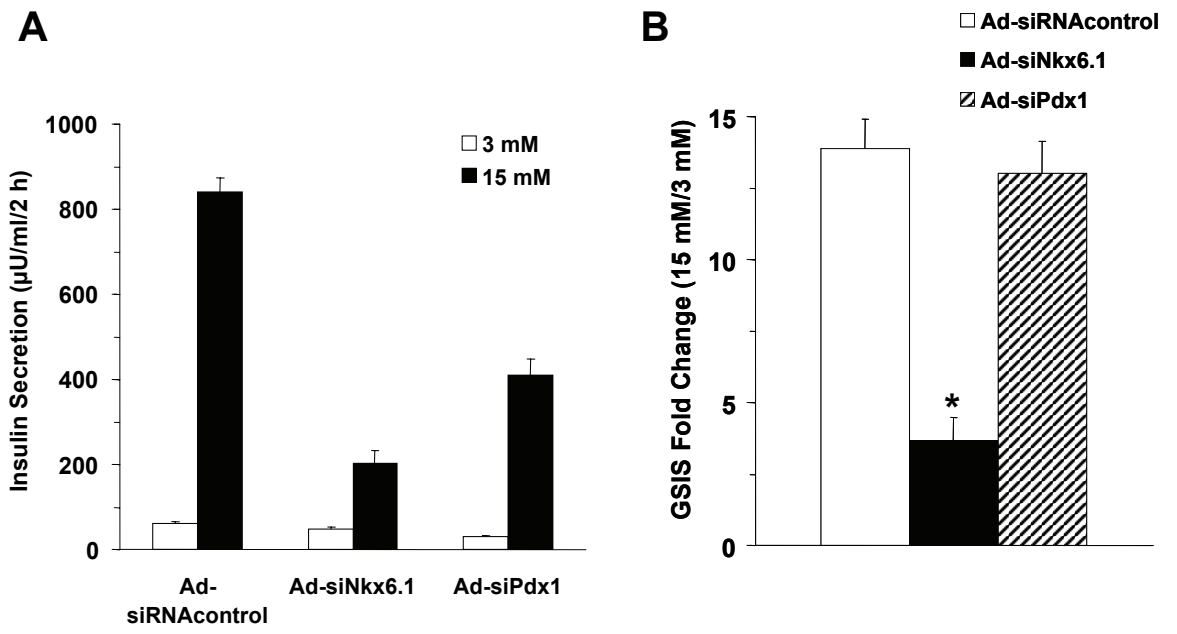


Figure 2-9. Effects of Nkx6.1 and Pdx1 suppression on GSIS in Class 3 cells. 832/13 cells were treated with the indicated siRNA-containing recombinant adenoviruses, followed by assay of insulin secretion in response to 3 or 15 mM glucose. **Panel A.** Insulin secretion expressed as µU/ml/2 h. **Panel B.** Insulin secretion expressed as fold response (insulin secretion at 15 mM glucose divided by insulin secretion at 3 mM glucose). Data represent the mean ± SEM for 3-5 independent experiments, each performed in triplicate. The symbol * indicates that fold response was decreased in Ad-siNkx6.1-treated cells, with $p < 0.001$.

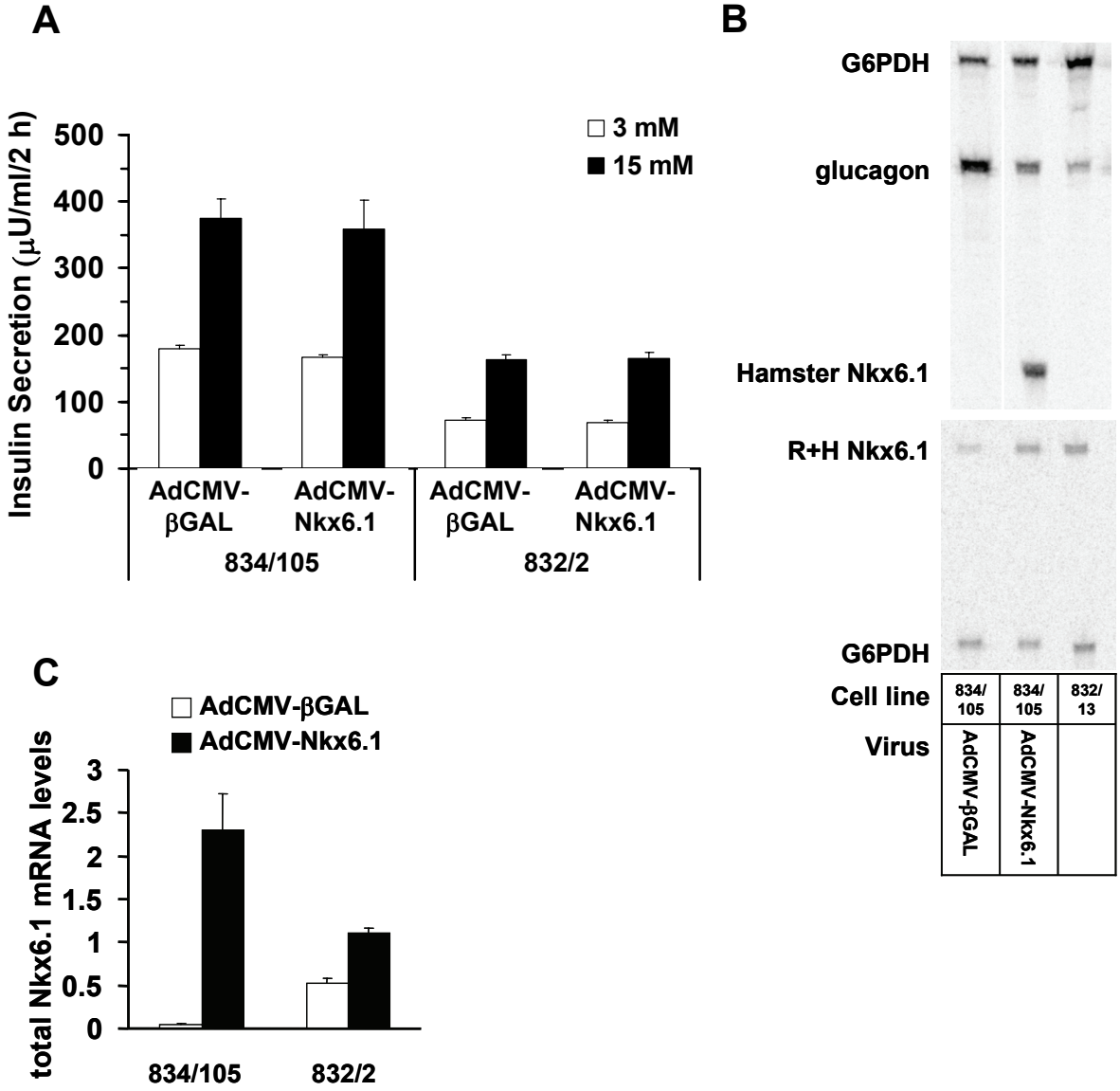


Figure 2-10. Overexpression of Nkx6.1 in Class 1 or Class 2 cells has no effect on glucose-stimulated insulin secretion.
Panel A. Class 1 (cell line 834/105) or Class 2 (832/2) cells were treated with AdCMV-βGAL or AdCMV-Nkx6.1, followed by assay of insulin secretion in response to 3 or 15 mM glucose. **Panel B**. Semi-quantitative multiplex PCR analysis of hamster Nkx6.1 mRNA (upper panel) and total (rat + hamster) Nkx6.1 mRNA (lower panel) in 834/105 cells treated with AdCMV-βGAL or AdCMV-Nkx6.1, and in untreated 832/13 cells. **Panel C**. Real-time PCR analysis of total Nkx6.1 mRNA in 834/105 or 832/2 cells treated with AdCMV-βGAL or AdCMV-Nkx6.1, relative to endogenous Nkx6.1 mRNA levels in untreated 832/13 cells.

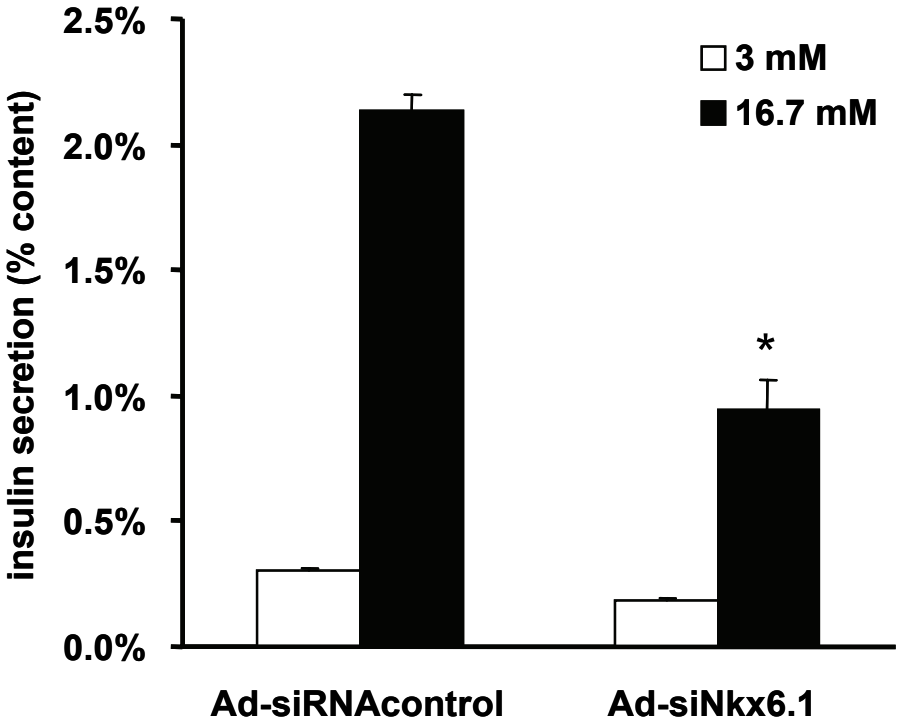


Figure 2-11. Suppression of Nkx6.1 expression impairs GSIS in rat islets.
Rat islets were treated with Ad-siNkx6.1 or Ad-siRNAcontrol viruses, resulting in a 46% decrease in Nkx6.1 mRNA in the Ad-siNkx6.1-treated cells compared to the controls. Data represent the mean ± S.E.M. for 3 independent experiments, with each insulin measurement performed in triplicate, and with data normalized to insulin content. The symbol * indicates that the fold response in Ad-siNkx6.1-treated cells was significantly reduced ($p = 0.012$). Moreover, insulin secretion at stimulatory glucose was decreased by 56% ($p = 0.001$).

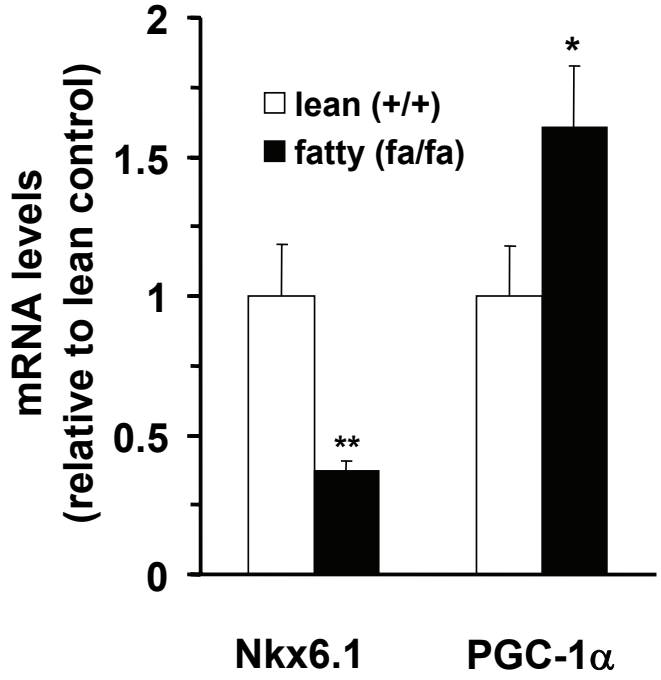


Figure 2-12. Real-time PCR analysis of Nkx6.1 and PGC-1α mRNA levels in islets from fatty (fa/fa) and lean (+/+) ZDF rats

Islets were isolated from 12-week old Zucker diabetic fatty (ZDF; fa/fa) or lean control (+/+) rats, and used for measurement of Nkx6.1 and PGC-1α mRNA levels by real-time PCR. Data represent the mean ± SEM for 6 independent measurements. The symbols * and ** indicate statistically significant changes with $p < 0.05$ and $p < 0.005$, respectively.

CHAPTER THREE

THE TRANSCRIPTION FACTOR NKX6.1 REGULATES ISLET BETA CELL PROLIFERATION AND REGULATES EXPRESSION OF CYCLIN B1

Introduction

The islets of Langerhans are comprised of at least four cell types that produce specific hormones, including insulin-producing beta cells and glucagon-producing alpha cells that play a critical role in maintenance of metabolic fuel homeostasis. Type 1 diabetes (insulin-dependent diabetes mellitus, IDDM) results from autoimmune destruction of the beta cells, whereas type 2 diabetes (non-insulin-dependent diabetes, NIDDM) involves loss of glucose-stimulated insulin secretion (GSIS) and a gradual diminution of beta cell mass (reviewed in (Weir and Bonner-Weir 2004). Insulin injection therapy has been the standard treatment for type 1 diabetes since the discovery of the hormone more than 80 years ago. Islet transplantation has been intensively investigated as an alternative approach to insulin replacement in patients with the type 1 disease, but a major obstacle to broad application of this therapeutic approach continues to be an inadequate supply of fully differentiated human islets (Hohmeier and Newgard 2005). Pharmacotherapy of type 2 diabetes includes administration of agents that enhance insulin secretion, but these drugs often lose efficacy over time and result in complications such as hypoglycemia (Lebovitz 2004). Thus, a more complete understanding of molecular and biochemical mechanisms involved in control of beta cell growth and function is required in order to develop more effective therapies for both major forms of diabetes.

Several members of the homeodomain family of transcription factors, including Pdx1, Hb9/Hlxb9, Nkx2.2, Nkx6.1, Isl-1, Pax6 and Pax4, are involved in the development of the endocrine pancreas, terminal differentiation of the various cell types, and maintenance of their differentiated functions (Reviewed in (Chakrabarti and Mirmira 2003; Melloul 2004; Habener *et al.* 2005). The transcription factor Nkx6.1 was initially isolated from a screen for homeodomain-containing transcripts in a hamster insulinoma cDNA library obtained from HIT cells (Rudnick *et al.* 1994). Subsequently, a functional role for Nkx6.1 was first described in development of the nervous system (Qiu *et al.* 1998), but also appears to be a critical factor in beta cell differentiation (Sander *et al.* 2000). Disruption of Nkx6.1 in transgenic mice results in a 90% decrease in functional beta cell mass with no apparent effect on development of other islet cell types (Sander *et al.* 2000; Henseleit *et al.* 2005). The dearth of beta cells in this model is attributed to the absence of the 'secondary transition', in which insulin-positive cells normally undergo rapid proliferation at approximately stage E15 in the developing mouse embryo.

The strong decrease in beta cell number that occurs in Nkx6.1 knock-out mice results in an inadequate number of cells for phenotypic characterization, leaving potential roles of Nkx6.1 in maintenance of mature beta cell function and growth unexplored. To address this issue, Chapter 2 described an alternative approach of adenovirus-mediated Nkx6.1 overexpression or delivery of a small interfering RNA (siRNA) specific for Nkx6.1 to mature beta cells, resulting in demonstration of new roles of the transcription factor in suppression of glucagon expression and control of GSIS (Schisler *et al.* 2005). In this chapter. we have expanded our analysis by investigation of the potential role of Nkx6.1 in beta cell

proliferation. We find that Nkx6.1 overexpression strongly stimulates thymidine incorporation, BrdU incorporation and cell replication in primary rat islets and beta cell lines, whereas siRNA-mediated suppression of Nkx6.1 has the opposite effect. We also demonstrate that the growth promoting effect of Nkx6.1 is mediated at least in part by an increase in cyclin B1 expression, caused by direct binding of Nkx6.1 to the cyclin B1 promoter.

Materials and methods

Cell culture conditions

The INS-1-derived cell lines 832/3, 832/13, 834/105, and 833/15 were prepared and cultured as previously described (Chen *et al.* 2000; Hohmeier *et al.* 2000). The culture medium was RPMI-1640 with 11.1 mM D-glucose supplemented with 10% fetal bovine serum, 10 mM HEPES, 2 mM L-glutamine, 1 mM sodium pyruvate, and 50 μM β-mercaptoethanol. Primary rat islets were maintained in RPMI-1640 with 8 mM D-glucose supplemented with 10% fetal bovine serum and 100 units/ml of penicillin, and 100 μg/ml streptomycin.

Use of recombinant adenoviruses for overexpression and siRNA mediated suppression of gene expression in INS-1-derived cell lines and primary islets.

For gene overexpression studies, recombinant adenoviruses containing the hamster Nkx6.1 cDNA (AdCMV-Nkx6.1), the bacterial β-galactosidase gene (AdCMV-βGAL), myc-tagged human cyclin B1 cDNA downstream of the tetracyclin operator (Ad-t-cyclin B1) and the tetracyclin activator (Ad-tA) were used as previously described (Becker *et al.* 1994; Jin *et al.* 1998; Schisler *et al.* 2005). These viruses were used to treat cell lines at a multiplicity of infection (MOI) ranging from 10 – 500 for 2 h followed by replacement of virus-containing media with normal growth media and further culture for 24 – 48 h.

For gene suppression studies, adenoviruses expressing small interfering RNAs (siRNAs) specific to rat Nkx6.1 (Ad-siNkx6.1) or a control siRNA with no known gene

homology (Ad-siRNAcontrol) were prepared as previously described (Bain *et al.* 2004; Schisler *et al.* 2005). The siRNA-expressing adenoviruses were use to treat INS-1-derived cell lines at an MOI of 10 – 30 for 16 h, followed by replacement of virus-containing media with fresh culture media and 80 additional h of tissue culture.

Pancreatic islets were harvested from male Spague-Dawley rats weighing approximately 250 g as previously described using the Liberase R1 enzyme (Roche) (Naber *et al.* 1980; Milburn *et al.* 1995). Approximately 200 primary rat islets per condition were cultured in 2 ml of RPMI treated with viruses at a concentration of 5×10^9 particles/ml media for 16 h. Virus containing media was replaced with fresh culture media and islets were cultured for an additional 80 h after treatment. All viruses were column purified (Vivapure AdenoPACK, Viva Science) and concentrations determined via OD_{260}.

Transfection of INS-1-derived cells with siRNA duplexes

To use AdCMV-Nkx6.1, AdCMV-βGAL, Ad-t-cyclin B1, and Ad-tA in combination with siRNA-mediated gene silencing in INS-1-derived cells, transfection of siRNA duplexes was used instead of siRNA-expressing adenoviruses to avoid viral toxicity. For siRNA-directed suppression of Nkx6.1 and Cyclin B1 expression, pre-annealed duplexes were obtained from Ambion (ID #195227 and 153133, respectively) and transfected into 832/3 cells via the Amaxa nucleofection system (Amaxa Inc) using 2 μg of duplex per 2×10^6 cells in T-solution. Cells were seeded at a density of 5×10^5 cells/ml and cultured for 48 h prior to treating with various adenoviruses as detailed in the figure legend. Suppression of the targeted genes was monitored by real-time PCR and immunoblotting.

^{3}H-thymidine incorporation and MTS assays

Relative change in DNA synthesis in INS-1-derived cell lines was measured similarly to previously described methods (Frodin *et al.* 1995; Hugl *et al.* 1998; Dickson *et al.* 2001). 2.5 x 10^{5} cells were seeded in 24-well plates and cultured in various conditions. ^{3}H-methyl thymidine (Amersham Biosciences) was added to the media at a final concentration of 1 μCi/ml during the last 4 h of cell culture. Cells cultured in 24-well plates were placed on ice and washed 3 times with cold serum-free RPMI-1640. DNA was precipitated by adding 1 ml of cold 10% trichloroacetic acid (TCA) followed by a 10 min incubation on ice and two additional precipitation steps with 0.5 ml of 10% TCA and 5 min incubation on ice. The precipitated DNA was solubilized by the addition of 250 μl of 0.3N NaOH and incubated at room temperature for 30-45 min. The amount of ^{3}H-thymdine incorporation into DNA was measured by liquid scintillation counting.

For primary rat islet studies, DNA synthesis rates were measured as described (Cozar-Castellano *et al.* 2004). ^{3}H-methyl thymidine was added to the media at a final concentration of 1 μCi/ml during the last 18 h of cell culture to pools of ~ 200 islets in various conditions. Groups of 30 islets were picked in triplicate from each condition and washed with 500 μl of cold RPMI-1640. Islets were centrifuged at 300 x G for 3 min at 4 °C and the wash was repeated twice. DNA was precipitated with 500 μl of cold 10% TCA and incubated on ice for 10 min. The precipitant was centrifuged at 15,000 x G for 3 min at 4 °C after which the precipitation step was repeated. The precipitated DNA was solubilized by the addition of 100 μl of 0.3N NaOH and incubated at room temperature for 30-45 min. The amount of ^{3}H-thymdine incorporated into DNA was measured by liquid scintillation

counting, and normalized to total cellular protein measured by the Bradford assay (Bradford 1976).

Cell viability was determined by the MTS assay as previously described (Malich *et al.* 1997). Briefly, 2.5 x 10^5 cells were seeded in 24-well plates and cultured in various conditions. The CellTiter 96 AQueous MTS Assay regent (Promega) was added directly to the culture media and incubated for the last hour of culture at 37°C. The reduction of the MTS tetrazolium compound was measured at an absorbance of 490 nm using a SpectraMax 340 plate reader (Molecular Devices). Data were normalized to the %MTS activity per well as well as %MTS activity per mg of protein of control conditions.

BrdU labeling and immunohistochemistry in primary rat islets

For BrdU labeling, a 1/100 dilution of BrdU labeling reagent (Invitrogen) was added to islet culture media in place of ^{3}H-thymidine for 18 h in various islet conditions as described above.

Preparation of islets for immunohistochemisty was performed as previously described (Cozar-Castellano *et al.* 2004). Pools of 200 islets were collected and washed three times with 1 ml of Dubelco's phosphate buffered saline solution (PBS) with centrifugation at 300 x G for 3 min between washes. Islets were fixed in Bouin's solution for 2 h 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 min and the majority of the formalin was removed by aspiration. 50 µl of Affi-Gel blue bead slurry (Biorad) was added to the islets to allow visualization of the islets 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 min 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 min. BrdU immunodetection was carried out using the BrdU detection kit (Invitrogen) which uses a biotinylated mouse anti-BrdU antibody (1 h incubation at room temperature). 3,3'-diaminobenzidine-tetra-hydrochloride (DAB) was used as the HRP substrate for colormetric detection.

For insulin and glucagon immunostaining, the HistoMouse-MAX kit (Invitrogen) was used with either prediluted guinea pig anti-insulin (Invitrogen) or rabbit anti-glucagon (Invitrogen) primary antibodies incubated for 1 h at room temperature. DAB (brown) or 3-amino-9-ethylcarbazole (AEC, red) were used as HRP substrates for secondary antibody colormetric detection. In the case of BrdU and insulin co-staining, detection of anti-insulin antibody was used with an alkaline phospatase-conjugated (AP) secondary antibody using Fast Red (Invitrogen) as the AP substrate. Where indicated in the figure legends, sections were lightly counterstained with hematoxylin.

Glucose-stimulated insulin secretion

Pools of 200 islets were treated with various recombinant adenoviruses and washed twice in 2 ml of HEPES balanced salt solution (HBSS) (114 mmol/l NaCl, 4.7 mmol/l KCl,

1.2 mmol/l KH_2PO_4, 1.16 mmol/l $MgSO_4$, 20mmol/l HEPES, 2.5mmol/l $CaCl_2$, 25.5 mmol/l $NaHCO_3$, and 0.5% bovine serum albumin [essentially fatty acid free], pH 7.2) containing 2.5 mM [2.5 mM what?]. Groups of 30 islets from each condition were picked in triplicate and placed in 2 ml of HBSS with 2.5 mM glucose in 6-well plates for 60 min. Islets were then placed in HBSS with 16.7 mM glucose for an additional 60 min. Media samples were analyzed by radioimmunoassay (RIA) with the insulin Coat-a-Count kit (Diagnostic Products, Los Angeles, CA) (Clark *et al.* 1997; Hohmeier *et al.* 1997).

Real-time PCR measurements of RNA levels at RT-PCR confirmation of adenoviral-mediated transgene expression

RNA was harvested from cells or from 20-50 primary rat islets using the RNeasy mini or micro kit (Qiagen), respectively, which included DNase treatment to eliminate genomic contamination. The iScript system (BioRad) was used for first strand synthesis of cDNA using 0.05 – 0.5 μg of RNA. Real-time PCR reactions were performed using the ABI PRISM® 7000 sequence detection system, software and reagents (An *et al.* 2004). Primers used with SYBR-Green PCR chemistry were used at final concentrations of 100 nM. Pre-validated primer and probe sets based on Taqman® chemistry (Applied Biosystems) were used as indicated in Appendix A. Triplicate reactions from independent RNA samples were carried out in a final volume of 25 μl containing 5 ng of cDNA template. RNA input was calibrated with 18S expression levels and relative mRNA levels were normalized to control conditions as indicated in the figure legends.

Confirmation of adenoviral-mediated overexpression of the hamster Nkx6.1 (AdCMV-Nkx6.1) and human cyclin B1 (Ad-t-cyclin B1) transgenes was performed via RT-PCR analysis. 5 ng of cDNA was used in 15 µl reactions (Invitrogen PCR blue supermix) with a final primer concentration of 100 nM. Primer sequences are listed in Appendix A. Reactions were activated for 2 min at 95 °C followed by 32 cycles at 95 °C for 15 sec, 53 °C for 15 sec, and 72 °C for 30 sec. 12 µl of each reaction was resolved on a 1.5% agaroge gel.

Immunoblot analysis

Aliquots of 5.0 µg of nuclear extract protein or 20 µg of whole cell extract protein were resolved on 10% Bis-Tris-HCl buffered (pH = 6.4) polyacrylamide gels (Invitrogen) and transferred to polyvinylidene difluoride (PVDF). Membranes were incubated with anti-Nkx6.1 (1:1000, Beta Cell Biology Consortium) or anti-Cyclin B1 (1:1000, Santa Cruz) antibodies overnight at 4^{o} C, or anti-γ-tubulin (1:10,000, Sigma) antibody for 2 h at room temperature. Antibodies were detected using appropriate HRP-linked secondary antibodies and visualized using ECL Advance (Amersham) on a Versadoc 5000 (Biorad).

Microarray analysis

RNA was prepared from the 832/13 cell line for microarray analysis. 5×10^5 cells were seeded into 6 cm plates and cultured overnight. Subsequently cells were treated with either Ad-siRNAcontrol or Ad-siNkx6.1 at an MOI of 20 for 16 h. Total RNA was harvested 72 h later using the RNeasy micro kit (Qiagen) which included DNase treatment to eliminate genomic contamination. Parallel experiments were performed to validate the GSIS

phenotype in cells treated with Ad-siNkx6.1 (Schisler *et al.* 2005) as well as to prepare protein samples.

Duplicate RNA samples (1 μg each) per condition from 3 independent experiments were used for one round of amplification and labeled with Cy5. These samples were hybridized with rat reference RNA labeled with Cy3 to a DNA chip containing the oligonucleotides from the rat operon v1.1 microarray (5600 rat genes, Operon) and the rat 10K OciChip (9715 rat genes, Ocimum) that was scanned on a Gene Pix 5000 scanner. Analysis of the data was performed using the Genespring v7.2 software (Silicon Genetics). Data was normalized using per chip and per spot intensity-dependent LOWESS normalization. Statistical analysis was performed using the software's cross-gene error model using 6 biological replicates per condition. Results of one-way ANOVA (parametric test, variances not assumed equal) were filtered for fold change (greater that 2-fold) and p-values less than 0.05 (Welch t-test).

Chromatin immunoprecipitation (ChIP) assays

ChIP assays were performed as detailed previously (Chakrabarti *et al.* 2002; Chakrabarti *et al.* 2003). Antisera used in the immunoprecipitations were either anti-Nkx6.1 antiserum or normal rabbit serum. Each ChIP assay was quantitated in triplicate by real-time PCR for recovery of either the rat cyclin B1 or myoD1 promoters. Three primer sets were used to amplify regions of the cyclin B1 gene. Forward and reverse primers (relative to the transcriptional start site) were, respectively: -1647 bp to -1521 bp 5'-GCTCTGCCATTTATCATCACTGG and 5'- TGACTGCCAAGCAAGGAAGC; -1606 bp

to -1467 bp 5'- CAGGCTTTCTGTAGCAGTGAGGTG and 5'-GGTTTCTGGTGTGTGTAGCGAAG; and -694 bp to -602 bp 5'-TCTCCTGCCCCTACCGTTTTAC and 5'- AACAGATAGCACCCAGACCCTCTC. Forward and reverse primers used to amplify the myoD1 gene were, respectively: 5'-CCACTTCGTCCTTGGCTCAAC and 5'- GGGATACCAGGCACAGCATAGG.

Statistical methods

Statistical significance was determined using a two-tailed Student's t-Test. P-values less than 0.05 were considered significant.

Results

Loss of Nkx6.1 expression decreases beta cell proliferation but not cell viability

To determine if Nkx6.1 expression is involved in the regulation of beta cell proliferation, the effect of siRNA-mediated silencing of Nkx6.1 mRNA on thymidine incorporation into genomic DNA was measured in the Class 3, robustly glucose-responsive INS-1-derived cell line, 833/15 (Chen *et al.* 2000; Schisler *et al.* 2005). Treatment with increasing amounts of Ad-siNkx6.1 resulted in an 84-96% decrease in Nkx6.1 mRNA compared to cells treated with Ad-siRNAcontrol, as measured by real-time PCR (Figure 3-1A). Thymidine incorporation decreased in proportion to the dose-dependent suppression of Nkx6.1 expression, with a maximum decrease of 86% (± 0.06) compared to control cells (Figure 3-1B). Similar findings were obtained when the same experiment was performed in an independent Class 3 cell line 832/3 (Hohmeier *et al.* 2000)(data not shown).

Thymidine incorporation is a direct measurement of DNA synthesis and an indicator of cell proliferation but may also reflect changes in cell viability. To determine if Nkx6.1 silencing altered cell viability, we employed the MTS assay (Malich *et al.* 1997), which measures viability via changes in mitochondrial function (Figure 3-1C). Under conditions where DNA synthesis was reduced by 69%, cell viability was unchanged relative to control cells (data normalized to %MTS/well). At the highest dose of Ad-siNkx6.1, which caused a decrease in thymidine incorporation of 86%, cell viability was decreased by 35% (%MTS/well) compared to control cells. Due to the large decrease in thymidine incorporation, the decrease in MTS activity may have been due to a significant decrease in

cell numbers. Consistent with this idea, normalization of the data to total protein (%MTS/mg protein) not only eliminates the decrease in MTS activity in response to Ad-siNkx6.1 treatment but actually causes a significant increase in MTS activity (145-174%) in all three Ad-siNkx6.1 conditions ($p < 0.001$). TThus, we conclude that Nkx6.1 has a specific effect on thymidine incorporation that is largely independent of effects on cell viability.

Nkx6.1 overexpression is sufficient to increase beta cell proliferation

Serum deprivation in cell culture systems, including INS-1-derived cell lines, have been shown to increase beta cell death and has been used to study mechanisms of beta cell proliferation (Hugl *et al.* 1998; Dickson *et al.* 2001; Ehses *et al.* 2003; Maestre *et al.* 2003). Class 1 INS-1-derived cell lines have relatively low levels of Nkx6.1 expression compared to Class 2 and Class 3 cell lines (Schisler *et al.* 2005). For this reason, the class 1 cell line, 834/105, was used to measure the effect of Nkx6.1 overexpression on beta cell proliferation.

As expected, the withdrawal of serum for 24 h resulted in a 402% ± 9.8% decrease in thymidine incorporation in cells treated with a control adenovirus (AdCMV-βGAL) (Figure 3-2A). To investigate the ability of Nkx6.1 expression to compensate for the decrease in proliferation seen with serum deprivation, cells were treated with a recombinant adenovirus containing the Nkx6.1 cDNA (AdCMV-Nkx6.1). Nkx6.1 protein is not detectable in control 834/105 cells, consistent with our prior work (Schisler *et al.* 2005), but a clear increase in Nkx6.1 protein is detected in whole cell extracts from cells treated with AdCMV-Nkx6.1 (Figure 3-2B). Overexpression was also validated via RT-PCR (Figure 3-2C), showing that the total amount of Nkx6.1 mRNA increases approximately 5-fold with AdCMV-Nkx6.1

treatment. As shown in Figure 3-2A, cells with overexpressed Nkx6.1 experienced only a 176.4% (± 7.4%) decrease in thymidine incorporation in response to serum withdrawal, a partial rescue relative to control (AdCMV-βGAL-treated) cells. The effect on Nkx6.1 overexpression to enhance thymidine incorporation was specific to serum-free conditions, as no increases were observed in Nkx6.1 overexpressing cells under normal culture conditions (10% serum).

Nkx6.1 overexpression strongly increases thymidine incorporation in primary rat islets

To begin to investigate the effect of manipulation of Nkx6.1 expression on DNA replication and cell growth in primary rat islets, we used recombinant adenovirus to overexpress Nkx6.1 expression. As shown in Figure 3-3A, treatment of primary rat islets with AdCMV-Nkx6.1 increased thymidine incorporation by 7.7 ± 0.7-fold versus cells treated with the AdCMV-βGAL control virus. RT-PCR analysis of RNA isolated from these islets confirmed an increase in Nkx6.1 mRNA of 5-fold in islets treated with AdCMV-Nkx6.1 compared to the AdCMV-βGAL-treated controls (Figure 3-3B). These results demonstrate a potent ability of Nkx6.1 to regulate thymidine incorporation in primary islets, which may reflect effects of the transcription factor on beta cell replication.

Overexpression of Nkx6.1 in islets increases BrdU immunoreactivity in beta cells

The increase in thymidine incorporation in Ad-Nkx6.1-treated rat islets in Figure 3-3A could be explained by stimulation of beta cell proliferation, but could also be due to replication of other islet cell types or non-islet fibroblastic cells. Also, whereas increases in

thymidine incorporation are generally indicative of activation of cell replication, more direct measures are required to validate this premise. To investigate these issues further, we measured uptake of the nucleotide analog 5'-bromo-2'-deoxyuridine (BrdU) into rat islets by immunohistochemistry. As shown in Figure 3-4A, islets treated with AdCMV-Nkx6.1 had a striking increase in the number of BrdU positive cells. Nearly all sections from islets overexpressing Nkx6.1 had multiple BrdU positive cells (range of 0-9 BrdU positive cells/section, average of 154 sections = 3.12) with many pairs of adjacent stained cells that appeared to have recently divided, whereas sections from untreated or AdCMV-βGAL-treated control islets usually had no BrdU positive cells (range of 0-4 BrdU positive cells/section, average of 213 sections = 0.39). The fold increase in the number of BrdU positive cells per section in islets treated with AdCMV-Nkx6.1 versus AdCMV-βGAL was 8.1 ± 1.3-fold. These data are well correlated with the large increase in thymidine incorporation shown in Figure 3-3A and support a robust increase in the number of islet cells undergoing cell division in response to Nkx6.1 overexpression.

To determine if the BrdU positive cells are beta cells, sections were co-stained with antibodies specific for BrdU and insulin. This analysis revealed that 79% of the BrdU positive cells in AdCMV-Nkx6.1-treated islets co-stained with insulin, showing that the majority of replicating cells were beta cells (Figure 3-4B). In contrast, the rare BrdU positive cells in control islets were comprised of a roughly equal number of insulin-positive and insulin-negative cell types. Taken together, the major effect of Nkx6.1 overexpression is to increase islet beta cell proliferation, consistent with the beta cell specific expression of the transcription factor in islet development.

Overexpression of Nkx6.1 does not impair GSIS or alter expression of key beta cell genes

In almost all cases reported to date, increases in beta cell replication caused by oncogene expression or growth factor and cell matrix manipulations are accompanied by loss of differentiated function, particularly decreases in insulin content and glucose-stimulated insulin secretion (Welsh *et al.* 1988; Beattie *et al.* 1991; Beattie *et al.* 1996; Yuan *et al.* 1996; Beattie *et al.* 1997; Laybutt *et al.* 2002). To determine if the growth promoting effects of Nkx6.1 overexpression are linked to similar functional derangements, we measured GSIS and insulin content in islets treated rat islets with AdCMV-Nkx6.1 or AdCMV-βGAL used in the ^{3}H-thymidine incorporation assay discussed previously (Figure 3-3A). Nkx6.1 overexpression was confirmed via RT-PCR (Figure 3-3B) as well as real-time PCR (data not shown). As seen in

Figure 3-5A, overexpression of Nkx6.1 caused no significant change in basal insulin secretion (measured at 2.5 mM glucose) compared to islets overexpressing β-galactosidase. However, Nkx6.1 overexpression caused a 46% increase in insulin secretion at a stimulatory glucose concentration (16.7 mM) (1398.5 ± 135.3 vs 958.8 ± 39.3 μU/mg protein, $p = 0.004$). Therefore, Nkx6.1 overexpression certainly does not impair beta cell function as measured by GSIS, and actually causes an increase in insulin secretion at stimulatory glucose concentrations.

It has been previously suggested that Nkx6.1 suppresses insulin expression (Mirmira *et al.* 2000; Iype *et al.* 2004; Taylor *et al.* 2005). These conclusions were based on the ability of Nkx6.1 to suppress a reporter gene with a portion of the insulin promoter cloned upstream

of a minimal promoter. In addition, chromatin immunoprecipitation (ChIP) analysis in beta cell lines has shown that Nkx6.1 interacts directly with the insulin promoter *in vivo*. Using primers that recognize *rat* insulin 2 gene products (Iype *et al.* 2005), we measured the effects of Nkx6.1 overexpression on insulin gene transcription. As shown in Figure 3-5B, neither mature or pre-mRNA species of insulin transcript are altered; the latter has been shown to reflect acute changes in insulin transcription due to its short half-life (8 min) (Iype *et al.* 2005). In addition, Nkx6.1 overexpression caused no significant changes in levels of Pdx1, GLUT2, or glucokinase mRNAs (data not shown). Taken together, these data indicate that overexpression of Nkx6.1 in primary rat islets does not impair expression of insulin or other key genes of the differentiated beta cell.

siRNA-mediated suppression of Nkx6.1 expression in primary rat islets suppresses thymidine incorporation and causes dramatic changes in islet cell morphology

To study the effects of Nkx6.1 suppression, we treated rat islets with a virus containing an siRNA specific to Nkx6.1 (Ad-siNkx6.1) or a control siRNA (Ad-siRNAcontrol) (Schisler *et al.* 2005). Treatment of islets with Ad-siNkx6.1 reduced the levels of Nkx6.1 mRNA by 56% ± 10% compared to islets treated with Ad-siRNAcontrol, as measured by real-time PCR. Strikingly, islets with reduced Nkx6.1 expression had a 58 ± 8% decrease in thymidine uptake relative to islets treated with Ad-siRNAcontrol (Figure 3-6A)

Despite the decrease in thymidine incorporation, islets with reduced Nkx6.1 expression were markedly ***enlarged*** relative to control islets (Figure 3-6B). To better understand this phenomenon, islet sections were stained with hematoxylin and eosin (Figure 3-6C). This analysis revealed that the larger, Ad-siNkx6.1-treated islets have a disorganized core of cells. In addition, there is an apparent increase in the size of individual cells in Ad-siNkx6.1-treated islets compared to Ad-siRNAcontrol-treated islets, suggesting that suppression of Nkx6.1 expression in mature beta cells results in islet hypertrophy, possibly secondary to interruption of the cell cycle. Immunohistochemical analysis of islets treated with Ad-siNkx6.1 revealed that the core cells of these islets do not express insulin (Figure 3-6C and Figure 3-6D) whereas control islets exhibit prominent insulin staining in the islet core (Figure 3-6D). Peripheral glucagon staining was consistent in comparing Ad-siRNAcontrol versus Ad-siNkx6.1 treated islets (Figure 3-6D). Additionally, the insulin-negative core of Ad-siNkx6.1-treated islets are also negative for glucagon expression. Taken together, these

results suggest that Nkx6.1 plays important roles in maintenance of normal islet cell architecture and islet cell size, in addition to its effects on islet cell replication.

Microarray analysis

To better understand the novel roles of Nkx6.1 to regulate GSIS (Schisler *et al.* 2005) and islet cell replication (this chapter), we performed microarray analysis on 832/13 cells treated with Ad-siNkx6.1 or Ad-siRNAcontrol. Duplicate RNA samples per condition from three independent experiments were collected and hybridized to a rat oligonucleotide array containing approximately 10,000 rat genes (details described in Methods). While originally described as a suppressor of gene expression, a recent study has indicated that Nkx6.1 may also serve as an enhancer, as Nkx6.1 stimulates its own expression (Iype *et al.* 2004). Our microarray analysis reveals that suppression of Nkx6.1 results in upregulated expression of 76 genes by two-fold or more, consistent with its role as a transcriptional repressor, but also results in suppression of an additional 38 genes by 50% or more, demonstrating a broader transcriptional activator role for this factor than previously realized (Table 3-1). Figure 3-7 confirms changes in expression in response to Nkx6.1 suppression for a number of selected transcripts, and also demonstrates that Nkx6.1 was effectively suppressed (by 66%) in these studies. The complete list of genes with the relative fold change and brief gene description is provided in Appendix B.

Nkx6.1 regulates cyclin B1 expression

In attempting to understand the effect of Nkx6.1 on islet beta cell replication, we noticed that suppression of Nkx6.1 results in a large decrease in cyclin B1 expression, in both the microarray and RT-PCR studies (Table 3-1 and Figure 3-7). Activation of cyclinB1-Cdk1 has been described as "the key molecular event required for initiation of mitosis", via its ability to promote the G2 to M phase transition (Nurse 1990; Pines and Rieder 2001). This led us to investigate whether modulation of Nkx6.1 expression regulates cyclin B1 expression. First, using samples described in Figure 3-1A and RT-PCR analysis, we found that Ad-siNkx6.1-mediated decreases in Nkx6.1 expression results in a dose-dependent decrease in cyclin B1 mRNA (Figure 3-8A). Second, serum deprivation results in a decrease in proliferation of 834/105 cells, as shown in Figure 3-2A. Compared to cells cultured in 10% serum, serum withdrawal resulted in a 73% decrease in cyclin B1 mRNA levels (Figure 3-8B). Also, cyclin B1 mRNA levels were increased in AdCMV-Nkx6.1-treated compared to AdCMV-βGAL-treated cells in both serum-free and 10% serum conditions, by 2.7 ± 0.2-fold and 1.7 ± 0.2-fold, respectively. Finally, overexpression of Nkx6.1 in serum-free conditions almost completely restored levels of cyclin B1 mRNA to levels found in control cells grown in 10% serum. Therefore, overexpression of Nkx6.1 increases cyclin B1 mRNA and restores the serum-dependent decrease in cyclin B1 mRNA levels in INS-1-derived cell lines.

To determine if changes in Nkx6.1 expression levels correlate to changes in cyclin B1 levels in primary rat islets, analysis of RNA samples from the islet proliferation experiments described in Figure 3-3 were analyzed for cyclin B1 transcript levels. As shown in Figure 3-

8C, overexpression of Nkx6.1 in primary islets results in a 9.4 ± 1.8-fold increase in cyclin B1 transcript levels. Taken together, these data suggest that Nkx6.1 is a positive regulator of cyclin B1 expression.

Chromatin immunoprecipitation of Nkx6.1 and the cyclin B1 promoter

The data in

Figure 3-8 suggests that Nkx6.1 contributes to the regulation of cyclin B1. To investigate this idea further, we first analyzed the sequence of the rat cyclin B1 promoter (GenBank Accession # F046121) for sequences homologous to a recently described Nkx6.1 enhancer sequence within the Nkx6.1 gene promoter (Iype *et al.* 2004). As shown in Table 3-2, there were multiple sequences that had the minimum -ATTT- core element necessary for Nkx6.1-mediated acitvation. However, three regions in the cyclin B1 promoter (-1787 to -1771, -1557 to -1541, and -1439 to -1423, relative to the start codon) had additional homology to sequence flanking the -ATTT- core found in the Nkx6.1 promoter (Table 3-2).

As shown in Figure 3-9A, these three regions were found to be flanked by sequence that was relatively high in GC content, another functional characteristic shared with the Nkx6.1 enhancer sequence (Iype *et al.* 2004). Conversely, the other targets (-518 to -502 and -119 to -103) are flanked by sequence relatively low in GC content. To determine which sites might interact with Nkx6.1, PCR primers were designed to target different regions of the cyclin B1 promoter (Figure 3-9B). The resulting ChIP analysis is shown in Figure 3-9C. There is a clear enrichment of a PCR product immunoprecipitated with Nkx6.1 antisera compared to control sera with primer set A and B (spanning the region from -1647 to -1467) , whereas no enrichment can be seen with primer set C (located in the region from -694 to -

602). Real-time PCR analysis controls for PCR efficiency and allows comparisons to be made across multiple PCR products (Chakrabarti *et al.* 2002; Chakrabarti *et al.* 2003). As shown in Figure 3-9D, real-time PCR analysis confirms a 2-fold enrichment in DNA only with primer sets A and B and no enrichment with primer set C.

Effects of cyclin B1 silencing and overexpression on beta cell proliferation

The foregoing results imply that Nkx6.1 may regulate beta cell proliferation via regulation of cyclin B1 expression. In order to investigate this putative growth regulatory pathway more directly, we transfected 832/3 cells with an siRNA duplex specific for rat cyclin B1 (siCyclin B1) or a control siRNA duplex with no known gene homology (siRNAcontrol). 48 h after the transfection, cells were treated with either AdCMV-βgal or AdCMV-Nkx6.1 and were maintained in 10% serum for the duration of the experiment. The effectiveness of the foregoing molecular manipulations was measured by real-time PCR. As shown in Figure 3-10A, cells transfected with siCyclin B1 exhibited a 55% decrease in cyclin B1 transcript compared to cell transfected with siRNAcontrol. A similar decrease in cyclin B1 transcript was measured in cells transfected with siNkx6.1 (46%), and in cells transfected with both siNkx6.1 and siCyclin B1 (56%). Levels of Nkx6.1 decreased 65% in cells transfected with siNkx6.1 compared to siRNAcontrol-transfected cells. Unexpectedly, Nkx6.1 mRNA was decreased by 46% in cells transfected with siCyclin B1. Immunoblot analysis of Nkx6.1 and cyclin B1 protein levels in nuclear extracts was consistent with the real-time PCR analysis (Figure 3-10B).

As shown in Figure 3-10C, silencing of cyclin B1 resulted in a 50% decrease in thymidine incorporation, comparable to the effects obtained with silencing of Nkx6.1. The effect of combined silencing of Nkx6.1 and cyclin B1 was similar to the effect of either targ*et al*one. In all cases, treatment with AdCMV-Nkx6.1 resulted in a partial rescue of thymidine incorporation.

Since treatment with AdCMV-Nkx6.1 rescues the impairment in proliferation seen in cells transfected with siCyclin B1 or siNkx6.1, we examined the effect of AdCMV-Nkx6.1 on cyclin B1 expression. Overexpression of Nkx6.1 is sufficient to restore levels of cyclin B1 mRNA in cells transfected with either siCyclin B1, siNkx6.1, or in combination, to levels found in control cells (Figure 3-10A). Thus, the effect of silencing Nkx6.1 on beta cell proliferation is mimicked by silencing of cyclin B1, and both effects can be overcome by overexpression of Nkx6.1. However, since Nkx6.1 expression also decreases with cyclin B1 silencing, this does not provide direct evidence that cyclin B1 mediates the effect of Nkx6.1 on proliferation.

We therefore tested the ability of cyclin B1 overexpression to rescue cells with decreased Nkx6.1 expression. To this end, 832/3 cells were transfected with an siRNA duplex targeting Nkx6.1. Subsequently, cells were treated with either a virus expressing the tetracycline activator (Ad-tA) alone, or Ad-tA in combination with a virus containing the human cyclin B1 cDNA (Ad-t-cyclin B1) under control of a tetracycline promoter (Jin *et al.* 1998). As shown in Figure 3-10, cells treated with Ad-tA and transfected with siNkx6.1 had a 32% decrease in thymidine incorporation relative to cells transfected with siRNAcontrol. In contrast, the siNkx6.1-mediated decrease in thymidine incorporation was only 3% in cells

overexpressing cyclin B1. Therefore, cyclin B1 overexpression compensates for the impaired proliferation in siNkx6.1-transfected cells. Confirmation of the knockdown of Nkx6.1 and overexpression of cyclin B1 from whole cell extracts is shown in Figure 3-10E.

Cyclin B1 overexpression can rescue proliferation in primary islets with reduced Nkx6.1 expression

Finally, we extended our analysis of the putative Nkx6.1/cyclin B1 growth regulatory pathway from INS-1-derived cell lines to primary cells. Rat islets were treated with Ad-siRNAcontrol or Ad-siNkx6.1 in the presence of Ad-tA or Ad-tA plus Ad-t-cyclinB1. As shown in Figure 3-11A, treatment of islets with Ad-siNkx6.1 decreased thymidine incorporation by 47% (± 3%), whereas overexpression of cyclin B1 was able to reverse the Ad-siNkx6.1-mediated decrease, bringing thymidine incorporation back to control levels. However, cyclin B1 overexpression in islets increased ^{3}H-thymidine incorporation by only 1.7-fold compared to islets treated with Ad-tA alone, as compared to a 7-fold increase in response to Nkx6.1 overexpression (Figure 3-3A). Overexpression of cyclin B1 was confirmed via RT-PCR. As shown in Figure 3-11B, human cyclin B1 can be detected only in RNA samples from islets treated with both Ad-tA and Ad-t-cyclin B1. A clear increase in total cyclin B1 levels can be seen with a primer set that recognizes both rat and human cyclin B1. These data suggest that cyclin B1 plays a major role in mediating the effect of Nkx6.1 to suppress islet proliferation, but that other factors may be involved in mediating the strong effect of Nkx6.1 overexpression on islet growth.

Discussion

Pancreatic islet beta cell mass is controlled by a dynamic balance between beta cell proliferation and cell death (apoptosis) (Topp *et al.* 2000; Bonner-Weir 2001). Diabetes occurs when this balance in disrupted by autoimmune-mediated beta cell destruction (type 1 diabetes) or a failure of beta cell mass to compensate for metabolic demand (type 2 diabetes). Gaining a better understanding of molecular mechanisms that regulate beta cell replication and survival is therefore of great relevance for development of new diabetes therapies. In the present study we provide evidence that the homeodomain transcription factor Nkx6.1 regulates beta cell proliferation, in part through transcriptional regulation of cyclin B1.

Prior studies provide evidence that homeobox transcription factors can regulate cell proliferation, although such a role has not been described previously for Nkx6.1 (reviewed in (Del Bene and Wittbrodt 2005)). These factors have heretofore been studied in the context of differentiation and development of other organ systems, including the nervous system, pituitary gland, and the eye. Both direct and indirect modes of regulation appear to be possible. For example, the homeobox gene Oct10 has been shown to regulate the cyclin D1 gene via direct interaction with its promoter (Magne *et al.* 2003), whereas the degradation of cyclin A, B1 and E proteins is mediated through protein-protein interactions with the homeobox protein ESXR1 (Ozawa *et al.* 2004). These studies provide broad precedent for the idea that Nkx6.1, a homeobox protein, might influence beta cell proliferation via direct or indirect regulation of cell cycle factors. Moreover, the generation of mice with targeted disruption of the Nkx6.1 gene resulted in a phenotype of pancreatic islets that retain only 6%

of the normal complement of beta cells (Sander *et al.* 2000). The decrease in beta cell mass was accompanied by a corresponding decrease in the number of BrdU+ cells, suggesting that the loss of Nkx6.1 resulted in a decrease in the capacity of beta cell precursors to proliferate and differentiate into insulin-expressing cells. However, the small number of mature beta cells in these animals precluded direct testing of a role for the transcription factor in control of beta cell replication.

In the current study, we show that Nkx6.1 regulates beta cell proliferation, as supported by several specific findings. First, siRNA-mediated silencing of Nkx6.1 expression results in a dramatic decrease in proliferation in both beta cell lines and primary rat islets, as measured by thymidine incorporation into genomic DNA. Second, overexpression of Nkx6.1 restores the proliferative capacity of serum deprived INS-derived cells, a condition that normally causes cessation of cell growth and beta cell death. Third, overexpression of Nkx6.1 in normal rat islets increases thymidine incorporation by 7-fold. Moreover, immunohistochemical measurements of BrdU incorporation confirm the increase in primary cell replication, and demonstrate that most if not all of the proliferating cells are beta cells. These data demonstrate a novel role of Nkx6.1 in control of islet beta cell growth.

Importantly, the Nkx6.1-mediated increase in beta cell proliferation does not impair beta cell function, as evaluated by studies of glucose-stimulated insulin secretion and measurement of expression levels (mRNA) of several important beta cell genes. In fact, overexpression of Nkx6.1 causes insulin secretion to be significantly increased at stimulatory glucose levels, with no change in insulin content, suggesting a specific enhancement of the secretory response. This is consistent with our previous work in which we demonstrated that

siRNA-mediated suppression of Nkx6.1 expression causes pronounced impairment of GSIS in both primary islets and beta cell lines, with this effect also being independent of changes in insulin mRNA or content (Schisler *et al.* 2005).

To identify potential Nkx6.1 target genes that contribute to proliferation and GSIS, we performed microarray analysis of 832/13 cells treated with Ad-siNkx6.1 compared to Ad-siControl. This analysis revealed changes in expression of 114 genes of ≥ 2-fold (both up- and down-regulated), of which 10 genes had ontology classifications related to proliferation (biological process or molecular function that included *cell cycle*, *cell division*, *proliferation* or *apoptosis*): Bnip3, Neuodap1, Ptma, Cdc10, cyclinB1, Itgb1, Nbl1, Sh3kbp1, Vdup1, and Bdnf. This analysis may not provide the full spectrum of proliferation-related and Nkx6.1-regulated genes, since the data were obtained from a transformed cell line. Similar experiments in primary cells are in progress, and may yield other relevant targets.

From the genes identified in the microarray study, we chose to focus on cyclinB1-Cdk1, as its activation has been described as "the key molecular event required for initiation of mitosis" (Nurse 1990; Pines and Rieder 2001). CyclinB1 is activated by a Cdk25 phosphatase, and is translocated from the cytosol to the nucleus in response to phosphorylation of several serine residues within its cytoplasmic retention sequence (Jackman *et al.* 2003). It functions to promote the G2 to M phase transition. Oddly, a literature search reveals no papers exploring the consequences of manipulation of cyclin B1 in islet beta cells. Moreover, whereas there are numerous examples of regulation of cell cycle regulatory genes, including cyclins, by homeodomain transcription factors (Del Bene and Wittbrodt 2005), such activity has never been described for Nkx6.1. Instead, recent

studies on insulin receptor-mediated events in beta cells have revealed a growth promoting effect of Akt-1/protein kinase B (Bernal-Mizrachi *et al.* 2001; Tuttle *et al.* 2001). The effects of Akt-1 seem to involve activation of the cyclin-dependent kinase Cdk-4, which interacts with the D cyclins. Other studies have confirmed that transgenic manipulation of D cyclins and/or Cdk-4 affect islet growth, and that cyclins D1 and D2 are essential for postnatal expansion of beta cell mass (Rane *et al.* 1999; Georgia and Bhushan 2004; Kushner *et al.* 2005). These findings do not preclude a critical role for cyclin B1, which sits upstream of the D cyclins in the process of mitotic cell division.

The studies summarized herein demonstrate that Nkx6.1 is an activator of cyclin B1 transcription and interacts directly with the cyclin B1 promoter. In addition, our findings strongly suggest that the stimulatory effects of Nkx6.1 on beta cell proliferation are mediated, at least in part, through the ability of the transcription factor to regulate cyclin B1 expression. Data supporting this statement include: 1) RNAi-mediated suppression of Nkx6.1 results in a decrease in cyclin B1 mRNA in beta cell lines; 2) overexpression of Nkx6.1 causes a dramatic increase in cyclin B1 mRNA levels in cell lines and primary islets; 3) in beta cell lines, siRNA-mediated suppression of cyclin B1 expression closely mimics the effect of Nkx6.1 silencing, in that both maneuvers causes equivalent decreases in thymidine incorporation; 3) furthermore, cyclin B1 overexpression partially restores the decrease in proliferation in response to suppression of Nkx6.1 in both cell lines and primary rat islets. The partial restorative effects observed in this last set of studies may indicate the involvement of factors in addition to cyclin B1 in Nkx6.1-mediated beta cell proliferation, and the identity of such factors is the topic of ongoing research in our laboratory.

In sum, we have described a novel role for the transcription factor Nkx6.1 in regulation of beta cell proliferation and have identified cyclin B1 as a direct Nkx6.1 target gene that participates in this response. In a broader context, these findings suggest that modulation of Nkx6.1 expression or activity, or alteration in Nkx6.1 target gene expression, could play a role in the etiologies of both type 1 and type 2 diabetes, where dramatic changes in beta cell mass and function are at the heart of both diseases. From the therapeutic perspective, development of methods for expansion of islet beta cell mass has been a long sought-after but highly elusive goal (Hohmeier and Newgard 2005). Strategies ranging from oncogenic transformation to application of growth factors and use of specific tissue culture matrices have been applied, but have resulted almost universally in loss of differentiated function in inverse proportion to success in promoting replication (de la Tour *et al.* 2001; Beattie *et al.* 2002). Surprisingly, Nkx6.1 seems to be a gene with growth promoting properties that also contributes to maintenance of the mature beta cell phenotype, an ideal combination for enhancing beta cell function and mass in type 2 diabetes. To date there have been only two other factors that has been ascribed these dual roles--hepatocyte growth factor (HGF) and Cyclin D1/Cdk4 have been shown to increase beta cell replication while enhancing or maintaining secretory function, respectively (Garcia-Ocana *et al.* 2000; Garcia-Ocana *et al.* 2001; Garcia-Ocana *et al.* 2003; Cozar-Castellano *et al.* 2004; Hino *et al.* 2004; Marzo *et al.* 2004). The relationship of the effects of HGF signaling to the new regulatory pathway described herein, the impact of Nkx6.1 overexpression on cyclin D1/Cdk4 activity, and the practical implications of gene regulatory networks that can regulate growth of mature islet cells seem deserving of further investigation.

Figures

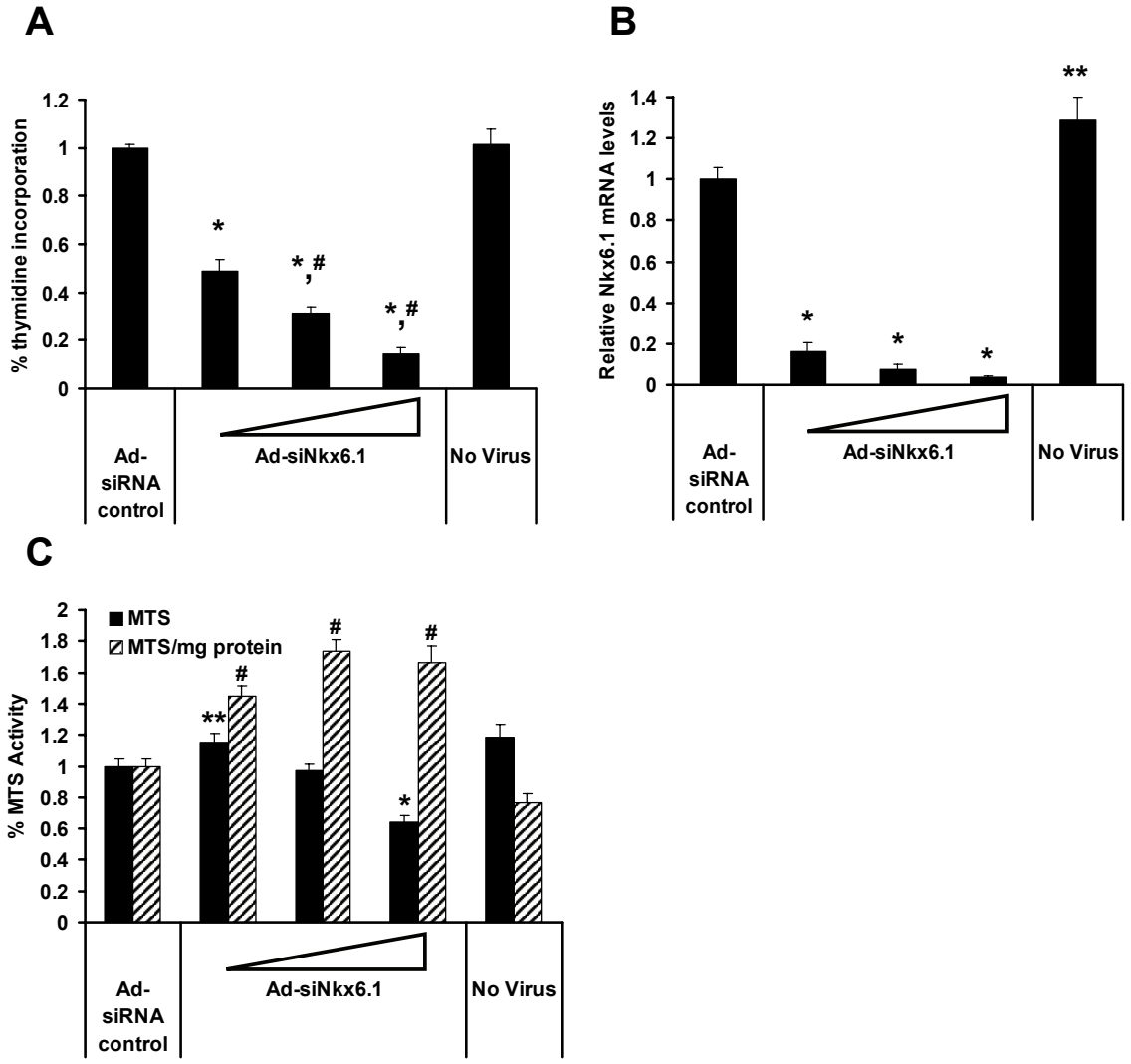


Figure 3-1. siRNA-mediated suppression of Nkx6.1 decreases beta cell proliferation. 833/15 cells were treated with Ad-siRNAcontrol, increasing amounts of Ad-siNkx6.1, or left untreated, followed by assays of proliferation, gene expression, and cell viability. **Panel A** shows that increasing amounts of Ad-siNkx6.1 results is a dose-dependent decrease in thymidine incorporation into genomic DNA. The symbol * indicates a statistically significant change between cells treated with Ad-siRNAcontrol compared to cells treated with Ad-siNkx6.1 with $p < 0.001$. The symbol # indicates a statistically significant change between increasing doses of Ad-siNkx6.1 with $p < 0.01$. **Panel B** shows the level of Nkx6.1 mRNA with increasing amounts of Ad-siNkx6.1 treatment relative to Ad-siRNAcontrol treated cells. The symbols * and ** indicate a statistically significant change compared to

Ad-siRNAcontrol-treated cells with $p < 0.0001$ and $p < 0.05$. **Panel C** shows the effect of Ad-siNkx6.1 treatment on cell viability as measured by the MTS assay. Data is represented as %MTS activity/well or %MTS activity/mg protein. The symbols * and ** indicate a statistically significant change in %MTS activity/well compared to Ad-siRNAcontrol with $p < 0.001$ and $p < 0.05$. The symbol # indicates a statistically significant change in %MTS activity/mg protein compared to Ad-siRNAcontrol with $p < 0.005$. All data represent the mean ± SEM from 3 independent experiments performed in duplicate.

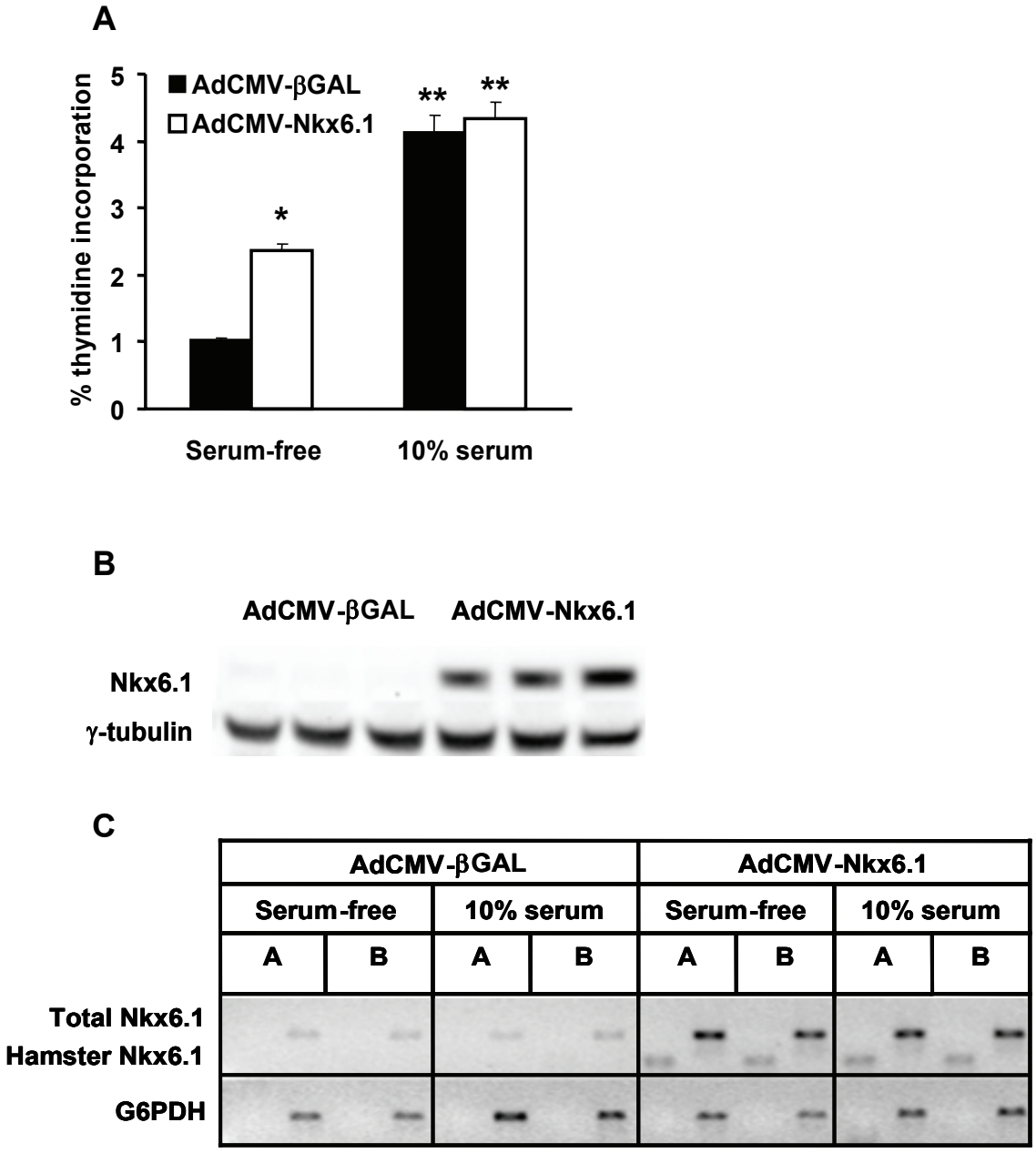


Figure 3-2. Nkx6.1 overexpression in sufficient to increase beta cell proliferation. 834/105 cells were treated with either AdCMV-βGAL or AdCMV-Nkx6.1, cultured in media with our without10% serum and assayed for changes in proliferation. **Panel A** shows the effect of serum deprivation and Nkx6.1 overexpression (AdCMV-Nkx6.1) on thymidine incorporation. The symbol * indicates a statistically significant change between AdCMV-βGAL and AdCMV-Nkx6.1-treated cells in serum-free conditions with $p < 0.0001$. The symbol ** indicates a statistically significant change in serum-free versus 10% serum

conditions with $p < 0.0001$. **Panel B** is a representative immunoblot confirming overexpression of Nkx6.1 protein in whole cell extracts, including γ-tubulin as an input control. **Panel C** shows the RT-PCR confirmation of Nkx6.1 overexpression and an increase in total Nkx6.1 from two independent RNA samples (A, B) in various conditions. The primer sets shown here detect hamster-Nkx6.1, rat plus hamster Nkx6.1, and G6PDH.

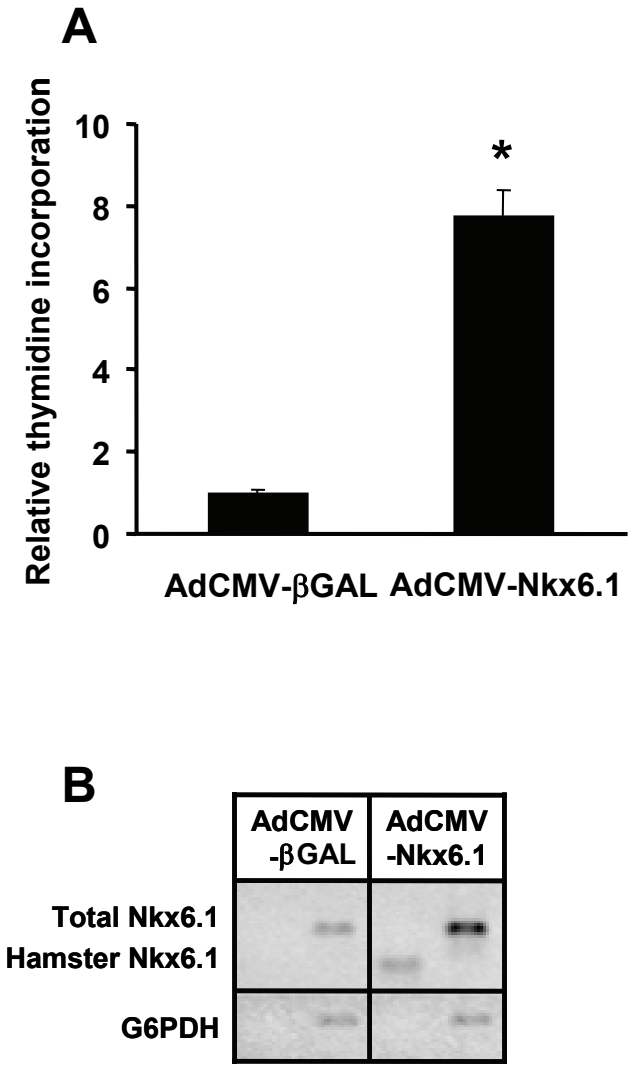
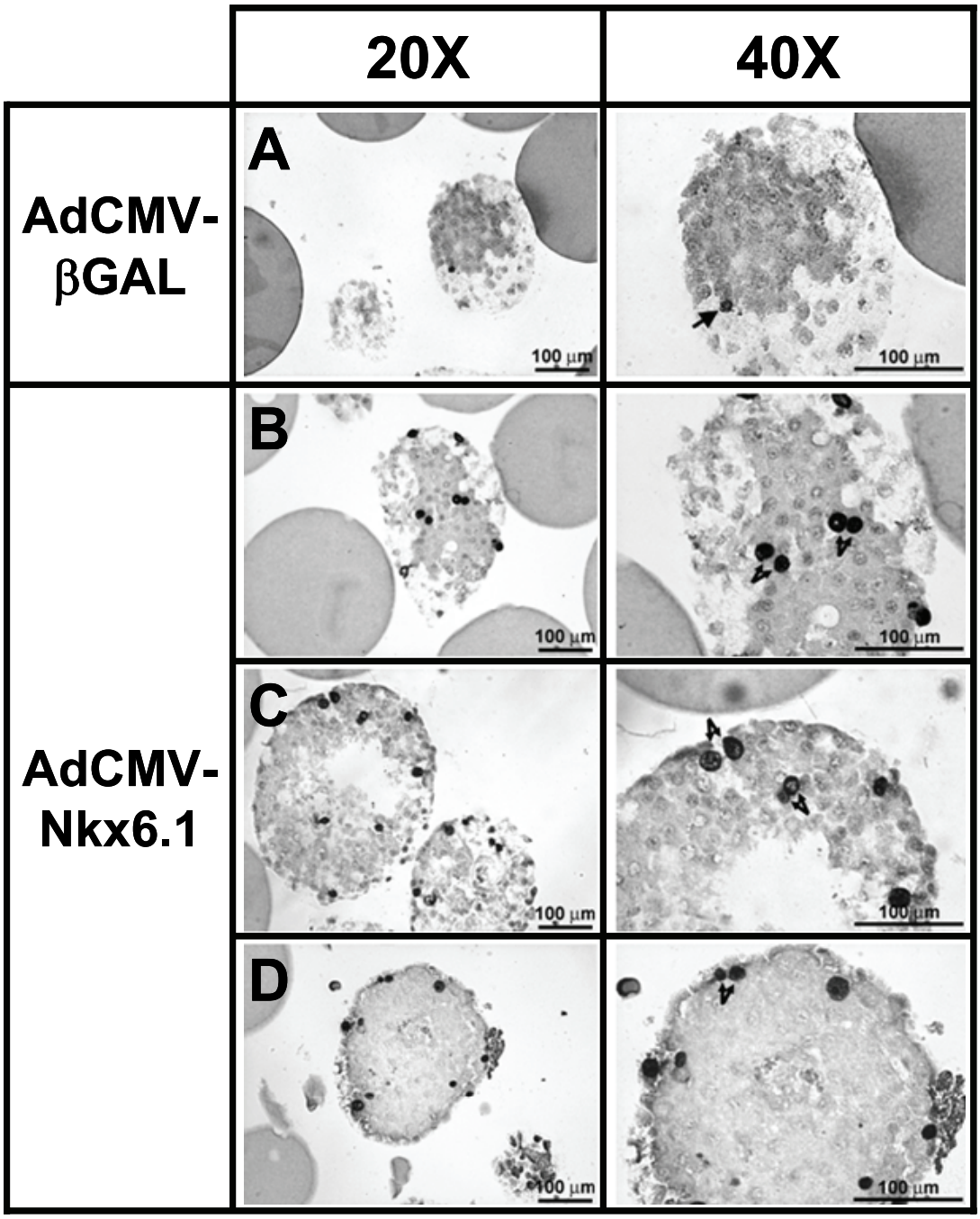


Figure 3-3. Nkx6.1 overexpression in primary rat islets increases proliferation. Primary rat islets were treated with either AdCMV-βGAL or AdCMV-Nkx6.1 and changes in proliferation were measured. **Panel A** shows the increase in thymidine incorporation into genomic DNA from rat islets treated with AdCMV-Nkx6.1 compared to islets treated with AdCMV-βGAL. The data represent the mean ± SEM from three experiments each involving triplicate pools of 30 islets per condition. The symbol * indicates a statistically significant change with $p < 0.0001$. **Panel B** is a representative RT-PCR on RNA from islets treated with either AdCMV-βGAL or AdCMV-Nkx6.1 using the indicated primer sets. G6PDH was used as an input control.

A



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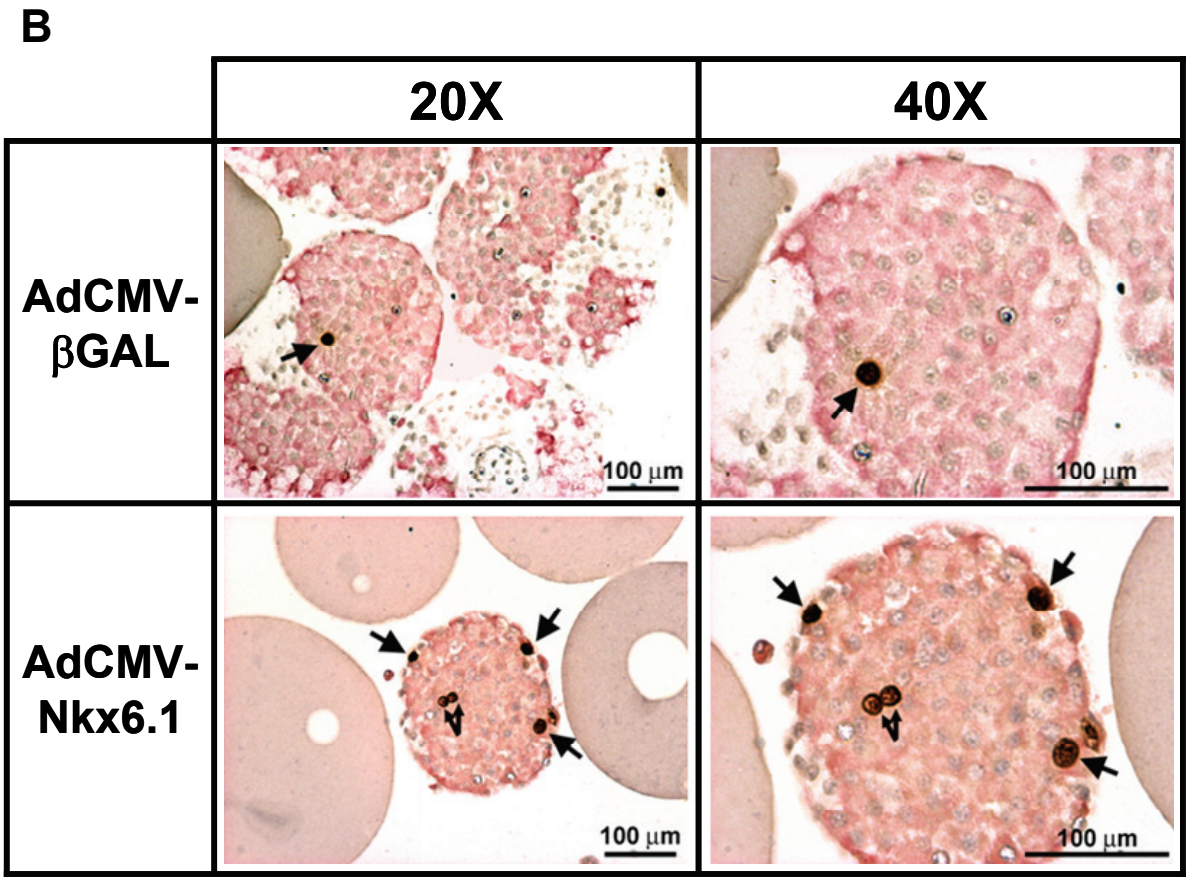


Figure 3-4. Immunodetection of BrdU incorporation in primary rat islets.
Panel A shows the immunodetection of BrdU from representative sections (5 μm) of primary rat islets treated with either AdCMV-βGAL or AdCMV-Nkx6.1 at either 20X or 40X magnification. The single BrdU-positive nuclei in the 40X AdCMV-βGAL section is indicated by the single-head arrow. Potential daughter cell formation is indicated by the double-headed arrow in the 40X AdCMV-Nkx6.1 sections. **Panel B** shows a representative co-stain of BrdU (brown) and insulin (red). BrdU and insulin co-positive cells are indicated by a single-head arrow. Double-headed arrows indicate potential insulin-positive daughter cell detection.

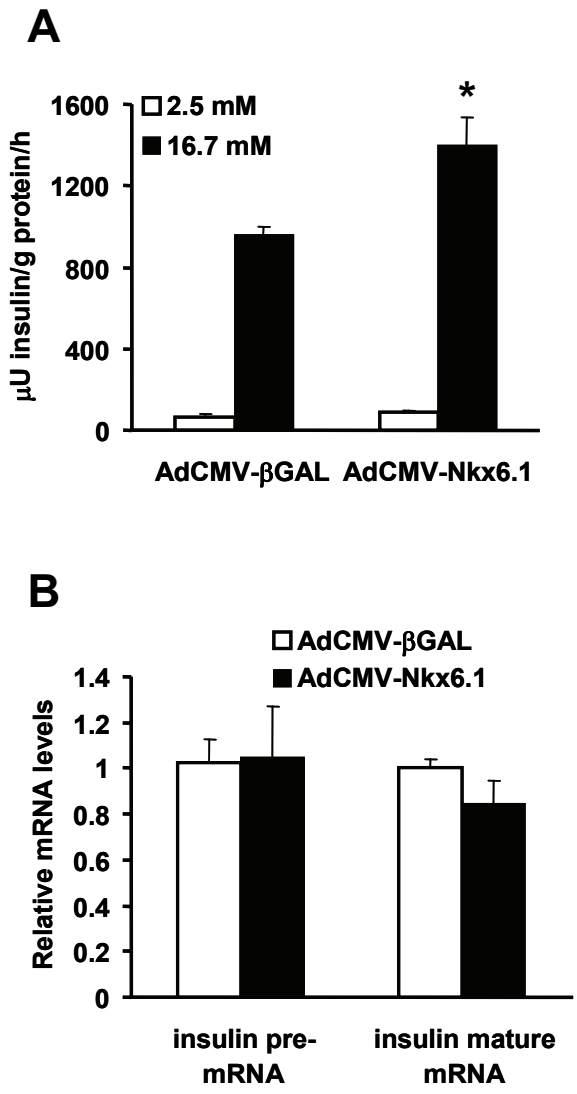
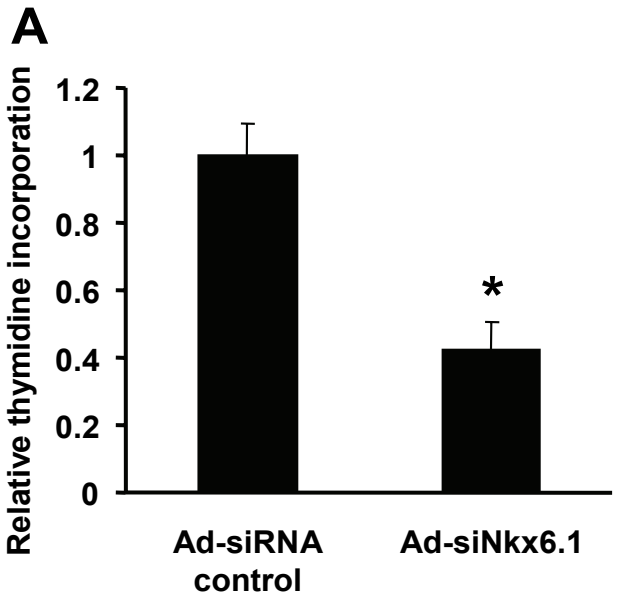
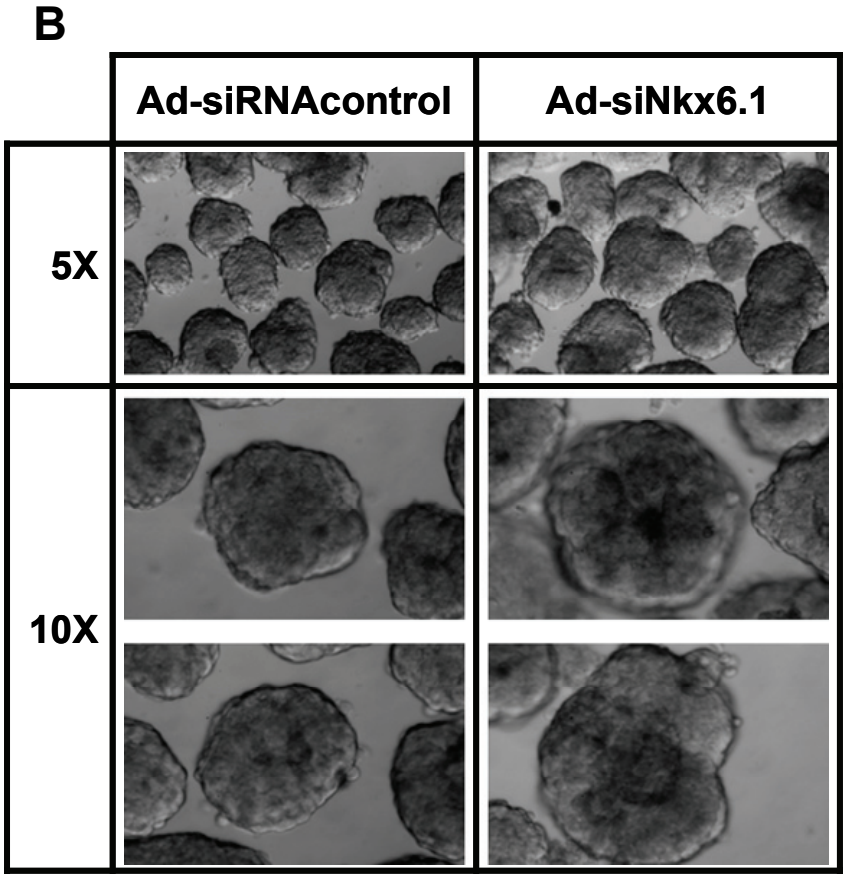
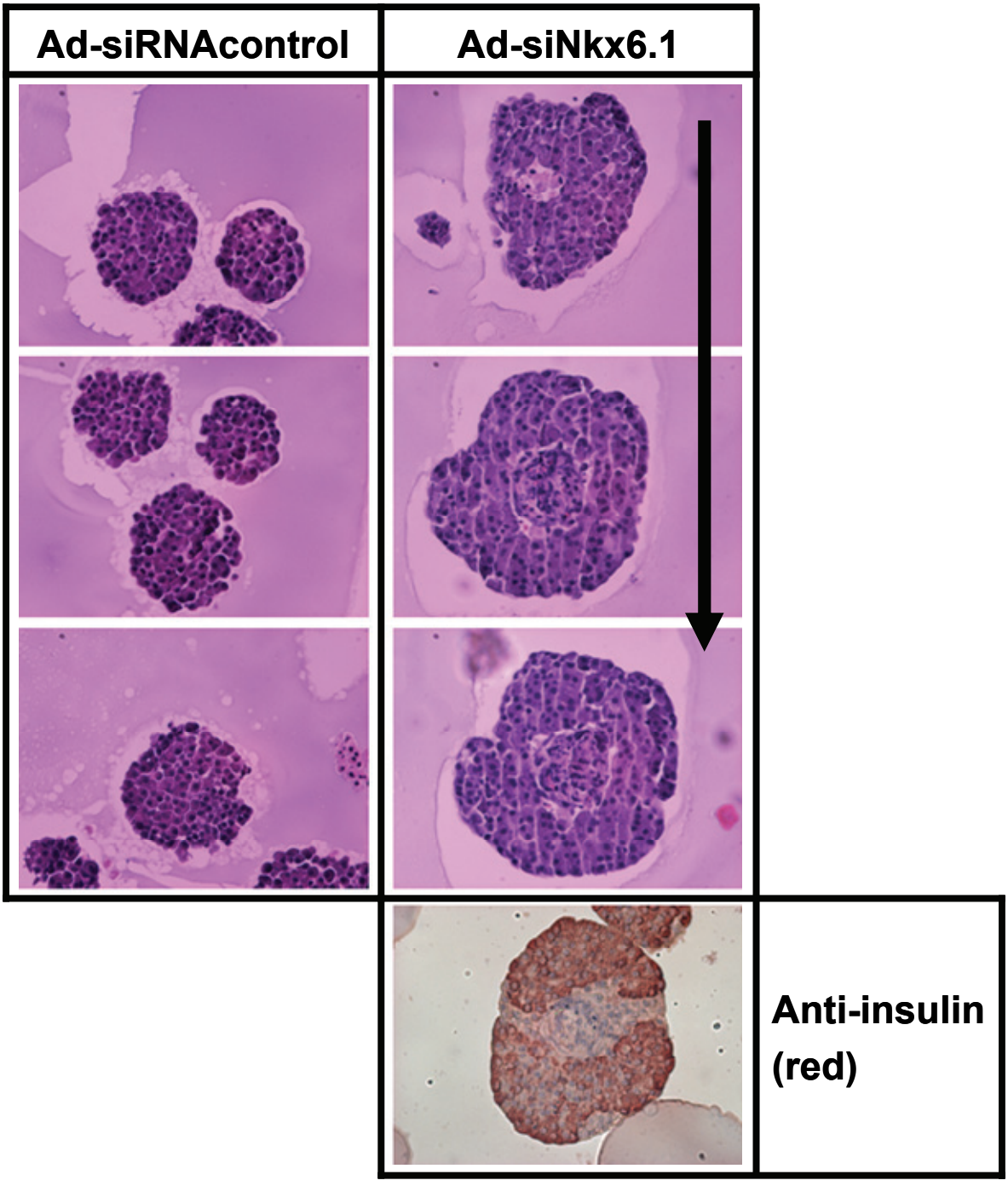


Figure 3-5. Overexpression of Nkx6.1 does not impair GSIS or insulin transcription. Primary rat islets were treated with either AdCMV-βGAL or AdCMV-Nkx6.1 and assayed for GSIS and changes in insulin transcription. **Panel A** shows insulin secretion measured in 1 h static incubations with either 2.5 or 16.7 mM glucose. The data are the mean ± SEM from three experiments, each involving triplicate pools of 30 islets per condition. The symbol * indicates a statistically significant change only in the 16.7 mM condition with $p < 0.005$. **Panel B** shows the effect of these conditions on both insulin pre-mRNA and mature mRNA levels as measured via real-time PCR analysis of RNA from three independent experiments. Data represented are the mean ± SEM normalized to AdCMV-βGAL mRNA levels. There was no statisicially significant effect on either transcript in comparing AdCMV-βGAL to AdCMV-Nkx6.1 treated islets.


A
Relative thymidine incorporation
1.2
1
0.8
0.6
0.4
0.2
0
*
Ad-siRNA control
Ad-siNkx6.1


B
Ad-siRNAcontrol
Ad-siNkx6.1
5X
10X

C



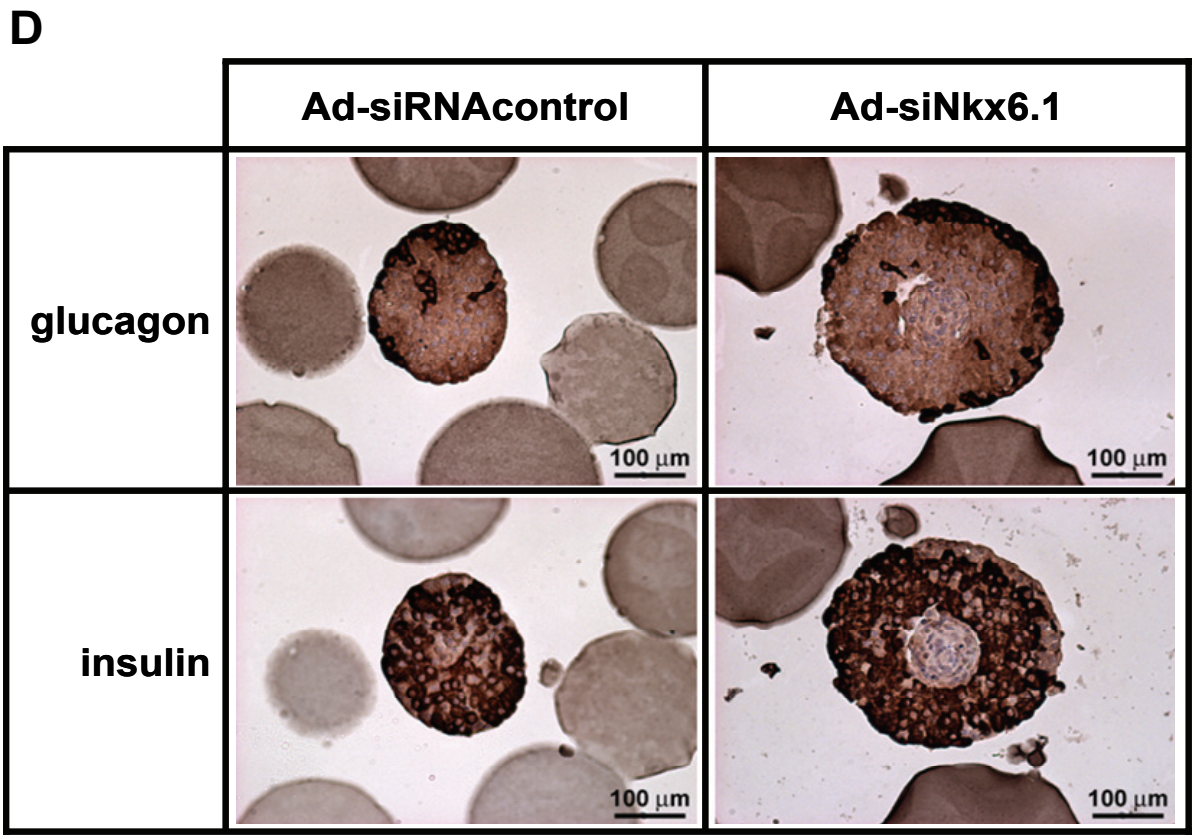
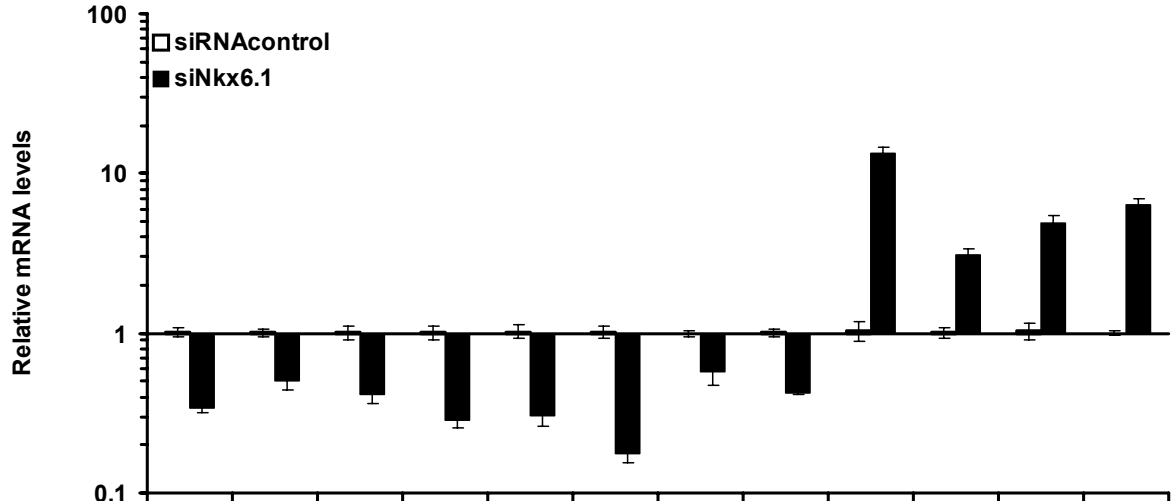


Figure 3-6. Silencing Nkx6.1 expression in primary rat islets decreases proliferation and changes islet morphology

Primary rat islets were treated with either Ad-siRNAcontrol or Ad-siNkx6.1. **Panel A** shows the effect of Ad-siNkx6.1 treatment of thymidine incorporation into genomic DNA. The data are the mean ± SEM from four experiments, each representing triplicate pools of 30 islets per condition. The symbol * indicates a statistically significant change with $p < 0.001$. **Panel B** shows representative 20X micrographs of primary rat islet cultures 90 h after treatment with the indicated viruses and following staining with hematoxylin and eosin. **Panel C** shows micrographs of several 5 μm sections of primary rat islets treated with Ad-siRNAcontrol (left column). A series of 20X micrographs from serial sections of an islet treated with Ad-siNkx6.1 is shown, indicated by the downward arrow (right column). Insulin immunostaining (red) of an islet treated with Ad-siNkx6.1 reveals an absence of immunoreactivity in the core of the islet (counterstained with hematoxylin). **Panel D** shows 20X micrographs of 5 μm serial sections of islets treated with either Ad-siRNAcontrol or Ad-siNkx6.1 and stained for either insulin or glucagon (brown, counterstained with hematoxylin).

Fold change	Up-regulated	Down-regulated
2-3	66	29
>3	10	9

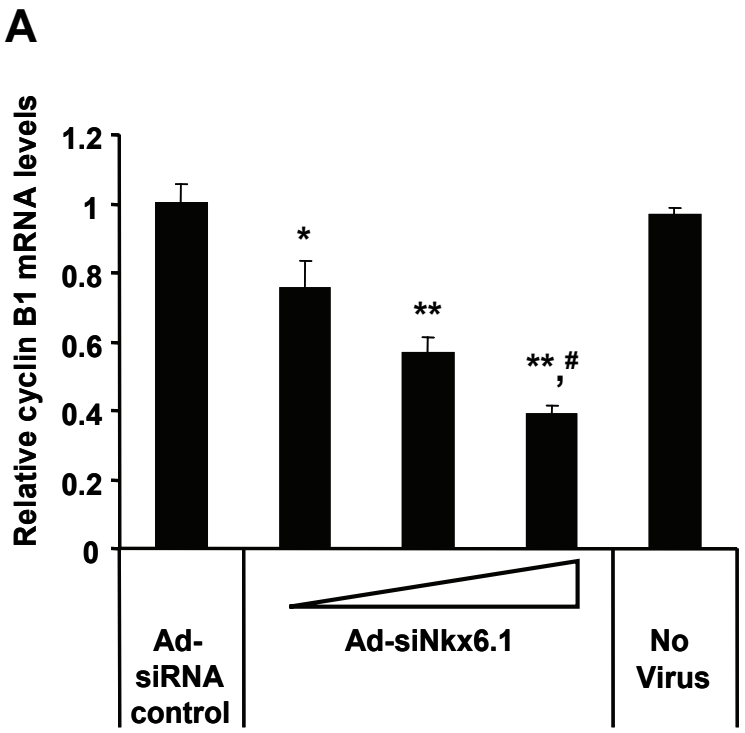
Table 3-1. Microarray analysis – summary of silencing Nkx6.1 expression.
The robustly glucose-responsive beta cell line 832/13 was treated with either an adenovirus expressing an siRNA targeting Nkx6.1 mRNA (Ad-siNkx6.1) or a control siRNA (Ad-siRNAcontrol). Duplicate RNA samples from 3 independent experiments were used for microarray analysis. This table summarizes the results comparing Ad-siNkx6.1-treated cells compared to Ad-siRNAcontrol cells, indicating that 76 and 38 genes were found to be up-regulated or down-regulated, respectively, greater than 2-fold with a statistical cut off of $p < 0.05$. The full list of genes can be found in Appendix B.



	Nkx6.1	Cyclin B1	p21	ProT-A	BNIP3	GAST	CDC25b	CDKN1B	slc22a1	Crbb3	PTPCe	UBC12
siRNAcontrol	1.01	1.01	1.02	1.02	1.02	1.02	1.00	1.01	1.03	1.01	1.03	1.00
siNkx6.1	0.34	0.50	0.41	0.28	0.31	0.18	0.58	0.43	13.40	3.07	4.95	6.37

Figure 3-7. Real-time PCR confirmation of selected genes identified in microarray analysis.

Selected genes identified in the microarray described in Table 3-1 were measured via real-time PCR on RNA samples from cells treated with either Ad-siRNAcontrol or Ad-siNkx6.1. The data represented are the mean ± SEM from the three independent experiments and are normalized to mRNA levels measured in Ad-siRNAcontrol-treated cells. The changes in gene expression in Ad-siNkx6.1 compared to Ad-siRNAcontrol-treated cells were all statistically significant, with $p < 0.005$.


A
Relative cyclin B1 mRNA levels
1.2
1
0.8
0.6
0.4
0.2
0
*
**
**,#
Ad-siRNA control
Ad-siNkx6.1
No Virus

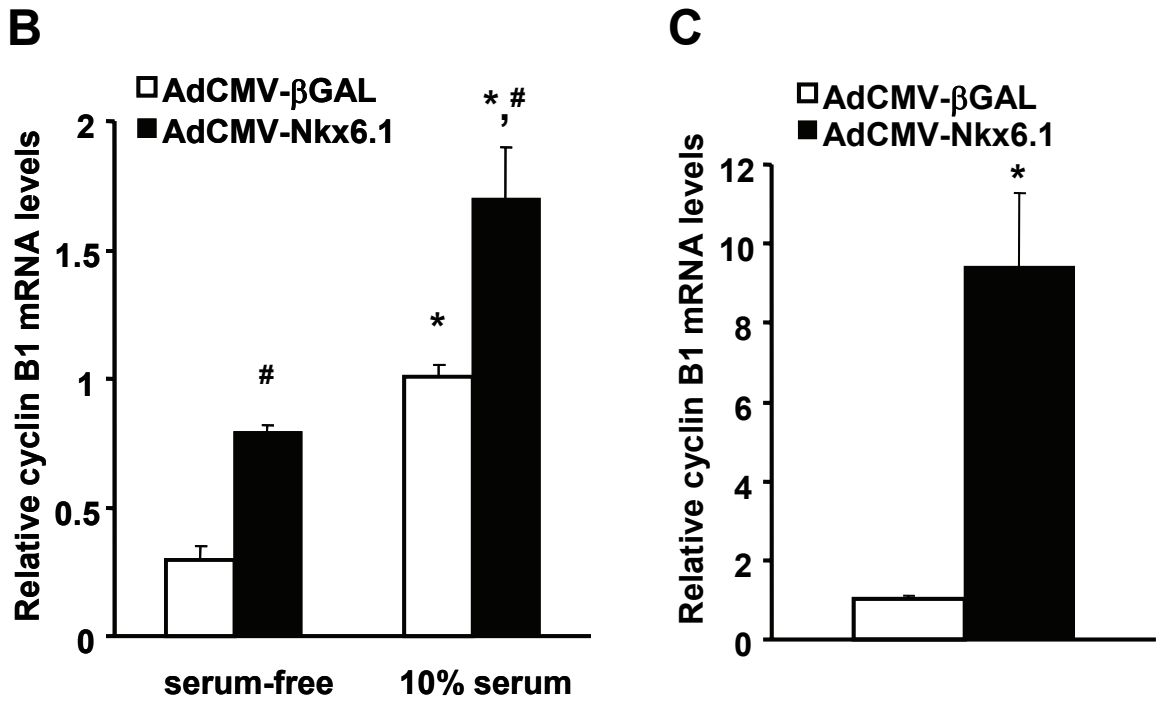
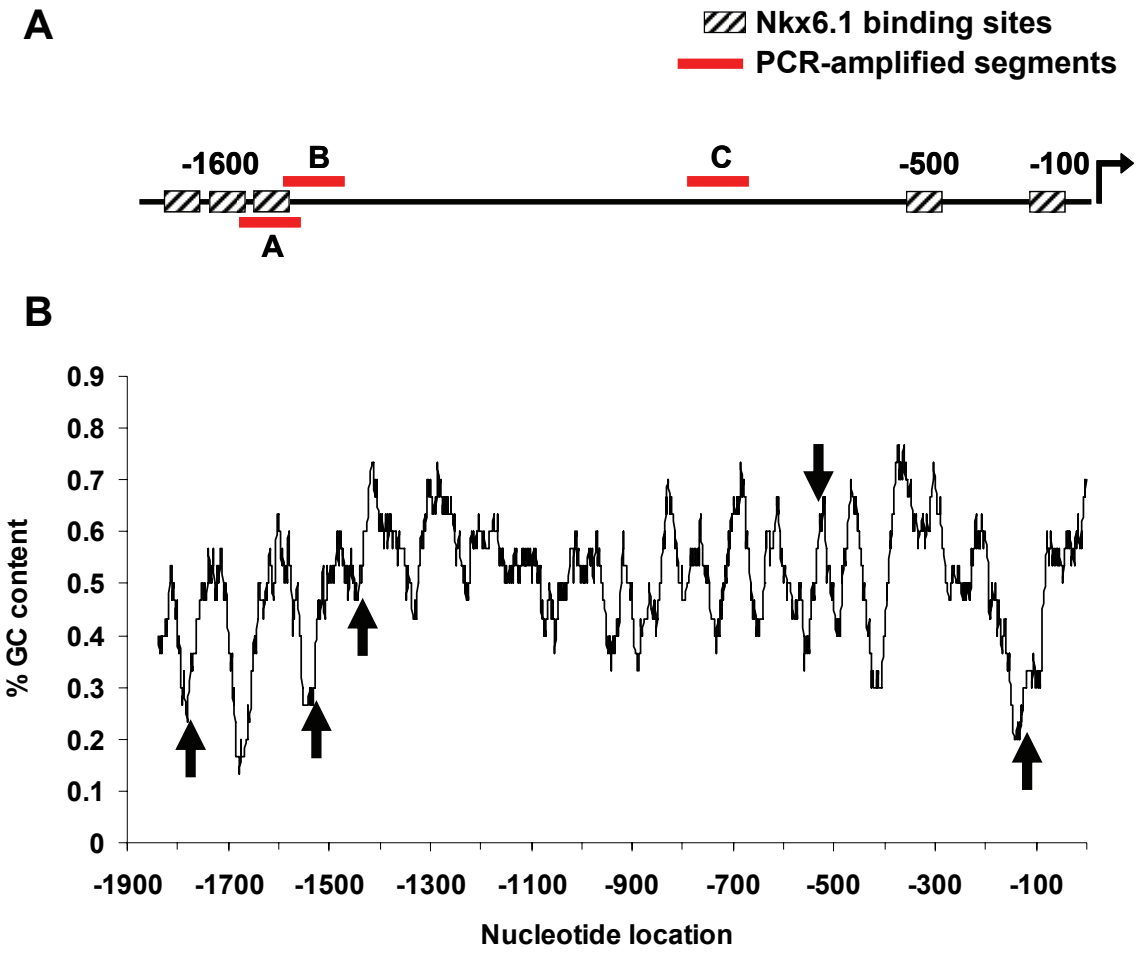


Figure 3-8. The effect of Nkx6.1 expression on cyclin B1 mRNA levels.
The level of cyclin B1 mRNA was measured via real-time PCR in several of the previously described experiments. Panel A shows that increasing amounts of Ad-siNkx6.1 decreases levels of cyclin B1 mRNA in 833/15 cells. Data represent the mean ± SEM from 3 independent experiments performed in duplicate. The symbols * and ** indicate statistically significant changes from the control condition with $p < 0.05$ and $p < 0.001$, respectively. The symbol # indicates a statistically significant change between the intermediate and high dose of Ad-siNkx6.1 with $p < 0.001$. Panel B shows the effect of serum deprivation and AdCMV-Nkx6.1 treatment on cyclin B1 mRNA levels in 834/105 cells. Data represent the mean ± SEM from 4 independent experiments performed in duplicate. The symbol * indicates a statistically significant change in cyclin B1 mRNA in serum-free versus 10% serum conditions in cells treated with either AdCMV-βGAL or AdCMV-Nkx6.1. The symbol # indicates a statistically significant change in cyclin B1 mRNA in cells treated with AdCMV-Nkx6.1 versus AdCMV-βGAL in both serum-free and 10% serum conditions. Panel C shows the relative increase in cyclin B1 mRNA levels in primary rat islets treated with AdCMV-Nkx6.1 compared to islets treated with AdCMV-βGAL. Data represent the mean ± SEM from 3 independent experiments performed in duplicate. The symbol * indicates a statistically significant change between the two conditions with $p < 0.001$.

Location upstream of start codon	Promoter sequence (5' – 3')
1787 - 1771	**TTGGCATTTTTGCCTTG**
1557 - 1541	**TTTTTATTTTCTAAGTA**
1439 - 1423	**GAAGGATTTTCCCCAGG**
518 - 502	**GGAGGATTTTGTGTGGT**
119 - 113	**GTTGTATTTTTCTTAAG**
Nkx6.1 PBE	**TTCTCATTTTGGCCCCC**

Table 3-2. Candidate Nkx6.1 binding sites in the rat cyclin B1 promoter.
Analysis of the rat cyclin B1 promoter revealed 5 candidate Nkx6.1 binding sites. The location (relative to the start codon) and sequence of 16 nucleotide segments containing the core binding motif -ATTTT- are listed here. Additionally, the Nkx6.1 proximal binding enhancer (**PBE**) element from the Nkx6.1 promoter is also shown. Nucleotides of the candidate binding sites that are identical to the Nkx6.1 PBE element are underlined.


A
Nkx6.1 binding sites
PCR-amplified segments
-1600
B
C
-500
-100
A
B
% GC content
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
-1900
-1700
-1500
-1300
-1100
-900
-700
-500
-300
-100
Nucleotide location

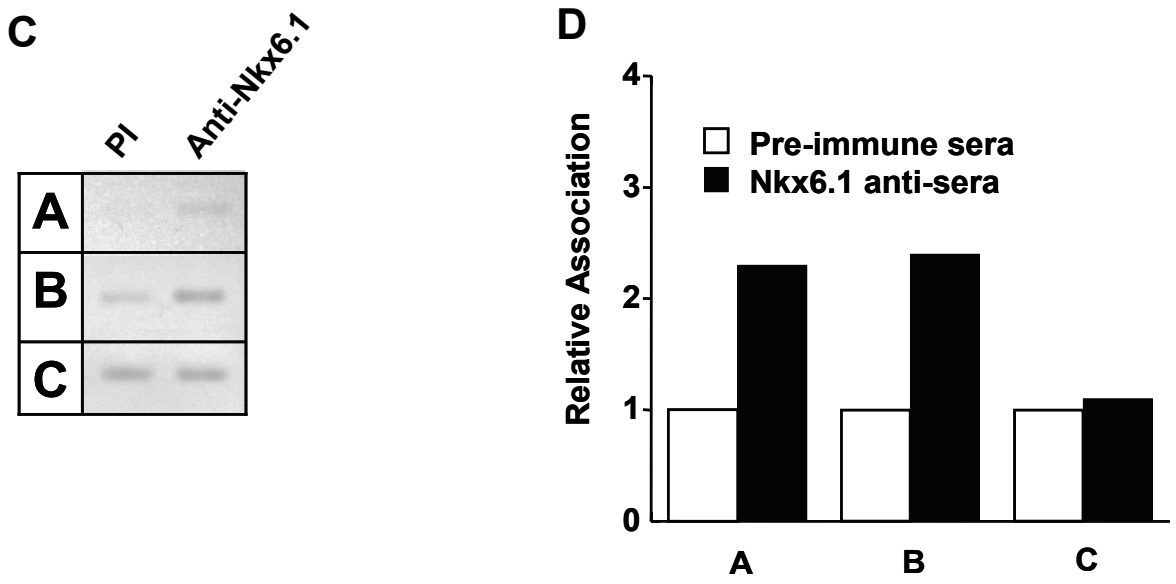


Figure 3-9. ChIP analysis of Nkx6.1 and the cyclin B1 promoter.
Panel A shows a diagram of the rat cyclin B1 prompter including 1800 base pairs upstream of the start codon. Sequence analysis indicated 5 potential Nkx6.1 binding sites (listed in Table 3-2) and their relative position is indicated by the hashed boxes. The amplicon of three PCR primer pairs used for ChIP analysis (A, B and C) is indicated by the solid red bars. **Panel B** is a plot of the %GC content of the cyclin B1 promoter using a window size of 30 base pairs. The solid arrows indicate the position of the candidate Nkx6.1 binding sites. **Panel C** is the result of the amplicon amplification with the indicated primer sets on genomic DNA precipitated with either pre-immune sera or Nkx6.1 anti-sera. **Panel D** is the real-time PCR analysis of the same conditions using all three primer sets. Data is represented as the relative association normalized to the amount of DNA recovered with pre-immune sera.

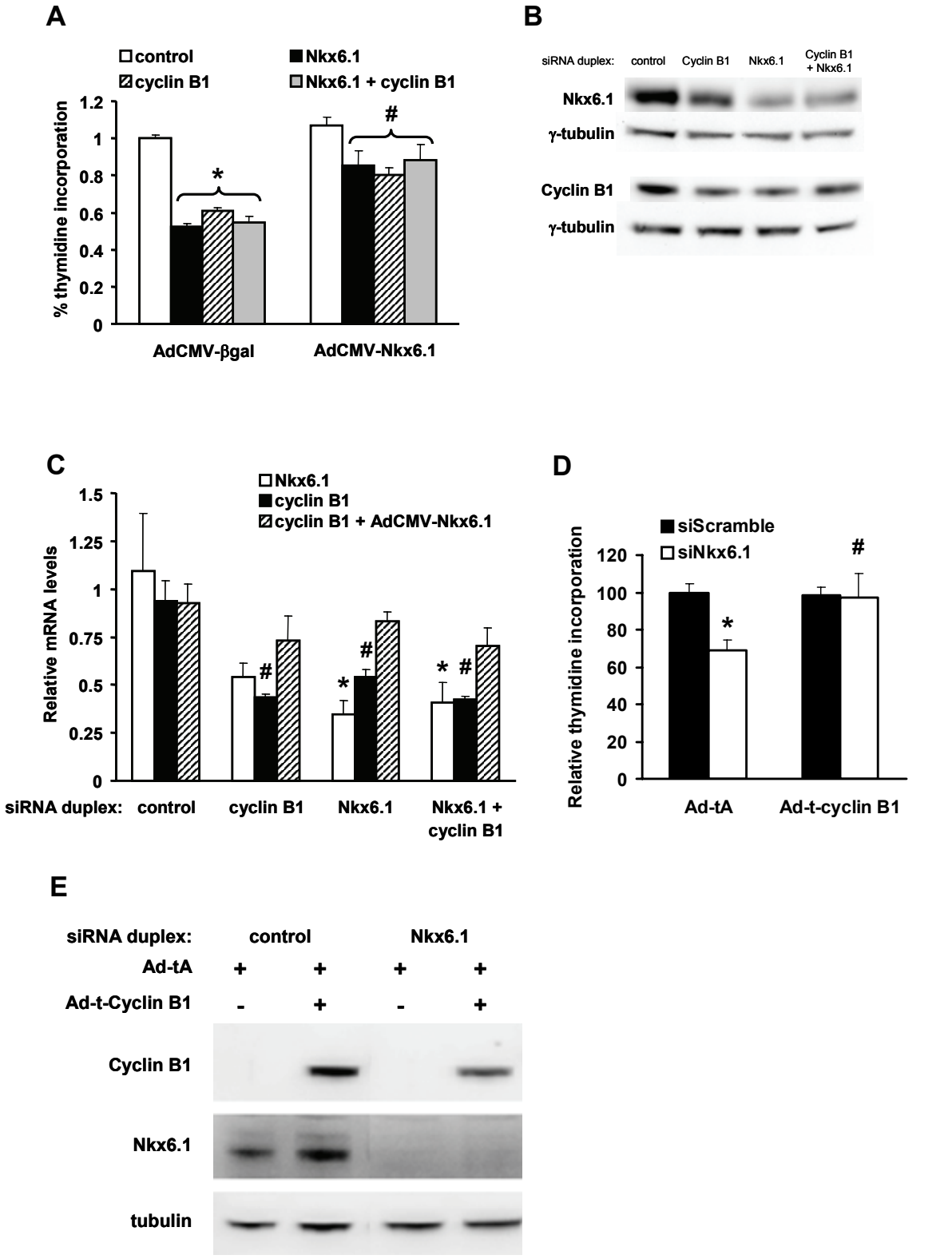

A
control
Nkx6.1
cyclin B1
Nkx6.1 + cyclin B1
% thymidine incorporation
1.2
1
0.8
0.6
0.4
0.2
0
*
#
AdCMV-βgal
AdCMV-Nkx6.1
B
siRNA duplex:
control
Cyclin B1
Nkx6.1
Cyclin B1 + Nkx6.1
Nkx6.1
γ-tubulin
Cyclin B1
γ-tubulin
C
Nkx6.1
cyclin B1
cyclin B1 + AdCMV-Nkx6.1
Relative mRNA levels
1.5
1.25
1
0.75
0.5
0.25
0
siRNA duplex:
control
cyclin B1
Nkx6.1
Nkx6.1 + cyclin B1
D
siScramble
siNkx6.1
Relative thymidine incorporation
120
100
80
60
40
20
0
Ad-tA
Ad-t-cyclin B1
E
siRNA duplex:
control
Nkx6.1
Ad-tA
+ + + +
Ad-t-Cyclin B1
- + - +
Cyclin B1
Nkx6.1
tubulin

Figure 3-10. The effect of cyclin B1 silencing and overexpression of proliferation. 832/3 cells were studied in a variety of conditions to measure the effect of cyclin B1 silencing and overexpression on proliferation. **Panel A** shows the relative mRNA levels of Nkx6.1 and cyclin B1 in cells that have been transfected with either siNkx6.1, siCyclin B1, or in combination, compared to cells transfected with siRNAcontrol. The effect of overexpressing Nkx6.1 (with AdCMV-Nkx6.1 treatment) on cyclin B1 transcript levels is also shown. Data represented are from 3 independent experiments measured from duplicate RNA samples. The symbol # and * indicate a statistically significant change in cyclin B1 or Nkx6.1 mRNA levels compared to cells transfected with siRNAcontrol with $p< 0.006$ or $p<0.05$, respectively. There was no significant change in cyclin B1 mRNA levels in the various transfection conditions compared to cells transfected with siRNAcontrol in cells treated with AdCMV-Nkx6.1. **Panel B** shows a representative immunoblot of nuclear extracts from cells transfected with the indicated siRNA duplexes reflecting the efficiency of the knockdown. **Panel C** shows the effect of these transfections on ^{3}H-thymidine incorporation into genomic DNA after treatment with either AdCMV-βGAL or AdCMV-Nkx6.1. Data are from 4 independent experiments, each performed in duplicate. The symbol * indicates a statistically significant change in thymidine incorporation compared to cells transfected with siRNAcontrol with $p< 0.0001$. The symbol # indicates a statistically significant change in thymidine incorporation in cells treated with AdCMV-βgal compared to AdCMV-Nkx6.1-treated cells, with $p<0.005$. **Panel D** shows the effect of treatment with Ad-tA or Ad-tA plus Ad-t-cyclin B1 on cells transfected with either siRNAcontrol or siNkx6.1. Data are from 5 independent experiments, each performed in duplicate. The symbol * indicates a statistically significant change in thymidine incorporation in cells transfected with siNkx6.1 compared to siRNAcontrol in Ad-tA treated cells with $p<0.0001$. The symbol # indicates a statistically significant change in thymidine incorporation in cells treated with Ad-tA compared to Ad-tA plus Ad-t-cyclin B1 in cells transfected with siNkx6.1 with $p<0.006$. **Panel E** is a representative immuoblot of whole cell extracts confirming the knockdown of Nkx6.1 protein and overexpression of cyclin B1 in the indicated conditions.

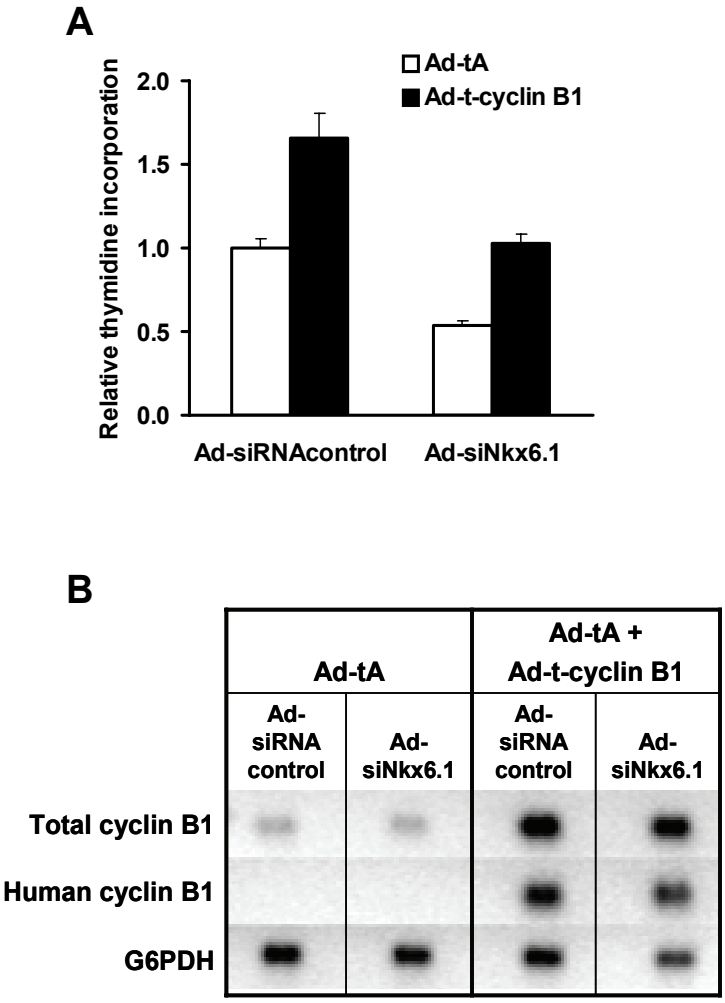


Figure 3-11. Cyclin B1 overexpression on proliferation in primary rat islets. Primary rat islets were treated with Ad-tA or Ad-tA plus Ad-t-cyclin B1 in the presence of either Ad-siRNAcontrol or Ad-siNkx6.1. The data represented are the mean ± SEM from three experiments each involving triplicate pools of 30 islets per condition. The symbol * indicates a statistically significant change in Ad-tA treated islets comparing Ad-siRNAcontrol to Ad-siNkx6.1 conditions with $p < 0.0005$. The symbol # indicates a statistically significant change in Ad-siRNAcontrol treated islets comparing to Ad-tA to Ad-t-cyclin B1 conditions with $p < 0.0001$. There was no significant change in Ad-siRNAcontrol/Ad-tA conditions compared to Ad-siNkx6.1/Ad-t-cyclin B1 conditions with $p = 0.74$. **Panel B** is a representative RT-PCR analysis on RNA from the various conditions using primer pairs that amplify either human cyclin B1, total cyclin B1 (rat and human), and G6PDH as an amplification control.

CHAPTER FOUR

CONCLUSIONS AND FUTURE DIRECTIONS

The role of Nkx6.1 and Pdx1 in the regulation of islet hormone gene expression

Alpha and beta cells develop from a common endocrine precursor cell type, but mature cells express either glucagon or insulin, respectively. Although much attention has been given to factors that influence insulin expression in beta cells, factors that contribute to control of glucagon expression have been less defined. Derangements in cell type-specific expression of hormones in islets have been observed in mouse models of Pdx1 haploinsufficiency (Johnson *et al.* 2003) and beta cell-specific inactivation of Pdx1 (Ahlgren *et al.* 1998) where a subset of beta cells also co-express glucagon. The finding of impaired beta cell function in these animals argues that in addition to gaining an understanding of the molecular mechanism of islet development, it is important to understand factors that maintain differentiated functions of the mature beta cell.

I have described how the manipulation of two transcription factors, Nkx6.1 and Pdx1, can impact glucagon expression in alpha and beta cell lines. Nkx6.1 overexpression is sufficient to suppress glucagon expression in both cell types, whereas silencing of either Nkx6.1 or Pdx1 results in the activation of glucagon expression in beta cells. Therefore, manipulation of these cell transcription factors in mature beta cell lines is sufficient to cause *transient changes* in hormone expression independent of developmental effects.

Interestingly, Pdx1 silencing has more potent effects on glucagon gene expression than silencing of Nkx6.1. EMSA analysis reveals that the interaction of these factors with a fragment of the glucagon promoter was diminished to a similar extent, suggesting that there may be indirect effects that contribute to the greater potency of Pdx1 on glucagon expression. The family of large Maf transcription factors has been implicated in the regulation of islet hormone expression (Kataoka *et al.* 2002; Olbrot *et al.* 2002; Matsuoka *et al.* 2003; Kataoka *et al.* 2004). Subsequent analysis revealed that silencing of Pdx1 resulted in suppression of MafA expression in addition to a robust increase in MafB expression. In contrast, silencing of Nkx6.1 only affects cMaf expression (Figure 2-8) (Raum J 2005). In collaboration with Roland Stein's lab, we have found that MafB is specifically expressed in alpha cells, and upon introduction into beta cells, it acts as a potent activator of glucagon gene expression (Artner *et al.* 2006). Therefore, the stronger effect of Pdx1 on glucagon gene activation is likely due to the added contribution of an indirect effect on MafB.

I have also presented data in Chapters 2 and 3 that demonstrate a lack of effect of modulation of Nkx6.1 on insulin gene transcription in both beta cell lines and in primary rat islets. These findings would not have been anticipated based on prior studies showing effects of Nkx6.1 on an insulin promoter-reporter (Mirmira *et al.* 2000; Iype *et al.* 2004; Taylor *et al.* 2005). However, plasmid-driven promoter-based assays do not recapitulate the endogenous promoter in the context of chromatin structure, which has been shown to play an important role particularly in insulin promoter accessibility (Chakrabarti *et al.* 2002; Chakrabarti *et al.* 2003; Francis *et al.* 2005). Additionally, other co-factors and cis-regulatory elements contribute to the overall regulation of insulin transcription (Melloul *et al.*

2002). Based on the region of the promoter used in the promoter-reporter assays (a 350 bp fragment directly upstream of the transcriptional start site) the effect of these factors and other elements may not be represented.

The *in vivo* physical interaction between Nkx6.1 and the insulin promoter identified via ChIP (Iype *et al.* 2005) also suggests that Nkx6.1 may influence transcription. Additionally, the DNA sequence of the insulin promoter that interacts with Nkx6.1 (containing a 5'-TAAT-3' element) is one previously associated with its suppressor function. Physical interactions between transcription factors and DNA targets are not always sufficient to influence transcription and may require other co-factors or post-translational modifications, as seen with the homeodomain protein CDP/cut (Li *et al.* 1999; Moon *et al.* 2001; Nepveu 2001; Santaguida *et al.* 2001) and the estrogen receptor (Lazennec *et al.* 1997; Wood *et al.* 2001). Taken together, despite a physical interaction between Nkx6.1 and the insulin promoter, my data has shown that transient changes in Nkx6.1 expression do not impact the overall transcription of the insulin gene.

However, my work has provided additional support for a role of Pdx1 as a transcriptional activator of insulin expression. In collaboration with the Mirmira lab, we demonstrated a potent effect of Pdx1 silencing on insulin pre-mRNA levels in both beta cell lines and in primary mouse islets (Iype *et al.* 2005). Additionally, MafA has also been identified as a direct activator of insulin gene expression. As discussed above, silencing of Pdx1 expression results in a decrease in MafA mRNA levels. Therefore, our studies provide new evidence of direct and indirect effects of Pdx1 on insulin gene expression.

Implications for identifying transcription factor target genes

The field of transcription factor biology uses a host of assays to determine direct relationships between these proteins and promoter regions of target genes, such as those discussed in the previous section and presented in the work described here. These assays commonly include: 1) promoter-reporter assays, which measure the transcriptional activity of a reporter gene downstream of a promoter fragment of the target gene; 2) electrophoresis mobility shift assay (EMSA) or gel shift assay, which measures the physical interaction between a transcription factor and a synthetic DNA fragment from the target promoter; 3) transient analysis of the target gene mRNA levels in combination with overexpression or silencing of the expression of the transcription factor; and 4) chromatin immunoprecipitation (ChIP) assays, which can detect the *in vivo* interaction between a transcription factor and a region of the target gene promoter in the context of the chromatin structure. The ChIP assay is also powerful since it can be combined with real-time PCR for quantitative analysis of this interaction, representing the 'gold standard' for target gene validation.

However, it is also clear that one of these assays alone is not sufficient to determine the functional relationship between a transcription factor and a target gene, especially in the case of a bi-functional factor such as Nkx6.1. As summarized in Table 4-1, Nkx6.1 has been found to interact *in vivo* (via ChIP analysis) with three different promoters: glucagon, insulin, and cyclin B1. As shown in this body of work, however, these interactions yield three different outcomes: suppression, no effect, and activation, respectively. Therefore, ChIP assays must be used to in combination with other assays to determine the net effect of a given

factor on transcriptional control. Moreover, chromatin structure and *in vivo* accessibility to promoter regions adds another level of complexity to the understanding of transcription factor biology and its relationship to beta cell function (Chakrabarti *et al.* 2002; Chakrabarti *et al.* 2003; Francis *et al.* 2005).

promoter	*In vivo* interaction	*In vitro* interaction	Reporter assay	Target gene mRNA
insulin	+	+	↓	No change
glucagon	+	+	↓	↓
cyclin B1	+	ND	ND	↑

Table 4-1. Summary of functional assays of Nkx6.1 target genes.
The result of various assays performed on the Nkx6.1 target genes insulin, glucagon, and cyclin B1: 1) *In vivo* interaction refers to ChIP analysis; 2) *in vitro* interaction refers to EMSA analysis; 3) reporter assays refer to plasmid-based promoter constructs driving reporter gene expression; and 4) target gene mRNA refers to the correlation of Nkx6.1 expression and real-time PCR analysis of the target gene mRNA levels. The symbol "+" indicates that an interaction is readily detectable. The symbol "↑" and "↓" represents either an increase or decrease, respectively, in either reporter gene activity or target gene mRNA

Nkx6.1 expression – implications in diabetes

Type 2 diabetes related to decreased Nkx6.1 function

The dramatic effect of Nkx6.1 silencing on GSIS in both beta cell lines and primary rat islets suggests a key role for this transcription factor in mature beta cell function and potentially in type 2 diabetes. Consistent with a potential role in disease development, Nkx6.1 expression is markedly reduced in two animal models of type 2 diabetes, the ZDF rat and partial pancreatectomy. Of course, two interpretations of these findings are possible: 1) the decrease in Nkx6.1 is a result of impaired beta cell function; or 2) the loss of Nkx6.1 contributes to the diabetic phenotype seen in both animal models. Given the strong effects of transient changes in Nkx6.1 on various islet phenotypes discussed here, the latter possibility is not unreasonable and worthy of further investigation. Also consistent with this concept, the human Nkx6.1 gene has been identified as a potential susceptibility locus in an unbiased genome-wide linkage analysis for genes conferring type 2 diabetes susceptibility in obese subjects (Parker *et al.* 2001). To determine if changes in Nkx6.1 are part of the progression of type 2 diabetes, further analysis of Nkx6.1 expression levels in various animal models of diabetes and obesity are necessary.

My analysis of the microarray study in Chapter 3 was focused on genes involved in beta cell proliferation and growth. This was a conscious decision, but I realize that the microarray study is also likely to provide important information about the mechanism by which Nkx6.1 regulates GSIS. As discussed in Chapter 1, cytosolic isocitrate dehydrogenase (ICDc) regulates pyruvate cycling activity and NADPH production, and is clearly involved in

regulation of GSIS (Ronnebaum *et al.* 2006). Of the 38 genes found to be decreased with Nkx6.1 silencing, ICDc was among the three most down-regulated (71% reduction in cells treated with Ad-siNkx6.1 versus Ad-siRNAcontrol), and this dramatic effect was subsequently confirmed by real-time PCR. An important experiment planned in the near future in our laboratory is to determine if ICDc overexpression can rescue the effect of suppression of Nkx6.1 on GSIS or vice versa. Interestingly, analysis of the ICDc promoter reveals three conserved Nkx6.1 enhancer elements, suggesting that the effect of Nkx6.1 on ICDc regulation may be direct. Further analysis of Nkx6.1-mediated regulation of ICDc expression, modeled on the studies that I have described herein that have established direct interactions of Nkx6.1 with the glucagon and cyclin B1 genes will reveal the nature of the relationship between these genes and how their expression regulates GSIS. Other members of the Newgard lab are currently evaluating other Nkx6.1 target genes that have been identified in my microarray studies for their potential role in GSIS via siRNA-mediated suppression and overexpression studies. This work may ultimately lead to new targets for stimulation of insulin secretion in diabetes

Nkx6.1 functions as a master regulator of beta cell proliferation

In addition to the strong effect on GSIS, Nkx6.1 expression appears to be necessary for maintenance of islet cell mass and basal beta cell proliferation. The striking effect of Nkx6.1 overexpression on islet proliferation also suggests a possible role of this transcription factor in beta cell expansion seen in various physiological conditions, such as pregnancy or response to injury. The identification of cyclin B1 as a direct target gene of Nkx6.1 will

provoke further investigation of the role of cyclin B1 in impacting beta cell proliferation in the Newgard laboratory.

What remains unclear is the apparent effect on islet morphology seen in islets with reduced Nkx6.1 expression. One possibility is that silencing of Nkx6.1 blocks the progression through the cell cycle, specifically at mitosis, thus creating enlarged cells that have already been through S phase. However, since the mitotic capacity of primary beta cells is very low, it would appear unlikely that a blockade of the cell cycle would have a dramatic effect throughout the entire islet. One way to examine this hypothesis is through histological analysis of islets that have been treated with both Ad-siNkx6.1 and Ad-t-cyclin B1 to see if cyclin B1 overexpression, in addition to resucing the Ad-siNkx6.1-medieated decrease in proliferation (Figure 3-11), can also impact the morphological effect. Alternatively, it is possible that the change in expression of a yet-to-be defined target gene of Nkx6.1 alters beta cell morphology independent of affecting the cell cycle.

Although the majority of attempts to increase primary beta cell proliferation have resulted in impaired function and less differentiated beta cells (see Chapter 1), the overexpression of Nkx6.1 avoids these pitfalls. Another exception is the overexpression of hepatocyte growth factor (HGF) (Garcia-Ocana *et al.* 2000; Garcia-Ocana *et al.* 2001; Garcia-Ocana *et al.* 2003), which had similar results to Nkx6.1 overexpression, as detailed in Chapter 3. In a follow up study analyzing the status of the cell cycle regulatory protein pRB (retinoblastoma) in primary beta cells, it was found that overexpression of cell cycle factors cyclin D1 and cdk4 have similar outcomes on proliferation and function to islets overexpressing HGF (Cozar-Castellano *et al.* 2004). Subsequently, analysis of transgenic

mice overexpressing an active form of Cdk4 (Hino *et al.* 2004; Marzo *et al.* 2004) confirmed a role of the cyclin D1/Cdk4 pathway in influencing proliferation while maintaining function in primary beta cells.

The phosphatidylinositol 3-kinase/Akt (PI-3/Akt) intracellular-signaling pathway has been shown to mediate the proliferative effects seen with HGF overexpression (Garcia-Ocana *et al.* 2003). Likewise, PI-3/Akt activation can directly stimulate beta cell proliferation (Bernal-Mizrachi *et al.* 2001; Tuttle *et al.* 2001), a process mediated in part due the inactivation of forkhead transcription factors leading to an increase in D cyclin activity and cell cycle progression (Medema *et al.* 2000; Kops *et al.* 2002; Alvarez *et al.* 2003). Therefore the impact of Nkx6.1 expression on PI-3/Akt signaling and changes in cyclin D1/cdk4 activity merits further investigation (see below). To address the possibility that HGF and Nkx6.1 converge on similar pathways in the beta cell, the effect of HGF and PI-3/Akt overexpression on Nkx6.1 levels can be determined by analyzing islets treated with an adenovirus overexpressing HGF or Akt (Garcia-Ocana *et al.* 2003) as well as islets isolated from mice transgenically overexpressing HGF (Garcia-Ocana *et al.* 2000). Furthermore, microarray analysis comparing the changes in gene expression induced by HGF, PI-3/Akt and Nkx6.1 in primary beta cells will provide a foundation to understand of how these factors modulate beta cell proliferation.

The use of a transformed cell line to screen for Nkx6.1-regulated genes involved in growth and proliferation may mask the identity of target genes that are critical to the strong proliferative phenotype seen in primary beta cells. Consistent with this idea Nkx6.1 overexpression has a much more dramatic effect on thymidine incorporation in primary rat

islets than in beta cell lines. Cyclin B1 appears to be a bona fide Nkx6.1 target gene, and plays a role in the basal proliferation of primary beta cells based on the experiments described in the previous chapter. However, the inability of cyclin B1 overexpression to recapitulate the proliferative effect seen with Nkx6.1 overexpression suggests that other targets of Nkx6.1 are important in regulating proliferation.

To that end, I have performed a microarray analysis of islets with and without Nkx6.1 overexpression, compared to islets overexpressing β-galactosidase. As expected, in addition to an increase in cyclin B1, a host of other cell cycle genes are upregulated with Nkx6.1 overexpression, including cyclin A2, B2, E1 and E2, and the cell division cycle genes Cdca3, Cdk1, Cdc20, Cdc6 and Cdca1. This result suggests that overexpression of Nkx6.1 can increase the expression of a host of genes involved in multiple stages of the cell cycle, consistent with a role of Nkx6.1 as a master regulator of beta cell proliferation. Although there was no indication of an increase in any of the D-cyclins, Cdk4, or PI-3/Akt factors, there was an increase in cyclin E1, cyclin E2 and Cdc6 expression. The D- and E- cyclins in conjunction with Ckd4 and Cdc6 coordinate the G1/S progression of the cell cycle (Sanchez and Dynlacht 2005). Although the effect of Nkx6.1 on these cell cycle genes requires real-time PCR confirmation, these data provide further clues in comparing the effect of Nkx6.1 and HGF on beta cell proliferation. The initial result of this microarray suggests that these two events do not converge at the level of PI-3/Akt or cyclin D/Cdk4.

Providing new tools for expanding beta cell mass and maintaining beta cell function – therapeutic considerations

Conversely, the studies presented in this dissertation may suggest that modulation of Nkx6.1 expression or selective activation of its critical target genes could protect against the diabetic phenotype. Delivery of Nkx6.1 *in vivo* could be tested in models of both type 1 and type 2 diabetes such as streptozotocin-induced diabetes and in the ZDF rat, respectively.

Type 1 diabetes, cytokine-induced impairment of beta cell function--implications for Nkx6.1

In the case of type 1 diabetes, the question to be addressed would be does Nkx6.1 promote the expansion of the limited number of beta cells that are not destroyed by the auto-immune response, thereby increasing the number of beta cells that are resistant to autoimmunity? Support for this approach has become evident in the analysis of Nkx6.1 expression in cytokine resistant versus cytokine sensitive beta cell lines, a two state model of beta cell function developed in the Newgard lab (Chen *et al.* 2000). Exposure of beta cells to cytokines results in a decrease in cell viability, cell proliferation, and loss of insulin secretion. A selection strategy allowed the procurement of a beta cell line (833/15) that was resistant to the cytokines interleukin 1β (Il-1β) and γ-interferon (IFNγ) as measured by cell viability (MTS activity). Remarkably, these cells also maintain robust GSIS in the presence of cytokines.

Given the effects of Nkx6.1 on GSIS and proliferation discussed in the previous chapters, the effects of Il-1β and IFNγ on Nkx6.1 expression were measured in these cell

lines and primary rat islets. Cytokine exposure resulted in a 38% decrease in Nkx6.1 mRNA in 832/13 cells and 32% decrease primary rat islets after 4 hours, and the effect persisted through longer time points (Figure 4-1A). The change in mRNA was also reflected at the protein level with a noticeable decrease in Nkx6.1 protein in the sensitive cell line 832/13 after 4 h of cytokine exposure (Figure 4-1B). In contrast, Nkx6.1 mRNA or protein levels did not decrease in response to Il-1β and IFNγ in the cytokine-resistant cell line 833/15 (Figure 4-1A and B).

To determine if Nkx6.1 expression contributes to either the sensitivity or resistance to cytokines in these cells, Nkx6.1 was *silenced* in cytokine-resistant cells and *overexpressed* in cytokine-sensitive cells (Figure 4-1C). Consistent with a role for Nkx6.1 in mediating this phenotype, silencing of Nkx6.1 expression in cytokine-resistant 833/15 cells resulted in a 30% decrease in viability in the presence of Il-1β and IFNγ as measured by MTS activity. Likewise, overexpression of Nkx6.1 in cytokine sensitive 832/3 cells rendered these cells resistant to cytokine-induced decreases in cell viability.

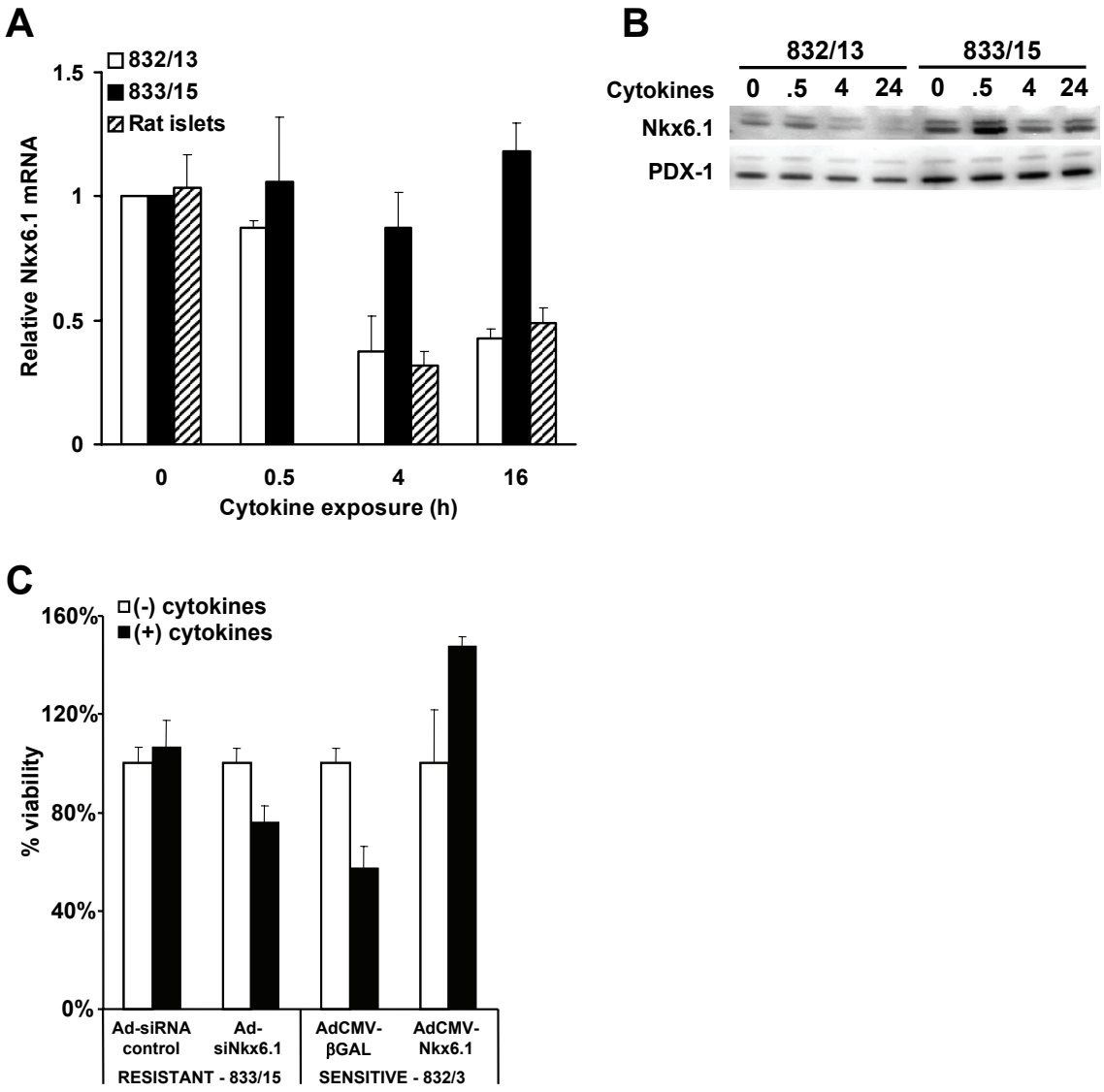


Figure 4-1. Nkx6.1 expression mediates cytokine resistance in beta cells.
Panel A shows the effect of Il-1β and IFNγ exposure on Nkx6.1 mRNA levels in cytokine-resistant cells (833/15) and cytokine-sensitive cells (832/13 and primary rat islets). **Panel B** shows the effect of cytokine exposure on Nkx6.1 protein levels in 833/15 and 832/13 cells. **Panel C** shows the effect of Ad-siNkx6.1 and Ad-siRNAcontrol or AdCMV-Nkx6.1 and AdCMV-βGAL treatment on cell viability in the absence (-) or presence (+) of exposure to Il-1β and IFNγ for 18 h in 833/15 cells or 832/3 cells, respectively. Data represented are the mean ± SEM for 2-3 independent experiments measured in triplicate.

Additionally, I examined the effects of Nkx6.1 overexpression on cytokine-induced impairments on other beta cell functions, including proliferation/DNA synthesis and GSIS. As shown in Figure 4-2A, exposing cytokine-sensitive cell lines to Il-1β and IFNγ for 18 h resulted in a 94% decrease in beta cell proliferation, as measured by thymidine incorporation into genomic DNA. This finding is consistent with the large drop seen in Nkx6.1 expression in the presence of cytokines (Figure 4-1A and 4-1B) and with the effect of direct manipulation of Nkx6.1 expression on beta cell proliferation (Chapter 3). Overexpression of Nkx6.1 was able to partially rescue the cytokine-induced decrease in proliferation, increasing thymidine incorporation 5.8-fold compared to cells overexpressing β-galactosidase in the presence of cytokines. Similar effects have also been observed in primary rat islets (preliminary, data not shown). Furthermore, 6 hour exposure to Il-1β and IFNγ was sufficient to impair GSIS in cytokine-sensitive cell lines (Figure 4-2B) again also consistent with a decrease in Nkx6.1 expression (Figure 4-1A and 4-1B), whereas overexpression of Nkx6.1 partially rescued GSIS. Further analysis in primary beta cells is ongoing and will better define a role of Nkx6.1 in preventing beta cell death and cytokine-induced impairment of beta cell function.

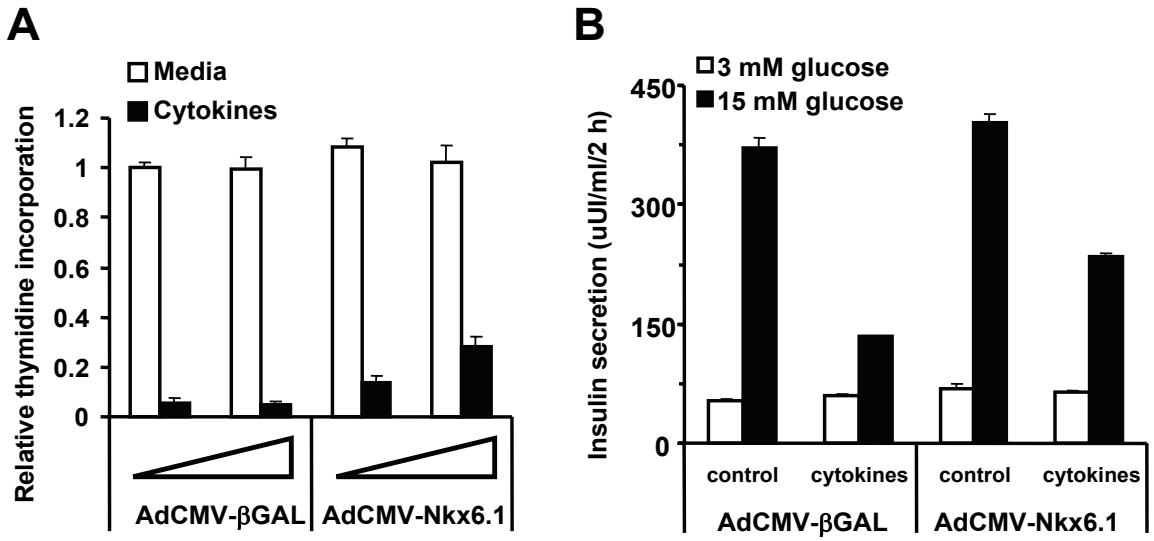


Figure 4-2. Nkx6.1 overexpression partially rescues cytokine-impaired beta cell function.

Panel A shows the effect of increasing doses of AdCMV-Nkx6.1 on thymidine incorporation compared to cells treated with equal doses of AdCMV-βGAL in the absence (open box) or presence (closed box) of Il-1β and IFNγ for 18 h in 832/3 cells. **Panel B** shows the effect of AdCMV-βGAL or AdCMV-Nkx6.1 treatment on GSIS in the absence (control) or presence (cytokines) of Il-1β and IFNγ for 6 h in 832/13 cells. Data represented are the mean ± SEM for 3 independent experiments measured in triplicate.

Improving beta cell function ex vivo--better islets for transplantation

The dramatic effects of Nkx6.1 on beta cell proliferation, insulin secretion, and resistance to inflammatory cytokines may have practical implications. Islet transplantation has proven to be a successful therapy for humans with type 1 diabetes. However, the utility of this therapy is restricted due to the limited source of donors, the need for multiple donor pancreases per transplant, and the administration of immunosuppressive drugs (Shapiro *et al.* 2000). If donor islets could be manipulated *ex vivo* to increase their insulin secretion and proliferation capacity through the overexpression of genes such as Nkx6.1, this would reduce the number of donors needed per transplantation. The added benefit of Nkx6.1 on cytokine-resistance may also increase cell survival in the transplant setting.

A recent report described the reversible immortalization of primary beta cells with simian virus 40 large T antigen and telemorease reverse transcriptase (Narushima *et al.* 2005). These cells, when transplanted into streptozotocin-induced diabetic mice, were able to reverse the diabetic phenotype. However, the use of oncogenes in these studies, even with the reversibility afforded by the cre recombinase system employed, raises questions about the suitability of these cells for human transplantation (Hohmeier and Newgard 2005). If a similar outcome can be achieved by overexpression of non-tumorgenic beta cell gene that promotes proliferation, like Nkx6.1, perhaps this could lead to a safer method for beta cell expansion.

Based on the studies summarized in this dissertation, I have initiated construction of a lentivirus containing the Nkx6.1 cDNA and a GFP reporter gene with Drs. Tom Becker and Hans Hohmeier in the Newgard laboratory, in order to evaluate the long term consequences

of Nkx6.1 overexpression in primary islet cells. In the context of a proliferating pool of cells, the effects of adenoviral and plasmid-based delivery of transgenes is ultimately limited since these reagents become diluted with replicating cells. We therefore will study the effect of stable overexpression of Nkx6.1 at a functional level, including measuring changes in proliferation, cytokine sensitivity and GSIS in both rat and human primary islets, as well as in dispersed cultures of primary islets. Additionally, the presence of the GFP reporter will also allow us to better visualize the effect on islet cell morphology and proliferation specifically on cells that are expressing the integrated Nkx6.1 gene. Dispersion of primary beta cells into monolayers results in a decrease in glucose responsiveness and insulin transcription, but a beta cell phenotype is regained upon re-aggregation into clusters of cells (Beattie *et al.* 1991; Beattie *et al.* 1996; Beattie *et al.* 1997) . Given the ability of Nkx6.1 to impact GSIS in primary beta cells, monolayers of primary beta cells may retain glucose responsiveness. These cells can then be transplanted into streptozotocin-induced diabetic SCID mice to analyze their *in vivo* performance (Narushima *et al.* 2005).

Nkx6.1 delivery in type 2 diabetes--curbing the Big Mac attack

The progression to type 2 diabetes involves first an increase in beta cell mass as compensation for insulin resistance, and later, a failure of this compensatory response and a loss of beta cell mass. Given the beneficial effects of Nkx6.1 on enhancing insulin output and proliferation, perhaps delivery of Nkx6.1 to beta cells of animals with type 2 diabetes can curb the dysfunctional pathologies. Initially this can be tested through the treatment of ZDF islets with AdCMV-Nkx6.1 and measurement of GSIS. Since the impairment of beta cell function in ZDF islets is progressive, the effect of Nkx6.1 overexpression can be analyzed at different stages, from the pre-diabetic stage to frank diabetes (Tokuyama *et al.* 1995).

The physiology behind the impaired function of ZDF is undoubtedly complex and involves derangements of gene expression and signaling pathways. This is exemplified by the sensitivity of ZDF islets to fatty acids in terms of impaired insulin secretion and increased beta cell death, the defects that appear well before the appearance of a diabetic phenotype (Hirose *et al.* 1996; Pick *et al.* 1998; Shimabukuro *et al.* 1998). Therefore, delivery of Nkx6.1 to pre-diabetic ZDF animals may prevent the development of the diabetic phenotype. The advances made in microbubble delivery of expression plasmids to selective tissues is an attractive approach for *in vivo* delivery of genetic cargo (Bekeredjian *et al.* 2003). To test this hypothesis, an expression plasmid encoding the Nkx6.1 cDNA under the control of the rat insulin promoter will mixed with lipid-based transfection reagents and encapsulated in albumin-coated microbubbles. Following infusion of the microbubbles, a directed ultrasound beam will disrupt the microbubbles in the area of the pancreas, allowing the delivery of the

expression plasmid to islet beta cells. Members of the Newgard lab in collaboration with Paul Grayburn (University of Texas Southwestern Medical Center, Dallas, TX) have demonstrated the successful delivery of the enzyme hexokinase 1 and reporter genes to islet beta cells (Chen 2005) providing the proof of principal for this experiment. Additional animal models of type 2 diabetes, including the parital pancreatecomized diabetic rat, previously shown to have reduced Nkx6.1 expression (Jonas *et al.* 1999), should also be considered as another model system to study the *in vivo* delivery of Nkx6.1 and its effects on diabetic progression.

Final thoughts – Nkx6.1, the gatekeeper to beta cell function

Analysis of a two-state model of beta cell function resulted in the identification of Nkx6.1 as a transcription factor whose expression positively correlated with beta cell function. Subsequent analysis of Nkx6.1 revealed a role for this transcription factor in the two holy grails of beta cell biology, *insulin secretion* and *proliferation*. Figure 4-3 is a model of how Nkx6.1 contributes to the beta cell phenotype through a myriad of mechanisms and provides a model for further understanding of the pivotal role of this transcription factor.

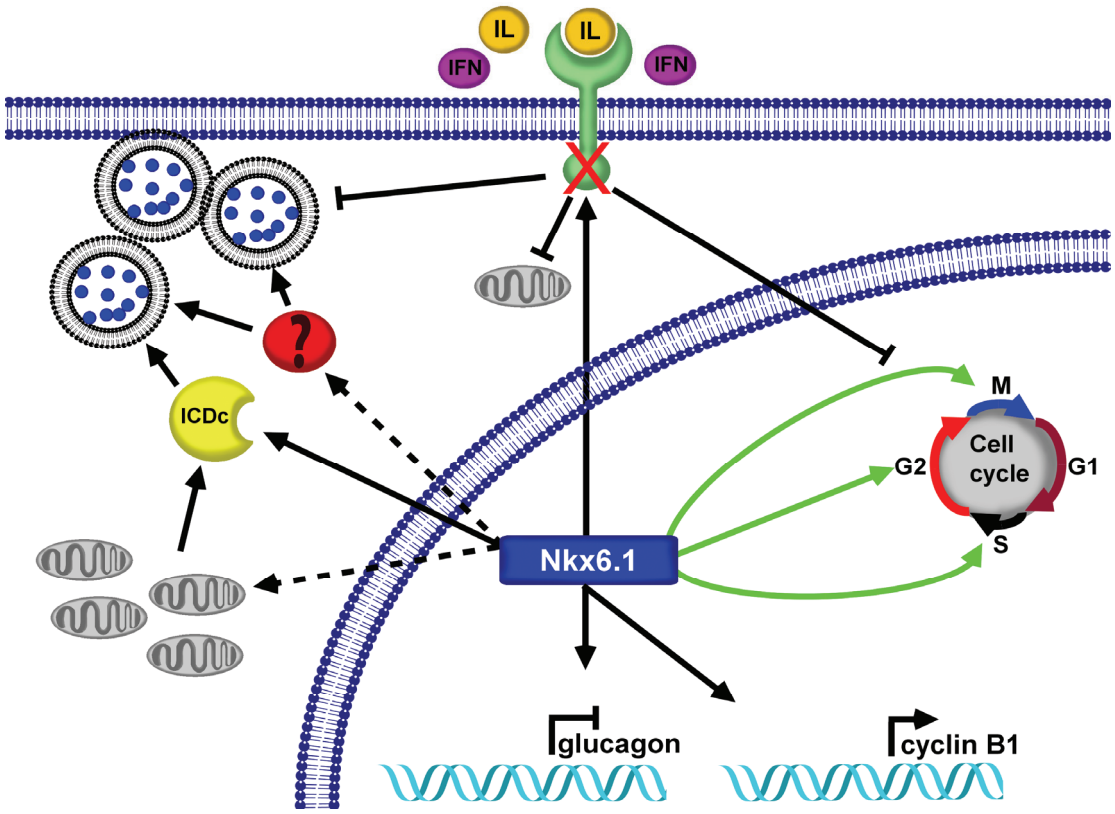


Figure 4-3. The functional role of Nkx6.1 in mature beta cell function.
Nkx6.1 impacts beta cell function through multiple cellular functions. **Nkx6.1** directly *suppresses* **glucagon** and *activates* **cyclin B1** gene transcription. The later effect, in addition to increasing the expression of other **cell cycle** related genes (indicated by the green arrows), impacts the cell cycle at multiple stages, mediating effects on beta cell proliferation. Additionally, Nkx6.1 expression is involved in glucose-stimulated insulin secretion, suggested in this model to be mediated through the regulation of cytolsolic isocitrate dehydrogenase (**ICDc**) which is coupled to glucose metabolism and directly impacts GSIS. The regulation of other Nkx6.1-target genes may also impact GSIS, either through changes in metabolic regulation or through mechanisms involved in the exocytotic machinery of the cell. Exposure to the pro-inflammatory cytokines Il-1β (**IL**) and IFNγ (**IFN**) results in a decrease in beta cell proliferation, mitochondrial function (cell viability), and GSIS. Nkx6.1 expression can block the effect of cytokines on all three mechanisms, improving the function of beta cells in the presence of these factors.

APPENDIX A
DNA oligonucleotides used in PCR analysis

Gene	Size (bp)	Forward Primer (5' – 3')	Reverse Primer (5' – 3')	# cycles
Internal standards				
Alpha-Tubulin	250	GCGTGAGTGTATCTCCATCCA	GGTAGGTGCCAGTGCGAACTT	12-18
G6PDH	214	GACCTGCAGAGCTCCAATCAAC	CACGACCCTCAGTACCAAAGGG	20-24
Glycogen targeting Proteins				
GI	232	CAAACTGAACAAGCCACTGAG	TCTGCTGTCGTCAGACTCAC	20
PTG	152	GACATTTTGTGAATAAACCGAAG	GTGGATGGCAGTGAGGGAG	20
Hormones				
Glucagon	161	ATCATTCCCAGCTTCCCAGA	CGGTTCCTCTTGGTGTTCAT	16
Insulin	312	TCCTGCCCCTGCTGGCCCTGC	AGTTGCAGTAGTTCTCCAG	12
Insulin2 pre-mRNA*	190	GGGGAGCGTGGATTCTTCTACACA	TGCCCACAGAAATGTGCCCCC	
Insulin2 mature mRNA*	81	GCACCTTTGTGGTTCTCACTTGGT	CGGGACATGGGTGTGTAGAAGAAT	
Somatostatin	232	TCTCTGCTGCCTGCGGACCT	GCCAAGAAGTACTTGGCCAGTTC	20
Enzymes				
G6Pase	256	CCTCTTCCCCATCTGGTTCC	TCACGTAGTATACACCTGCTG	20
Glucokinase	161	CAGATCCTGGCAGAGTTCCAG	ACTTCTGAGCCTTCTGGGGTG	20
Glut-2	270	GACACCCCACTCATAGTCACAC	CAGCAATGATGAGAGCATGTG	18
Hexokinase I	224	TGTACGCCATGCGGCTCTC	CTCGTGGTTCACCTGCACC	22
Hexokinase II	234	GGAACAAACTTCCGGGTCCTGC	GTCTAGGCTGTTCTGCTGG	22
Glutamate Dehydrogenase	258	TGGAGGTGCTAAAGCAGGC	ATGCCTCCTTGGCTGATGG	22
Transcription factors				
HNF1-alpha	236	TCACGCCCACCAAGCAGGTC	GTTGGATGGCAGCAGGTGG	24
Islet-Brain 1	220	GCTCACCGTGCACTTTAACC	GCAGGCAAACCGGTGGTC	24
NeuroD	235	CTTGGCCAAGAACTACATCTGG	CTGGTAGGAGTAGGGATGC	24
Nkx6.1 (rat)	273	GCCTGGGGTGATGCAGAG	CTTCTTTCTCCACTTGGTCC	18
Nkx6.1 (rat and hamster)*	181	CACCAGACCCACATTCTCC	CTCGGCTGCGTGCTTCTTT	24
Nkx6.1 (hamster)	180	CACGAGACCCACGTTCTCT	TCAGCCGCGTGCTTCTTC	18

The cycle number used in semi-quantitative multiplex-PCR in indicated for the primer sets used in that application. The symbol * indicates the primers were used in real-time PCR analysis using SYBR-green PCR chemistry.

Nkx2.2	195	AAACTATGTTTGTGTGAGTAGC	CAGAACGTCATTTGGCAACG	24
PDX-1	160	CCGTGCGCATTCCAGAGG	CTTCGTATGGGGAGATGTCC	22
Pax4	254	CCAGTGTGAAGCATGCAGC	GTGGCCAGACGTGGTTTGC	24
E47/Pan-1	185	GCCTGGTCAGCGGAAGC	TCTCCTCCCGCTTGATCTC	24
Brn4	188	CCTCTGATGAGTTGGAACAG	GATCTTGTCAATGCTGGTCG	24
Microarray confirmation genes				
BAP29*	136	TCGTTGAGAAGAACTCAGCCAGCA	AGCGTAACCAGACGTCTCAACACT	
cdc25b*	174	TCAAAGGCGGCTACAAGGAGTTCT	AGAACTCCTTGTAGCCGCCTTTGA	
cdkn1b(p27)*	125	AGCTTGCCCGAGTTCTACTACAGA	TTTGCCTGAGACCCAATTGAAGGC	
crybb3*	96	TTAGAGAACTTCCAGGGCAAGCGA	TTCCACTTGGATAGAGCCCACCTT	
Me2GlyDH*	177	TCGAGTCCAAGTGTTCCATGGGTT	ACGGAGACAGGTCAATCACACCAA	
nDAP*	192	AGAGTTACGCCAAGGGAAGCAGAA	AAATCGGTGAGGCAGAGAAGCAGA	
P21*	99	TACTTGGCTGGTGGCTCTTTGACT	ATCCAAAGCTTGGAGGCACTCTCT	
proT-A*	192	TTGTGGAGGAGGCAGAGAATGGAA	TAGGAGCCTCAGCTTCCTCATCTT	
PTPCe*	129	AGAATGGTCTGGGAGCAAAGGTCA	ACACACCCGGATGTTCCCATAAGT	
Slc22a1*	87	AACCACGCGAGCTGTCAGGATAAT	TCAAGGCAGAGCATCTTCAGGTCA	
ubc12*	143	TTAACGAGCTGAACCTGCCCAAGA	ACCCTGTCCCACCTTAAAGCTGAA	
Cyclin B1 primers				
cyclin B1 (rat and human)	183	ACTGGGTCGTGAAGTCACTGGAAA	AACATGGCAGTTACACCAACCAGC	
cyclin B1 (rat)*	175	TGTGTCAGGCTTTCTCCGATGTGA	TTTCCAGTGACTTCACGACCCAGT	
cyclin B1 (human)	179	GTCAGTGAACAACTGCAGGCCAAA	TTAACAGGCTCAGGTTCTGGCTCA	
Real-time PCR probe sets		***ABI Assay ID***		
Pdx1		Rn00755591_m1		
Nkx6.1		Rn00581973_m1		
Glucagon		Rn00562293_m1		
Foxo1		Rn01769619_m1		
Pax6		Rn00443072_m1		
GLUT2		Rn00563565_m1		
GK		Rn00561265_m1		
Cyclophilin B		Rn00574762_m1		
Ribosomal 18S		#4319413E		

The cycle number used in semi-quantitative multiplex-PCR in indicated for the primer sets used in that application. The symbol * indicates the primers were used in real-time PCR analysis using SYBR-green PCR chemistry.

APPENDIX B
Microarray analysis of Nkx6.1 silencing in 832/13 cells

FC	Common	Genbank	Product
-4.4	Gast	NM_012849	gastrin
-4.2	Bnip3	NM_053420	BCL2/adenovirus E1B 19 kDa-interacting protein 3
-3.9	Bcap29	NP_031556	B-cell receptor-associated protein 2 (Bap29)
-3.6	Bnip3	AF243515	BCL2/adenovirus E1B 19 kDa-interacting protein 3
-3.4	Idh1	NM_031510	isocitrate dehydrogenase 1 (NADP+), soluble
-3.3	Pak3	NM_019210	p21 (CDKN1A)-activated kinase 3
-3.3	Neurodap1	D32249	ring finger protein 131
-3.2	Ptma	NM_021740	prothymosin alpha
-3.0	Gucy1b3	NM_012769	guanylate cyclase 1, soluble, beta 3
-2.9	CHPT1	NP_064629	choline phosphotransferase 1
-2.9	Ptma	NM_021740	prothymosin alpha
-2.6	Pir	AW918049	ESTs, Highly similar to PIR_MOUSE Pirin [M.musulus]
-2.6	Soat1	NM_031118	sterol O-acyltransferase 1
-2.5	Scd2	U67995	stearoyl-Coenzyme A desaturase 2
-2.4	P4ha1	X78949	procollagen-proline, 2-oxoglutarate 4-dioxygenase (proline 4-hydroxylase)
-2.4	Hmmr	AF133037	hyaluronan mediated motility receptor
-2.4	Nkx6a	NM_031737	NK homeobox (Drosophila), family 6, A
-2.4	Rpl4	NM_022510	ribosomal protein L4
-2.3	Pir	NP_081429	pirin
-2.3	Zcchc12	AK012833	Zinc finger CCHC domain containing protein 12(Smad-interacting)
-2.3	Rab12	M83676	RAB12, member RAS oncogene family
-2.3	Scd2	NM_031841	stearoyl-Coenzyme A desaturase 2
-2.3	Pak3	NM_019210	p21 (CDKN1A)-activated kinase 3
-2.3	PFDN4	NM_002623	ESTs, Highly similar to prefoldin 4 [Homo sapiens] [H.sapiens]
-2.2	Cstn2	NM34377	calsyntenin 2
-2.1	Rpl4	NM_022510	ribosomal protein L4
-2.1	Cdc10	NM_022616	CDC10 (cell division cycle 10, S.cerevisiae, homolog)
-2.1	P4ha1	X78949	prolyl 4-hydroxylase alpha subunit
-2.1	Atp2a2	J04022	ATPase, Ca++ transporting, cardiac muscle, slow twitch 2
-2.1	Cacna2d1	AF400662	calcium channel, voltage-dependent, alpha2/delta subunit 1
-2.1	Pdcl	NM_022247	phosducin-like
-2.1	57606	BAA95982.1	kiaa1458 protein (fragment) - homo sapiens
-2.1	Ccnb1	X64589	cyclin B1
-2.0	Senp2	NM_023989	SUMO/sentrin specific protease 2
-2.0	Pawr	U05989	par-4 induced by effectors of apoptosis
-2.0	PFDN4	AL133335	prefoldin 4 (c-1) - homo sapiens
-2.0	Cd47	NM_019195	CD47 antigen (Rh-related antigen, integrin-associated signal)
-2.0	Itgb1	NM_017022	integrin beta 1

FC	Common	Genbank	Product
2.0	Nbl1	NM_031609	neuroblastoma, suppression of tumorigenicity 1
2.0	Serf1	BF522362	ESTs, Highly similar to small EDRK-rich factor 1
2.0	Hpgd	NM_024390	NAD-dependent 15-hydroxyprostaglandin dehydrogenase
2.0	Fabp5	U13253	fatty acid binding protein 5, epidermal
2.0	Nme3	NM_053507	non-metastatic cells 3, protein expressed in
2.0	Slfn4	Y17327	CDK107
2.0	Txnip	NP_076208	similar to thioredoxin interacting factor - mus musculus
2.1	Slc25a10	AJ223355	solute carrier family 25 (mitochondrial carrier; dicarboxylate transporter), member 10
2.1	Vsp45a	U81160	vesicular transport protein rvps45
2.1	Ggtp	M33821	gamma-glutamyl transpeptidase
2.1	Sh3kbp1	AF255888	SH3 domain-containing adapter protein
2.1	Vsp45a	U81160	vacuolar protein sorting 45
2.1	Zfp106	NP_035873	SH3-domain binding protein 3 - zinc finger protein 106 - mus musculus
2.1	Bk	U30831	B/K protein
2.1	Pde9a	NM38543	phosphodiesterase 9A
2.1	C1galt1	NM_022950	core1 UDP-galactose:N-acetylgalactosamine-alpha-R beta 1,3-galactosyltransferase
2.1	Asah	NM_053407	N-acylsphingosine amidohydrolase 1
2.1	Mrpl36	BC009166	mitochondrial ribosomal protein l36 - mus musculus
2.1	S100a4	NM_012618	S100 calcium-binding protein A4
2.1	Acac	X53003	acetyl-coenzyme A carboxylase
2.2	Acac	NM_022193	acetyl-coenzyme A carboxylase
2.2	Bdnf	NM_012513	brain derived neurotrophic factor
2.2	Lin7c	NM_021851	lin-7 homolog C
2.2	Asah	AF214647	N-acylsphingosine amidohydrolase (acid ceramidase)
2.2	Atp5f1	M35052	ATP synthase, H+ transporting, mitochondrial F0 complex, subunit b, isoform 1
2.2	Gsto1	AB008807	glutathione S-transferase omega 1
2.2	Ift57	AF274590	huntingtin-interacting protein-1 protein interactor - mus musculus
2.2	Jundp2	NM_053894	Jun dimerization protein 2
2.3	75564	AK006440	Protein C6orf206 homolog - mus musculus
2.3	Hsbp1	AF522937	heat shock factor binding protein 1
2.3	Hsbp1	BC002153	similar to heat shock factor binding protein 1 - mus musculus
2.3	Slc12a4	NM_019229	solute carrier family 12, member 4
2.4	Sh3kbp1	AF255884	SH3 domain-containing adapter protein
2.4	Nelf	NM_057190	nasal embryonic LHRH factor
2.4	Cyp4f14	NM_019623	cytochrome P450 4F1
2.4	Atp6v0e2	NP_598525	similar to ATPase, H+ transporting, V0 subunit - mus musculus
2.4	Lklf	AF181251	ESTs, Moderately similar to Kruppel-like factor 6
2.4	Vdup1	U30789	upregulated by 1,25-dihydroxyvitamin D-3
2.4	S100a4	NM_012618	S100 calcium-binding protein A4

FC	Common	Genbank	Product
2.4	Hsbp1	BC002153	similar to heat shock factor binding protein 1 - mus musculus
2.4	AF146738	AF146738	testis specific protein
2.4	Ache	S50879	Acetylcholinesterase
2.4	Lcat	NM_017024	lecithin cholesterol acyltransferase
2.4	Guca2b	NM_022284	guanylate cyclase activator 2b
2.4	S100a3	AF140231	S100 calcium binding protein A3
2.5	BRD1	AW525127	Highly similar to bromodomain containing protein 1; BR140-like
2.5	Cspg5	NM_019284	chondroitin sulfate proteoglycan 5
2.5	AF146738	AF146738	testis specific protein
2.5	Ocm; OM	X15836	Oncomodulin
2.5	LOC94168	U19485	spp-24 precursor
2.5	Pcp4	NM_013002	Purkinje cell protein 4
2.5	Ldhb	NM_012595	lactate dehydrogenase B
2.6	Camk4	NM_012727	calcium/calmodulin-dependent protein kinase IV
2.6	Matk	NM_021859	non-receptor protein kinase protein
2.7	Fgb	U05675	fibrinogen, beta polypeptide
2.7	Slc2a4	NM_012751	solute carrier family 2 , member 4
2.7	Klf2; Lklf	AF181251	Kruppel-like factor
2.7	Inexa	NM_019128	internexin, alpha
2.8	Nelf	AJ293697	nasal embryonic LHRH factor
2.8	S100a3	NM_053681	S100 calcium binding protein A3
2.8	Matk	NM_021859	non-receptor protein kinase protein
2.9	Gipc2	NP_058563	similar to semaf cytoplasmic domain associated protein 2
2.9	Rgs19	AF068136	ESTs, Moderately similar to regulator of G-protein signaling 19
2.9	Fgb	M27220	fibrinogen, beta polypeptide
2.9	Bv8	NM38852	homolog of mouse Bv8; prokineticin 2 precursor
2.9	Prkaa2	NM_023991	AMP-activated protein kinase alpha 2 catalytic subunit
3.0	Tac1	NM_012666	tachykinin 1
3.1	Ldhb	NM_012595	lactate dehydrogenase B
3.3	Crybb3	NM_031690	crystallin, beta B3
3.3	Crybb3	NM_031690	crystallin, beta B3
3.8	Ube2m	NP_663553	similar to ubiquitin-conjugating enzyme e2m (ubc12 homolog)
3.8	Dmgdh	X55995	dimethylglycine dehydrogenase precursor
3.9	Ptpre	Y07834	protein tyrosine phosphatase epsilon-like 2
4.1	Ube2m	NP_663553	similar to ubiquitin-conjugating enzyme e2m (ubc12 homolog)
4.6	Ptpre	D78610	protein tyrosine phosphatase epsilon C
5.2	Slc22a1	NM_012697	solute carrier family 22 (organic cation transporter), member 1

Fold change (FC) represents the relative level of expression from samples treated with Ad-siNkx6.1 versus Ad-siRNAcontrol. Therefore, a negative value or positive value indicates that the relative expression of that gene was lower (-FC) or higher (+FC), respectively, in cells treated with Ad-siNkx6.1 compared to cells treated with Ad-siRNAcontrol. Also provided are the common gene name, GenBank identifier, and a description of the gene product.

APPENDIX C
Phage display applications in identifying beta cell binding peptides

Phage display libraries have been engineered to express cell-surface peptides of various lengths for use in numerous biological screens. I was interested in using this technology to identify beta cell-specific binding peptides, or peptides that can detect different states of cellular stress, such as cells that are contaminated with mycoplasma bacterium, a common contaminant in cell culture systems. In collaboration with Stephen A. Johnston, Kathlynn C. Brown, Tsukasa Oyama, and Kausar N. Samli (University of Texas Southwestern Medical Center, Dallas TX) we successfully isolated individual phage clones that bound to and promoted uptake in beta cell lines and primary rat islets with high specificity. Additionally, we isolated phage clones that bind to cells only when infected with a specific species of mycoplasma bacteria, *Mycoplasma arginini*. A summary of the selection screen used for these studies is shown in Figure C-1.

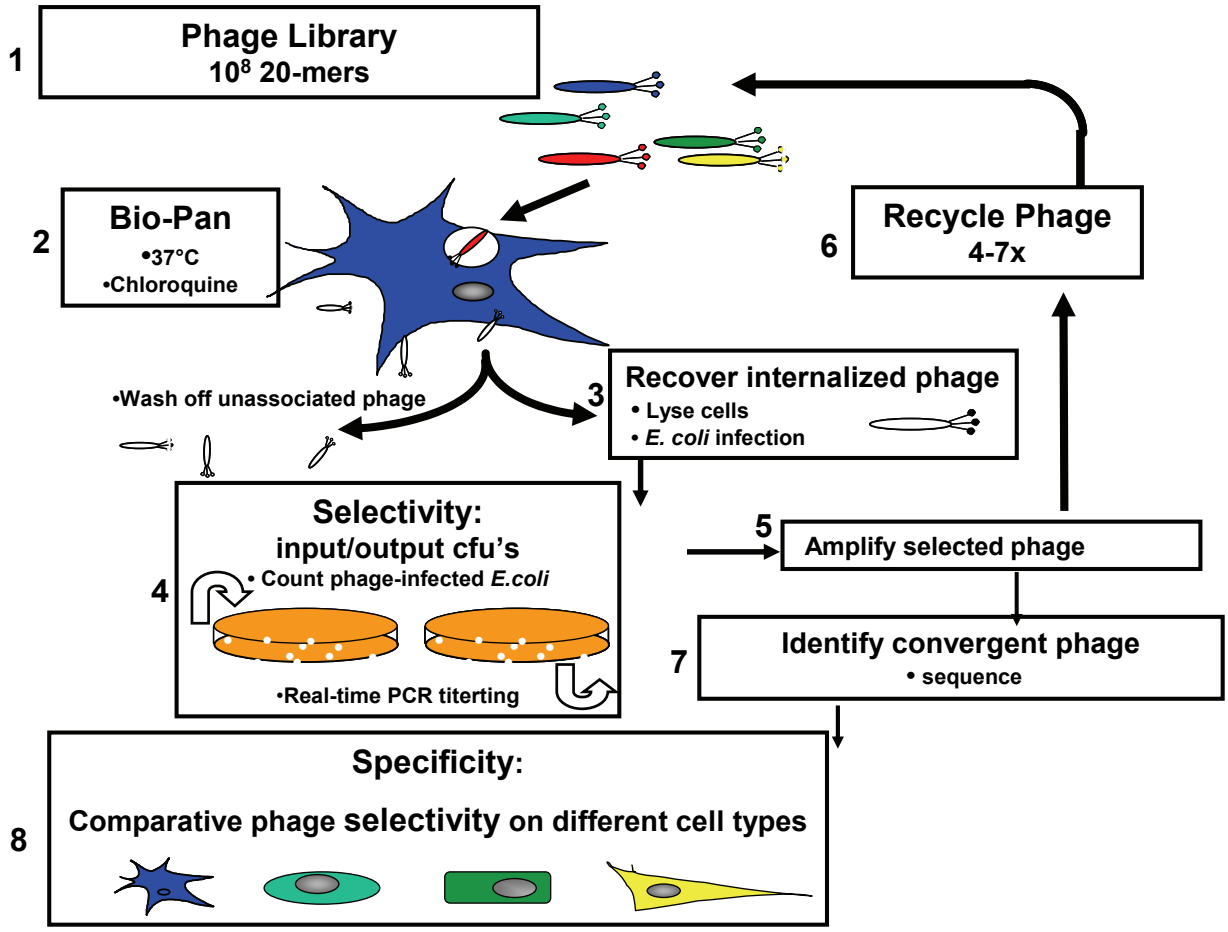


Figure C-1. Outline of phage library screening for cell-specific peptide isolation. Panning a phage library for isolation of individual phage clones that bind to and promote uptake into pancreatic beta cells was carried out using the steps described herein. After a round of panning, only phage that were taken up into the cell or tightly bound to the cell surface are amplified and subsequently used in the next round of panning. In later rounds of panning, sequence analysis of the 20-mer peptide expressed on the surface of selected phage identifies the relative abundance of individual clones. Selected phage clones are isolated, amplified, and further analyzed for their **selectivity** (uptake compared to a random control phage) and **specificity** (selectivity value on the target cell type compared to different cell types).

I successfully isolated phage clones from a 20-mer surface peptide library through a selection screen on two independently derived beta cell lines (832/13 and 833/15) infected with the mycoplasma *M. arginini*. Interestingly, the same two clones were selected for at high frequency during the panning of both infected cell lines (TP 832/13.1 and TP 832/13.2). A third clone also appeared in both cell lines during later rounds of panning with a lower frequency (TP 832/13.3). A fourth targeting phage (TP 832/13.4) only appeared in screening the *M. arginini*-infected 833/15 cell line. Subsequent analysis demonstrated that TP 832/13.1 bound to mycoplasma-infected cells with high selectivity (comparing targeting phage binding versus random phage binding) and high specificity (comparing the selectivity of the targeting phage on mycoplasma-infected cells versus mycoplasma-free cells, analysis performed by K.N.S. and K.C.B). These initial findings and follow up experiments have been published (De *et al.* 2005) and I have included below data that I generated as well as data that was not included in the manuscript.

In parallel experiments using the same library and screening approach, T.O. isolated a targeting phage from the beta line 832/1 (TP 832/1.1) which was not infected with mycoplasma. Subsequently, we were able to show that TP 832/1.1 bound to beta cell lines and primary rat islets with high selectivity. Additionally, testing this phage against various rat cell lines and primary rat hepatocytes demonstrated that TP 832/1.1 also exhibited high specificity for beta cells (J.C.S., K.N.S., and K.C.B.).

Mycoplasma^{+} *832/13 cells*

Phage Clone	Sequence	Rd 3	Rd 4	Rd 5	Rd 6
TP 832/13.1	AMDMYSIEDRYFGGYAPEVG	0/10	2/11	4/13	5/13
TP 832/13.2	AYRVDGGYIYADLDEATDAG	2/10	2/11	5/13	1/13
TP 832/13.3	EHVRDIVVDIGAFTNLPEGR	0/10	1/11	0/13	0/13
TP 832/13.4	VKLNSGLSREVGPRWEDHVFP	0/10	0/11	0/13	0/13

Mycoplasma^{+} *833/15 cells*

Phage Clone	Sequence	Rd 4	Rd 5
TP 832/13.1	AMDMYSIEDRYFGGYAPEVG	3/11	4/13
TP 832/13.2	AYRVDGGYIYADLDEATDAG	2/11	2/13
TP 832/13.3	EHVRDIVVDIGAFTNLPEGR	1/11	1/13
TP 832/13.4	VKLNSGLSREVGPRWEDHVFP	2/11	0/13

Mycoplasma^{-} *832/1 cells*

Phage Clone	Sequence	Rd 5	Rd 6
TP 832/1.1	MSKSPEEGRATVQPSTQPHY	2/6	2/6

Table C-1. Sequence analysis of display peptides on selected phage from various beta cell screens.
Identification of display peptides were determined by DNA sequence analysis of phage clones isolated during different rounds of screening. The resulting 20-mer peptide sequence and the abundance of clones with identical display peptides that occurred twice or more are listed. Included in this table are the four targeting phage selected from two different beta cell lines infected with *Mycoplasma Arginini* (832/13 and 833/15) as well as a single targeting phage selected on a beta cell line free of any mycoplasma infection (832/1, performed by T.O.).

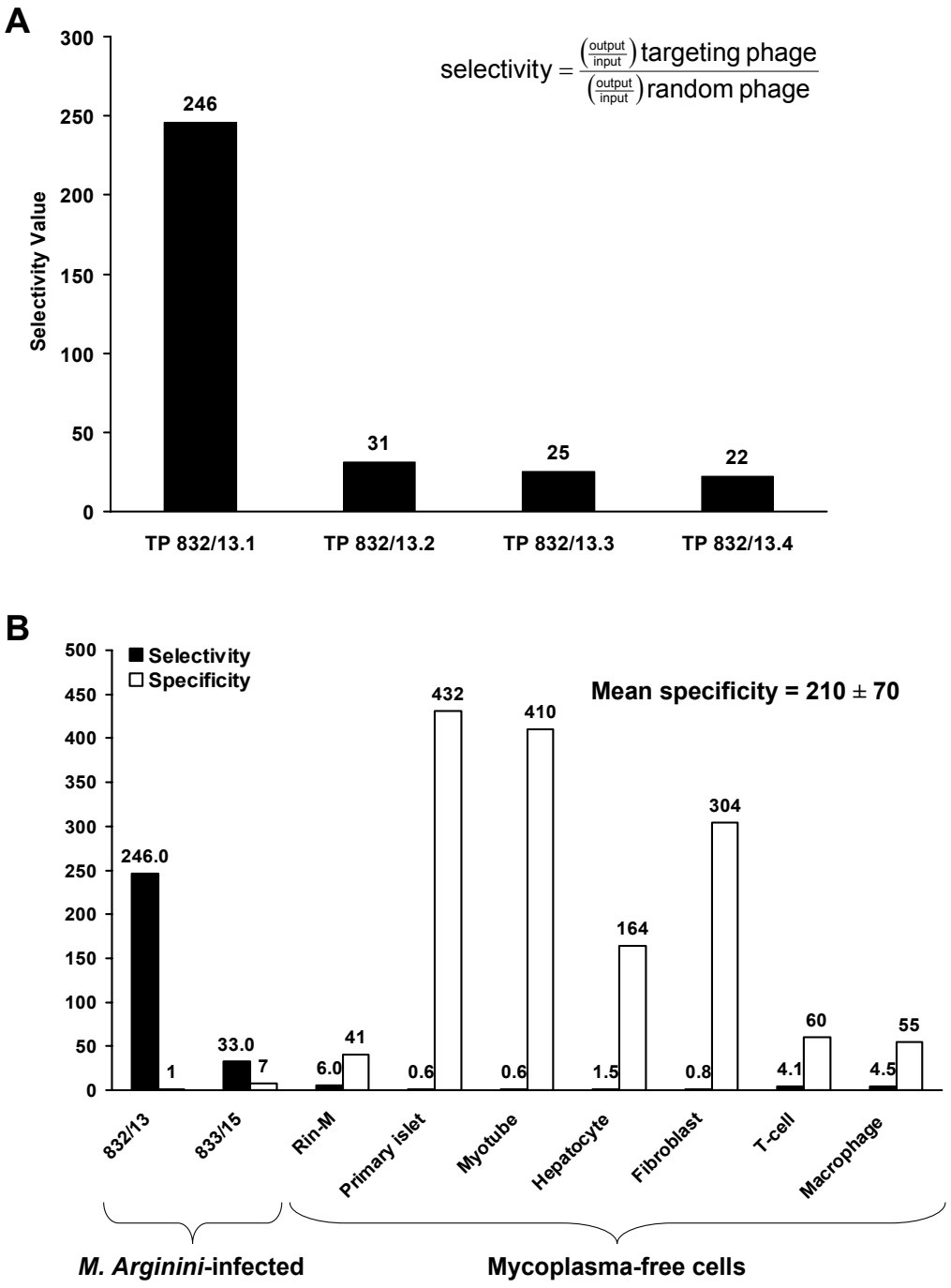


Figure C-2. Selectivity analysis of targeting phage selected from *M. arginini*-infected 832/13 cells and specificity determination of TP 832/13.1.
Targeting phage or random phage were incubated for 1 h prior to recovery of internalized phage. **Panel A** shows the *selectivity* values of the 4 targeting phage isolated from the *M. arginini* screen (Table C-1) on *M. arginini*-infected 832/13 cells. **Panel B** shows the *selectivity* (■) and *specificity* (□) of TP 832/13.1 on various cell lines not infected with *M. arginini* or any other mycoplasma bacteria (performed by K.N.S and K.C.B.). The *specificity* value of TP 832/13.1 is the ratio of *selectivity* values (cell line 832/13 : other cell type).

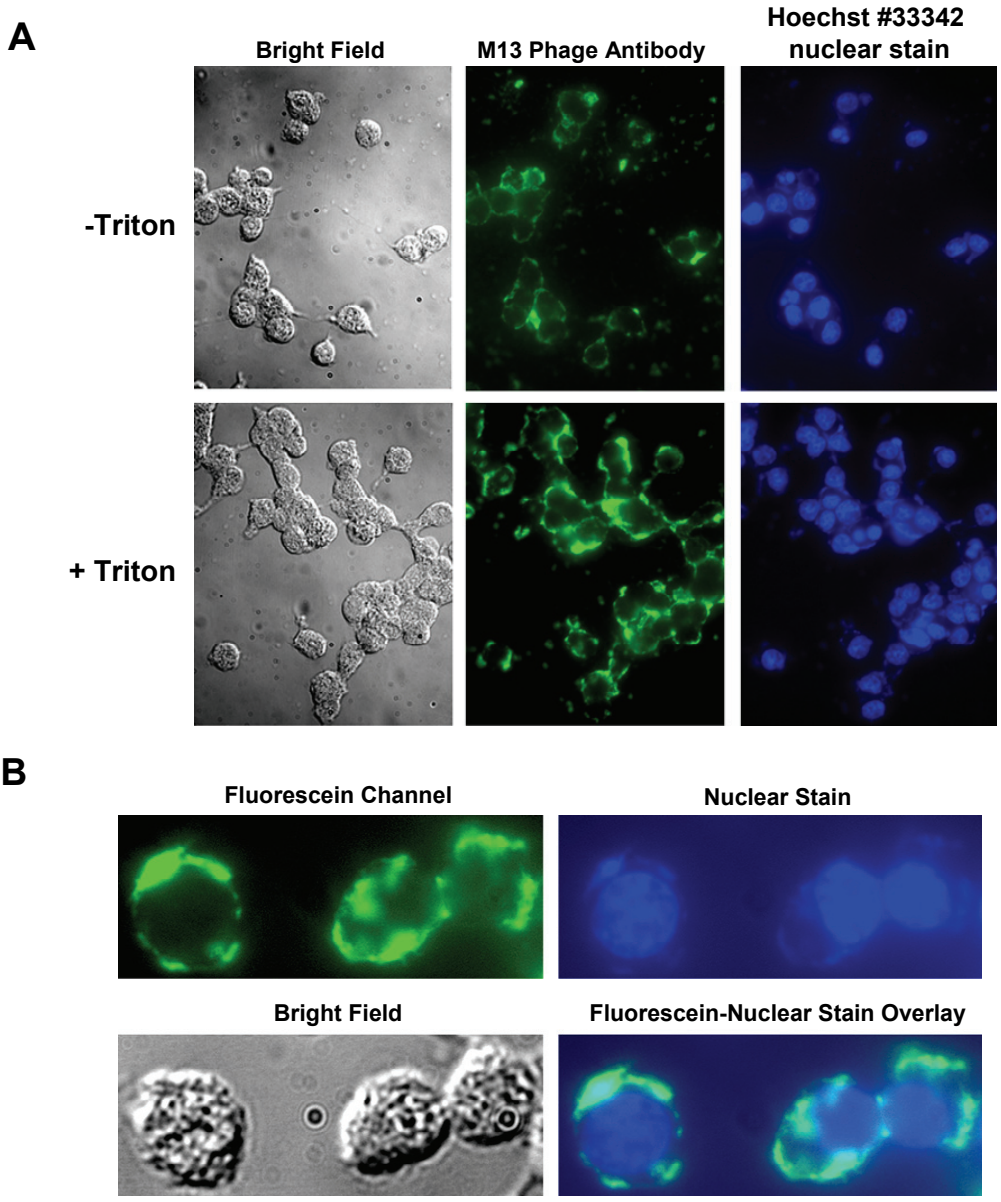


Figure C-3. Immunodetection of TP 832/13.1 on *M. arginini*-infected 832/13 cells. Indirect immunofluorescence was used to visualize TP 832/13.1 binding and uptake into *M. arginini*-infected 832/13 cells. Differences between TP 832/13.1 and random phage binding via immunohistochemistry can be found in (De *et al.* 2005). **Panel A** shows compares TP 832/13.1 binding (M13 phage antibody – green) with (- Triton) and without (+ Triton) permeabilization at 20X magnification. Notably, phage binding to the external plasma membrane of the cell can be detected (- Triton). Upon permeabilization (+ Triton), M13 phage antibody intensity increases on the cell surface in addition to some cytoplasmic localization. These data suggest that TP 832/13.1 binds abundantly to the plasma membrane of the cell and gains entry into the bilayer and cytosol. **Panel B** shows a 60X magnification of permeabilized cells. Extra nuclear DNA staining from the phage can clearly be seen in regions of high phage binding (fluorescein channel). Performed by K.N.S..

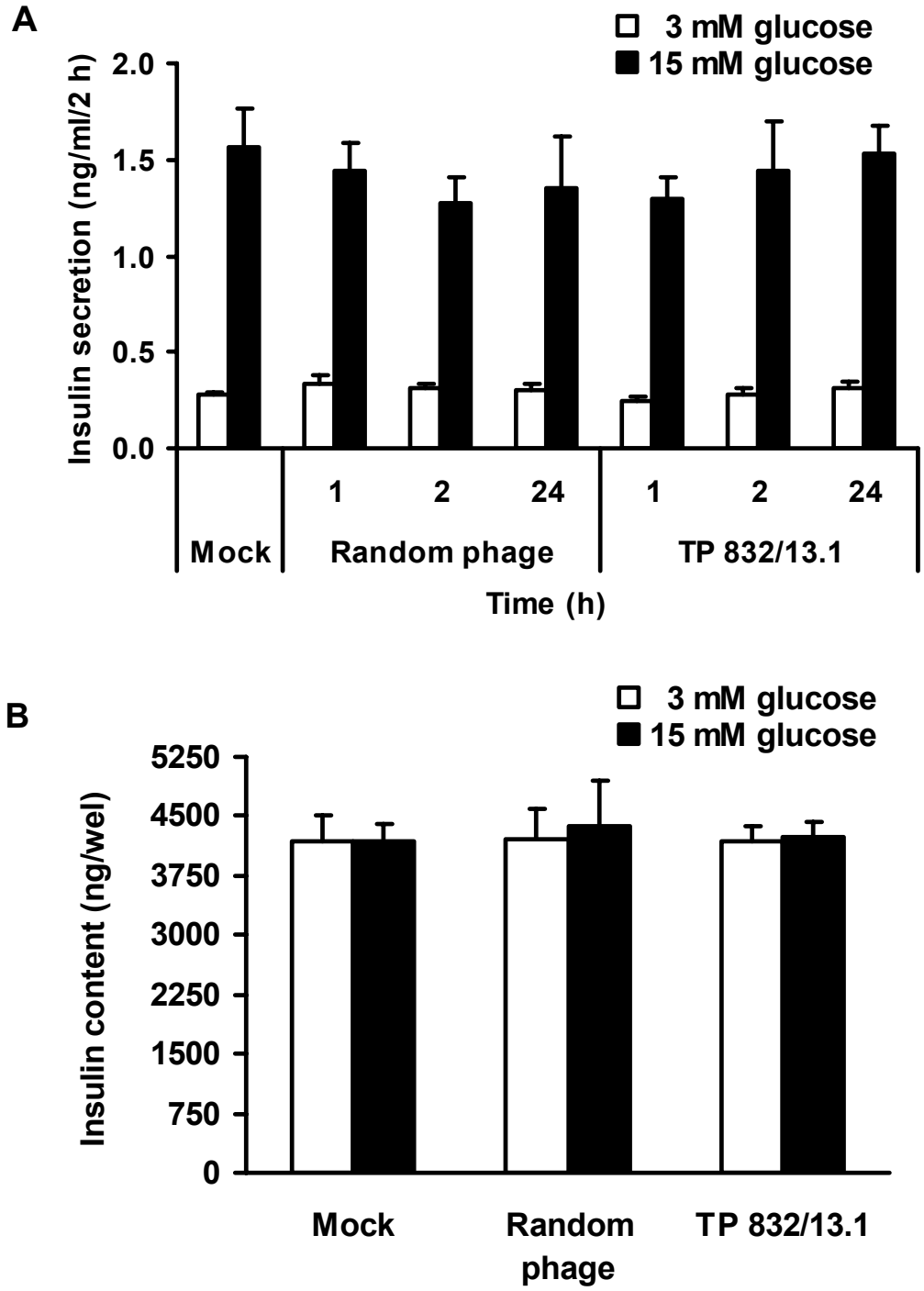


Figure C-4. TP 832/13.1 does not affect GSIS or insulin content in 832/13 cells. **Panel A** shows the GSIS of *M. arginini*-infected 832/13 cells that were treated with either TP 832/13.1 or a random phage for the indicated time points prior to the start of the GSIS assay. Neither TP 832/13.1 nor the random phage has an effect on insulin secretion in low and high glucose conditions. **Panel B** shows that a 24 h exposure to either TP 832/13.1 or a random phage does not alter insulin content.

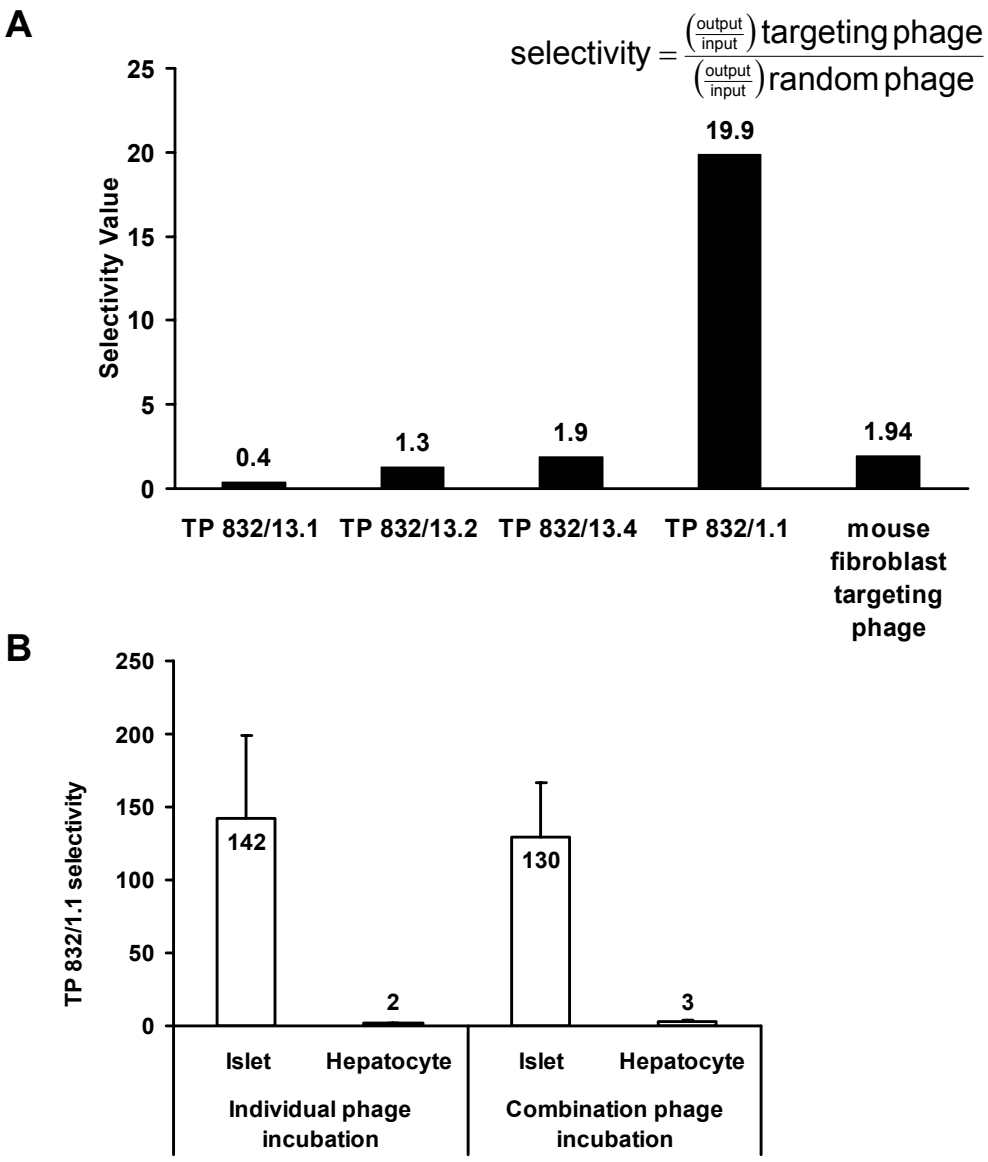


Figure C-5. TP 832/1.1 binds to primary rat islets.
Panel A shows the selectivity of various targeting phage compared to random phage on intact primary rat islets. TP 832/1.1 was the only targeting phage with significant selectivity for primary rat islets. **Panel B** compares the selectivity of TP 832/1.1 (compared to random phage) on splayed primary rat islets and hepatocytes (titering for this assay was done via real-time PCR versus traditional colony counting). Phage were incubated individually or in combination with similar results. These data demonstrate the high selectivity of TP 832/1.1 on primary rat islets with no selectivity on hepatocytes. Additionally, TP 832/1.1 uptake appears to increase in splayed islets compared to intact islets, consistent with the increase in the number of exposed beta cells.

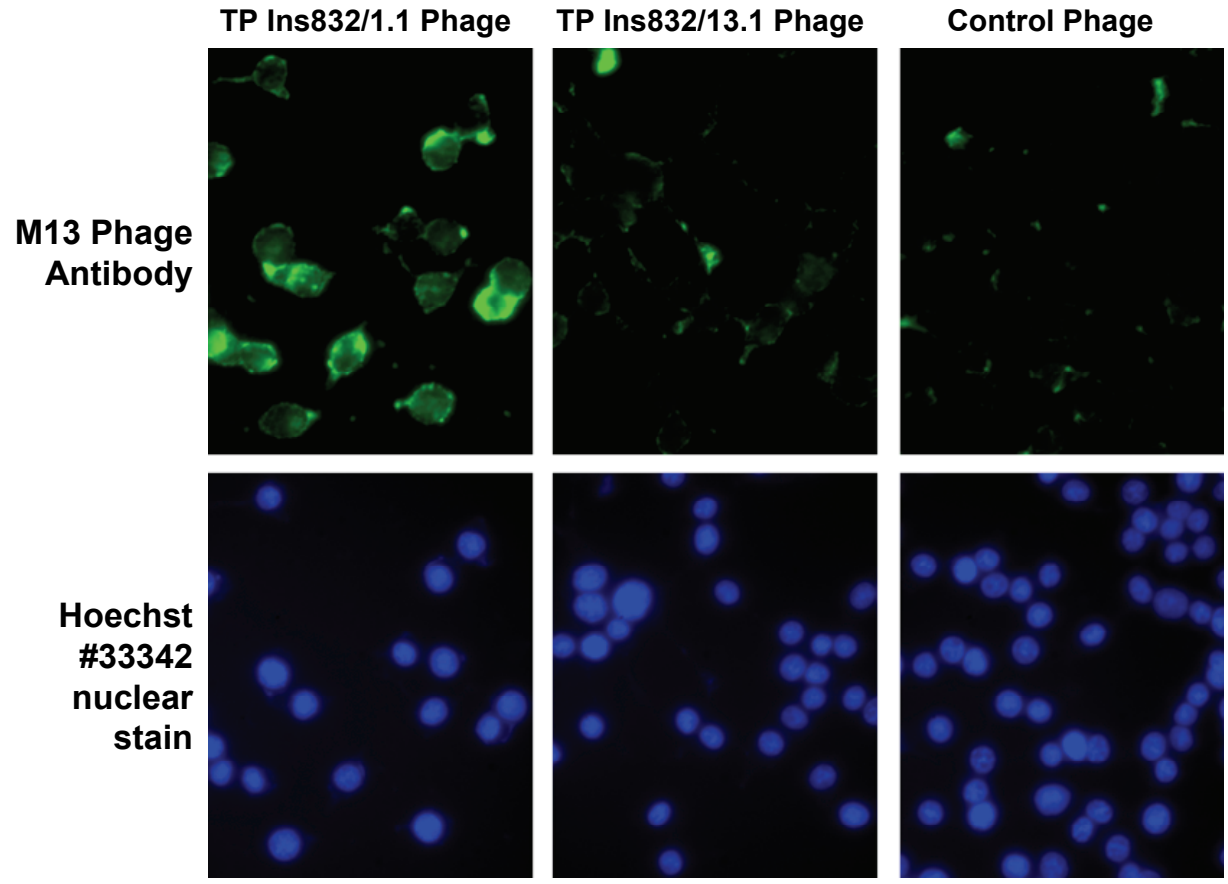


Figure C-6. Immunodetection of TP 832/1.1 on the 832/1 beta cell line.
832/1 cells were incubated for 1 h with TP 832/1.1, TP 832/13.1 or random phage. Indirect immunofluorescence was used to visualize phage binding to cells (M13 phage antibody – green) and counterstained for DNA. TP 832/1.1 exhibits strong localization to the plasma membrane as well as detection throughout the entire cell, in contrast to TP 832/13.1 and a random phage. These data are consistent with TP 832/1.1 being a bona fide beta cell-binding phage and TP 832/13.1 being non-beta cell, *M. arginini*-specific cell-binding phage.

APPENDIX D
Glucagon receptor expression and its role in glucose-stimulated insulin secretion

The identification of genes important to GSIS and maintenance of the mature beta cell through the candidate gene screen described in Chapter 2 was a biased analysis. To complement this approach, a subtractive hybridization method called Representation Difference Analysis (RDA) was performed on the same set of beta cell lines, providing an unbiased screen for differentially expressed genes. The RDA was done in the Newgard lab by Per Bo Jensen (UTSW). The glucagon gene was found to be expressed at higher levels in poorly glucose responsive cells (Class 1 and Class 2) relative to robustly glucose responsive cells (Class 3), confirming the results from the candidate gene screen (Chapter 2). Interestingly, the glucagon receptor (GR) was found to be expressed higher in Class 3 cells relative to Class 1 and Class 2 cells. Although the expression of the glucagon receptor has been documented both in INS-1 cells and primary beta cells (Kieffer *et al.* 1996) a functional role for the receptor in beta cell biology is poorly understood. Therefore adenoviral vectors were constructed to overexpress or silence glucagon receptor expression in beta cell lines and primary rat islets. The human glucagon receptor cDNA (provided by Erica Nishimura, Novo Nordisk) was used to make a recombinant adenovirus (AdCMV-GR or Ad-GR) expressing GR under control of the CMV promoter (created by Katsu Murase, UTSW). Additionally, I used the human glucagon-like-peptide-1-receptor cDNA (GLP-1-R) to make the recombinant adenovirus, AdCMV-GLP-1-R. To complement the overexpression studies, an adenovirus expressing an siRNA targeting rat GR mRNA was constructed to study the effect of GR silencing on beta cell function.

The main findings of the following experiments are: 1) Overexpression of the glucagon receptor but not the glucagon-like-peptide 1 receptor (GLP-1-R) in poorly glucose responsive Class 2 beta cell lines resulted in an improvement in GSIS; 2) Overexpression of GR and GLP-1-R increased the insulin output in Class 3 cell lines and responsiveness to glucagon and GLP-1 peptides, respectively; 3) Endogenous GR mRNA levels were effectively silenced via RNAi, and decreasing GR expression resulted in a loss of GSIS in Class 3 cells and primary rat islets; 4) Manipulation of GR expression levels, either through overexpression or silencing, did not change cAMP levels, suggesting the effect of GR signaling on GSIS is independent of cAMP-mediated signaling.

A

Robustly glucose responsive Class 3	Poorly glucose responsive Class 1 & Class 2
Human leucyl tRNA synthetase	Osteocalcin
HSP 90	***Glucagon***
Sialylyl transferase	Proteasome subunit RC10-11
Rat glucagon receptor	Oxytocin receptor
Mouse ras-related homolog	Ribosomal protein L22
Rat evectin 1	Fetuin-like protein IR1685
Phosphofructokinase Isozyme c	Thymosin beta-4
Rat plasma glutathione peroxidase precursor	Potassium channel beta 2 subunit

B

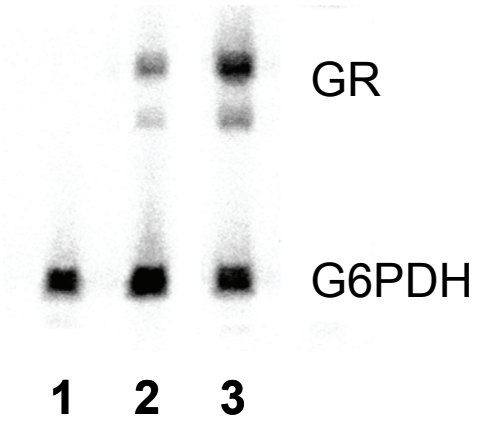


Figure D-1. Differentially expressed genes identified by RDA and QM-PCR analysis of glucagon receptor expression in beta cell lines.
The genes identified to be expressed at relatively higher levels in either Class 3 cells (relative to Class 1 and Class 2 cells) or Class 1 and Class 2 cells (relative to Class 3 cells) are listed (**Panel A**). Genes identified by this screen included higher *glucagon* expression in poorly glucose responsive cells as well as higher *glucagon receptor* expression in robustly glucose responsive cells. **Panel B** confirms the differential expression of GR in the 3 classes of beta cell lines. Class 3 cell lines had approximately 5-fold higher GR expression relative to Class 2 cells whereas Class 1 cells lacked significant GR expression.

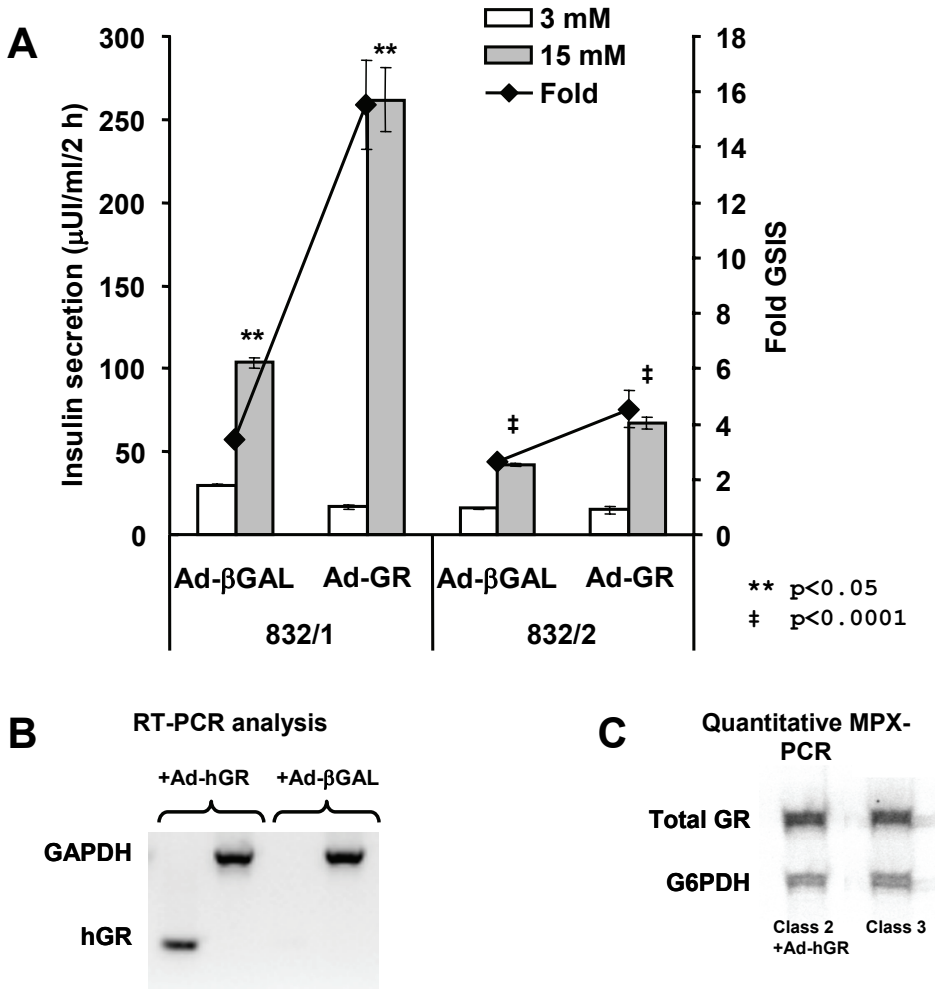
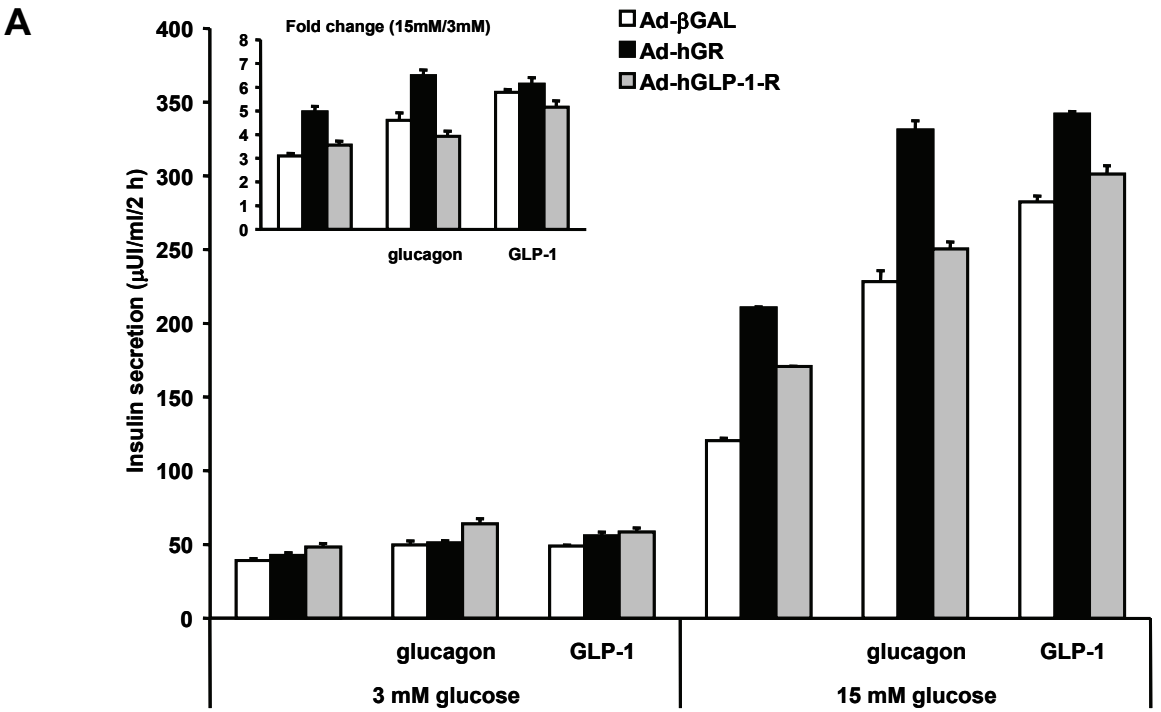


Figure D-2. Overexpression of GR improves GSIS in poorly glucose responsive cell lines. **Panel A** shows the GSIS in two poorly responsive class 2 cell lines treated with adenovirus overexpressing beta galactosidase (Ad-βGAL) or human glucagon receptor (Ad-GR). Overexpression of GR increased the secreted insulin fold response (15 mM versus 3 mM glucose) from 3.5 to 15.5-fold in 832/1 cells and 2.7 to 4.7-fold in 832/2 cells (compared to Ad-βGAL-treated cells). **Panel B** shows the RT-PCR confirmation of expression of the human GR transgene only in cells treated with Ad-GR. **Panel C** shows the QM-PCR analysis of total glucagon receptor expression in Class 2 cells treated with Ad-GR compared to the endogenous GR expression in Class 3 cells. The dose of Ad-GR virus used on Class 2 cells in these studies resulted in an increase in total GR expression approximately equal to endogenous GR expression in Class 3 cells. Data represented are the mean ± SEM from 2-4 experiments per cell line with each condition performed in triplicate.


A
Fold change (15mM/3mM)
Ad-βGAL
Ad-hGR
Ad-hGLP-1-R
Insulin secretion (μUI/ml/2 h)
glucagon
GLP-1
3 mM glucose
15 mM glucose

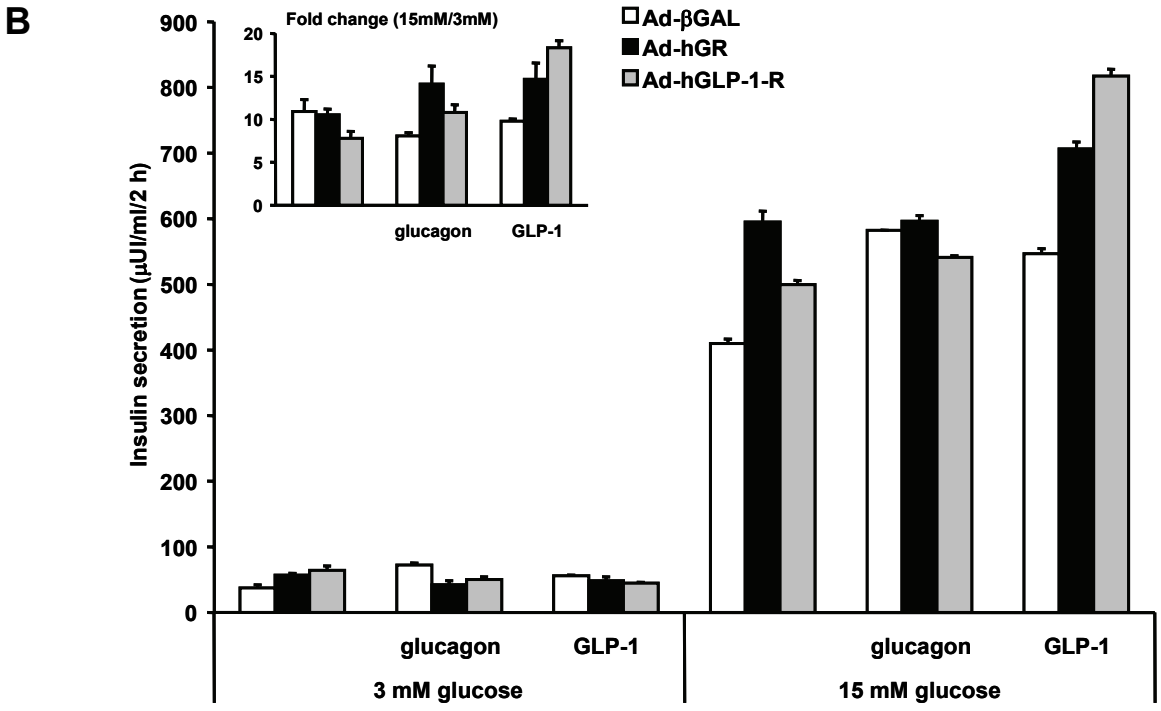

B
Fold change (15mM/3mM)
Ad-βGAL
Ad-hGR
Ad-hGLP-1-R
Insulin secretion (μUI/ml/2 h)
glucagon
GLP-1
3 mM glucose
15 mM glucose

Figure D-3. The differential effects of GR and GLP-1-R overexpression on GSIS in response to ligand in poorly and robustly glucose responsive beta cell lines.
Poorly glucose responsive cells (832/2 – **Panel A**) or robustly glucose responsive cells (832/13 – **Panel B**) were treated with adenoviruses overexpressing either β-galactosidase (Ad-βGAL) human glucagon receptor (Ad-hGR) or human glucagon-like-peptide receptor (Ad-hGLP-1-R) for 24 h prior to GSIS analysis. Where indicated, the peptide ligands glucagon and GLP-1 were included in the secretion conditions at a concentration of 100 nM. **Panel A** shows both GR and GLP-1-R overexpression increases insulin secretion in 15 mM glucose conditions, however, only GR overexpression results in a significant increase in fold secretion (*inset*). The glucagon and GLP-1 peptides increased insulin secretion in 15 mM glucose with all virus treatments. Overexpression of GR did not increase the effect of glucagon peptide on insulin secretion at 15 mM glucose relative to the effect on Ad-βGAL-treated cells. **Panel B** shows both GR and GLP-1-R overexpression increases insulin output in 15 mM glucose in 832/13 cells, although the fold response is not increased (*inset*). Similarly, glucagon and GLP-1 peptides significantly increase insulin output with no increase in fold secretion. In contrast to poorly glucose responsive cells, both GR and GLP-1-R overexpression increases the fold response to glucagon and GLP-1 peptides, respectively, compared to Ad-βGAL-treated cells. In summary, overexpression of GR (but not GLP-1-R) in the absence of any exogenous ligand increased GSIS in poorly glucose responsive beta cells but did not improve the secretagogue effect of exogenous glucagon peptide. Overexpression of GR in robustly glucose responsive beta cells increased insulin output, but not fold secretion; however GR overexpression increased the secretagogue effect of glucagon peptide. Data represented are the mean ± SEM from 3 experiments per cell line with each condition performed in triplicate.

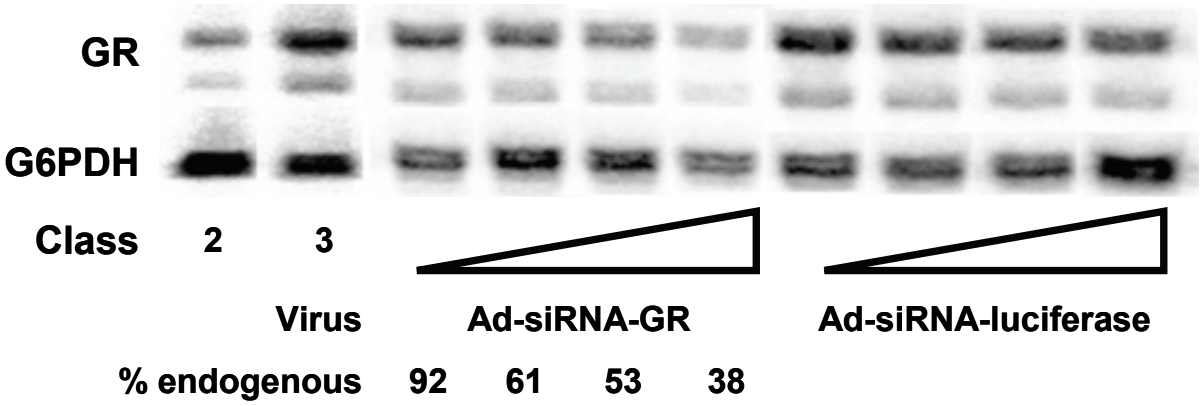


Figure D-4. siRNA-mediated silencing of rat glucagon receptor expression in Class 3 cells. Class 3 cells (832/13) were treated with increasing amounts of adenovirus expressing an siRNA targeting the rat glucagon receptor (Ad-siRNA-GR) or a control virus (Ad-siRNA-luciferase). The amount of glucagon receptor mRNA was measured via semi-quantitative multiplex-PCR. GR mRNA decreased in a dose-dependent manner with increasing amounts of Ad-siRNA-GR; in contrast, there was no change in GR mRNA with Ad-siRNA-luciferase. The percent knockdown was calculated via densitometry. As reference, the levels of GR in Class 2 and Class 3 cells is shown (left two lanes). Note that the higher two doses of Ad-siRNA-GR resulted in lowering GR expression in Class 3 cells to that found in Class 2 cells. The Ad-siRNA-GR-mediated reduction in glucagon receptor mRNA levels was confirmed in multiple independent experiments, shown here is a representative analysis.

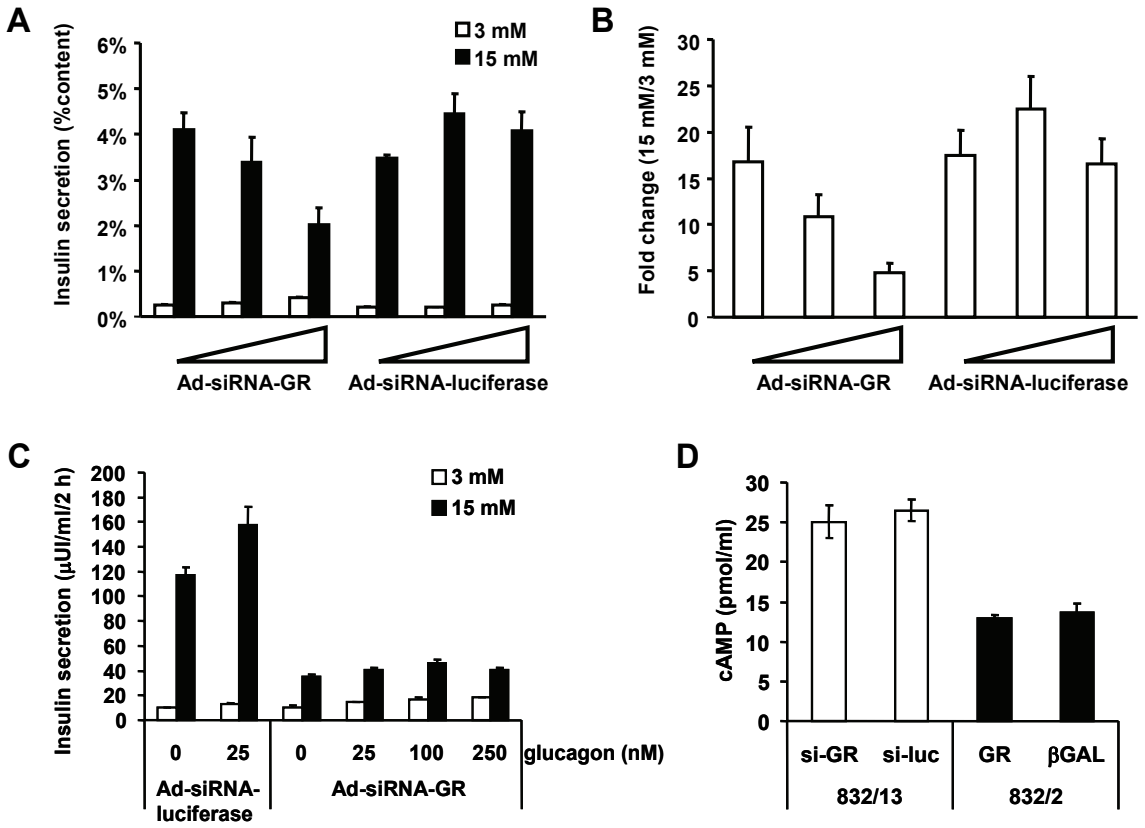


Figure D-5. Glucagon receptor expression is necessary for GSIS in beta cell lines. Class 3 cells (832/13) were treated with increasing amounts of Ad-siRNA-GR or Ad-siRNA-luciferase for 18 h followed by 3 days of culturing prior to GSIS analysis. **Panel A** shows that insulin secretion at 15 mM glucose decreased in a dose-dependent manner with increased amounts of Ad-siRNA-GR. **Panel B** shows the fold change comparing insulin secretion at 15 mM versus 3 mM glucose and reflects the decrease in GSIS with increasing amounts of Ad-siRNA-GR. **Panel C** shows that exogenous glucagon peptide can increase insulin secretion in Class 3 cells at concentrations as low as 25 nM. Silencing GR expression results in a loss of GSIS (compare Ad-siRNA-luciferase and Ad-siRNA-GR, 0 nM glucagon condition) and also blocks any increase in insulin secretion mediated by exogenous glucagon peptide (compare Ad-siRNA-luciferase and Ad-siRNA-GR, 25 nM glucagon condition). Increasing the concentration of exogenous glucagon peptide up to 250 nM could not rescue the effect of Ad-siRNA-GR. **Panel D** shows the measurement of cAMP levels after cells were switched from 3 mM to 15 mM glucose for 30 minutes. Class 3 cells (832/13) treated with Ad-siRNA-GR (si-GR) or Ad-siRNA-luciferase (si-luc) and Class 2 cells (832/2) treated with AdCMV-βGAL (βGAL) or AdCMV-hGR (GR). Despite the effect seen on GSIS with the manipulation of GR expression, there does not appear to be any change in cAMP accumulation, suggesting that the effect of GR expression on GSIS may be independent of cAMP-mediated signaling. Data represented are the mean ± SEM from 2-4 experiments with each condition performed in triplicate.

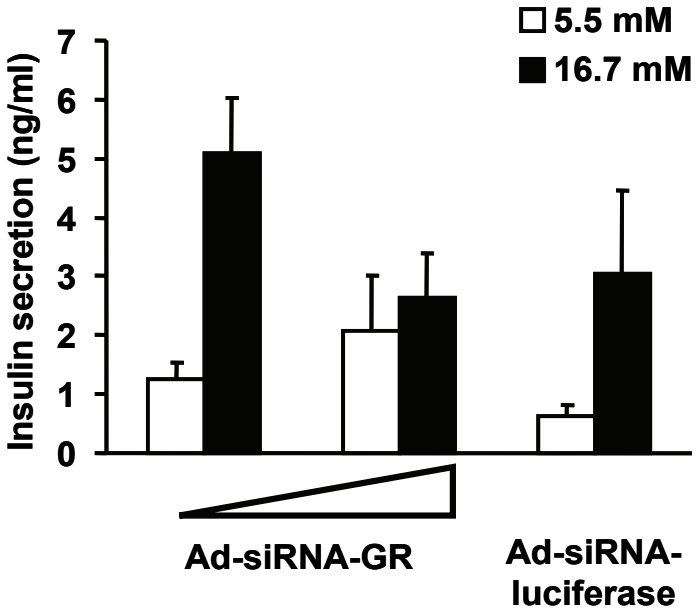
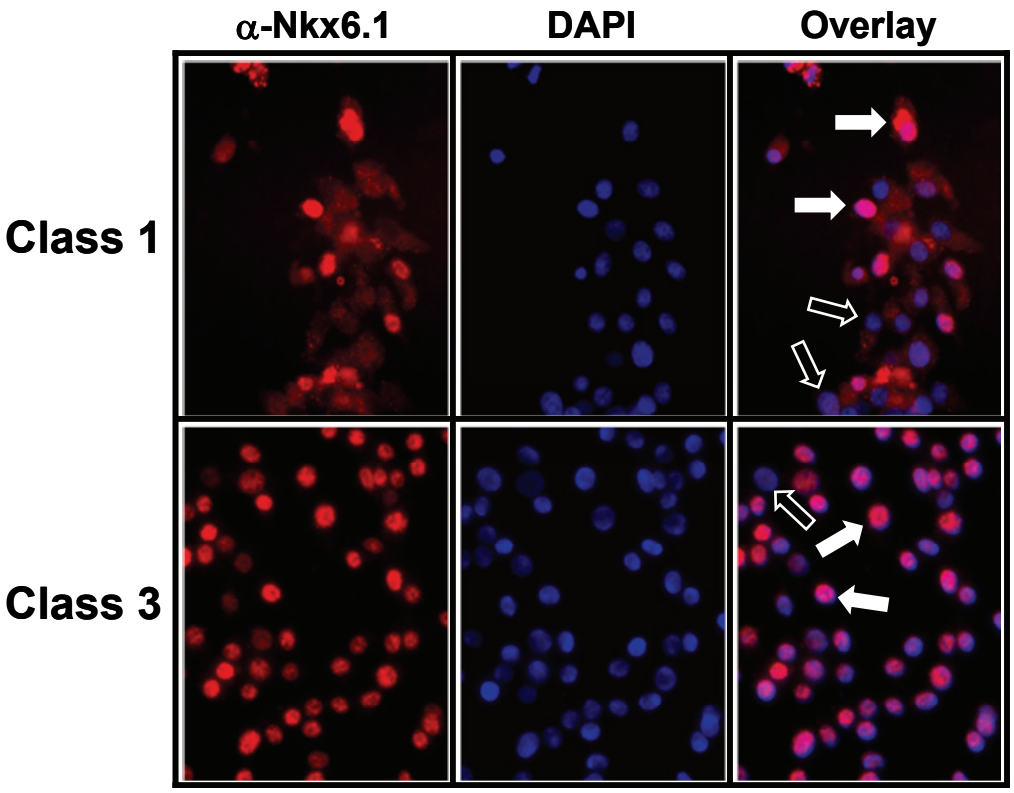


Figure D-6. Glucagon receptor expression is necessary for GSIS in primary rat islets. Two different amounts of Ad-siRNA-GR or a control virus (Ad-siRNA-luciferase) were added to primary rat islets for 18 h and maintained in culture for 72 h followed by GSIS analysis. The low dose of Ad-siRNA-GR had a mild effect on GR mRNA levels (25% reduction relative to Ad-siRNA-luciferase-treated cells). Although raw insulin output was slightly elevated in this condition, there was no change in fold secretion. However, the higher dose of Ad-siRNA-GR resulted in a 55% reduction in GR mRNA levels as well as a loss of GSIS, attributed to a large increase in basal insulin secretion. Results are the mean ± SEM from two independent islet experiments done in duplicate.

APPENDIX E
Indirect immunofluorescence of Nkx6.1 and Pdx1 expression

Chapter 2 described the observation that Class 3 cells have higher levels of Nkx6.1 expression relative to Class 1 cells. This observation has been observed both on an mRNA level (QM-PCR and real-time PCR) and a protein level (immunoblot analysis). However, this did not preclude that these differences were due to differential expression of Nkx6.1 within the cell line, *i.e.* do all Class 1 cells have reduced Nkx6.1 expression, or is there subpopulations of cells with varying Nkx6.1 expression? To address this issue, indirect immunofluorescence was used to detect the localization and expression level of Nkx6.1 in a Class 1 and Class 3 cell line. Nkx6.1 expression varied in Class 1 cells, with the majority of cells expressing little Nkx6.1 protein. However, there was a subpopulation of Class 1 cells that had strong nuclear localization of Nkx6.1, similar to nearly all Class 3 cells. Additionally, although there was no change in Pdx1 expression on an mRNA and protein level between Class 1 and Class 3 cells, indirect immunofluorescence was also used to analyze Pdx1 expression. Interestingly, there was a heterogeneous Pdx1 expression *within* a Class 1 cell line but not in a Class 3 cell line.


α-Nkx6.1
DAPI
Overlay
Class 1
Class 3

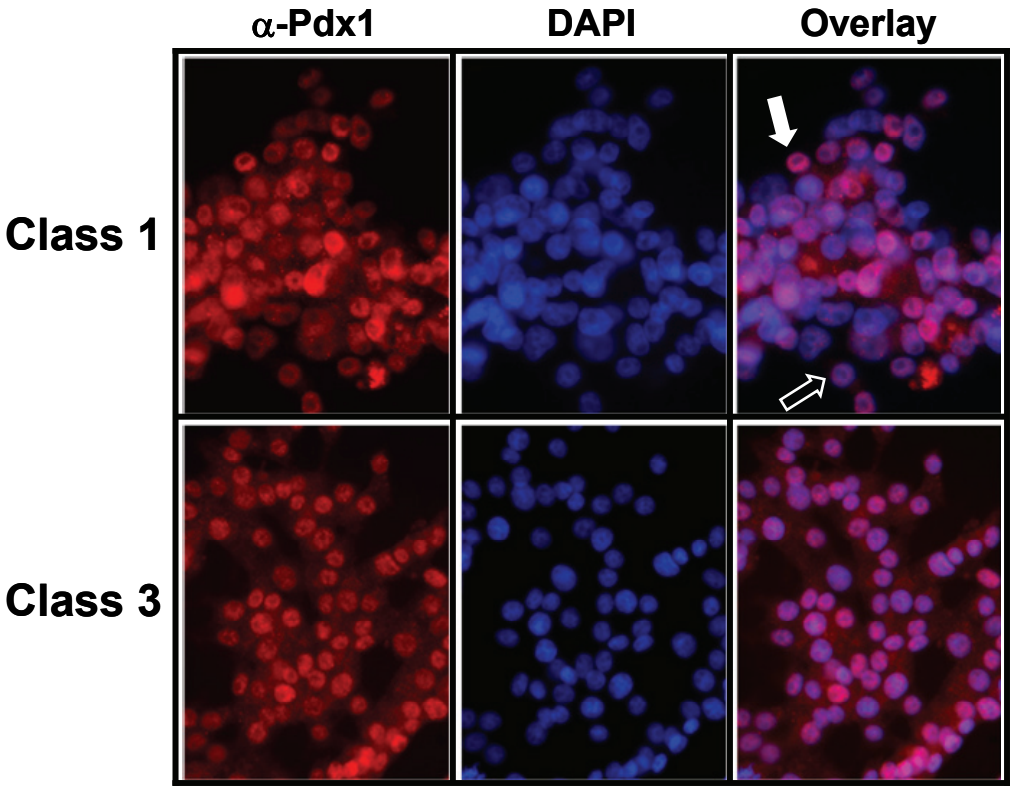

α-Pdx1
DAPI
Overlay
Class 1
Class 3

Figure E-1. Indirect immunofluorescence of Nkx6.1 and Pdx1 expression in Class 1 and Class 3 cells.

Cells with relatively low (Class 1 – 834/105) or high (Class 3 – 832/3) Nkx6.1 expression were seeded onto chamber slides and analyzed for Nkx6.1 or Pdx1 expression via indirect immunofluorescence. Briefly, cells were fixed with ice-cold 4% paraformaldehyde/PBS on ice for 15 min. Slides were washed with 25 mM NH4Cl/PBS for 5 min and washed in PBS for 5 min. Samples were permeabilized with 0.3% Triton X-100/PBS for 5 min, twice, and washed twice in PBS for 5 min. Blocking was done with 5% normal donkey serum/PBS for 30 min and removed by wiping. Primary antibodies: mouse anti-Nkx6.1 (1:1000, BCBC) or rabbit anti-Pdx1 (1:5000, C. Wright) was added in 5% normal donkey serum/PBS and incubated overnight at 4ºC. Slides were washed three times in PBS for 5 min. Secondary antibodies (Rhodamine Red-X-conjugated Donkey IgG, Jackson Labs) were diluted 1:25 in 5% normal donkey serum and incubated for 1 h at room temp in the dark. Arrows identify cells with high levels (closed arrows, ➡) or minimal levels (open arrows, ⇨) of nuclear Nkx6.1 immunoreactivity. Note that the majority of Class 1 cells appear to have little to no Nkx6.1 expression, however there are Class 1 cells with levels of Nkx6.1 expression equal to that found in Class 3 cells. Nearly all Class 3 cells have high nuclear Nkx6.1 expression, relative to the Class 1 cells. Additionally, in cells from both Classes that express high levels of Nkx6.1, the localization of Nkx6.1 is properly localized to the nucleus. The pattern of Pdx1 expression was also different between these two classes of cells, although the effect was not as dramatic as Nkx6.1 expression. On an mRNA and protein level, there was no significant difference in Pdx1 expression between Class 1 and Class 3 cells (Chapter 2 and data not shown). Consistent with that observation, the fluorescent intensity of Pdx1 levels on a per cell basis was not changed (data not shown). However, whereas the Pdx1 intensity in Class 3 cells was consistent on a cell-to-cell basis, in Class 1 cells there was a wider distribution of Pdx1 intensity, thus Class 1 cells consist of subpopulations of cells with differential Pdx1 expression. Arrows identify cells with high levels (closed arrows, ➡) or minimal levels (open arrows, ⇨) of total Pdx1 immunoreactivity.

APPENDIX F
Comparisons between Nkx6.1 and Pdx1 expression in maintaining beta cell function

The majority of this dissertation focuses on the role of Nkx6.1 expression in mediating several important beta cell functions, such as GSIS and beta cell proliferation. In most cases where Nkx6.1 expression was manipulated, either through overexpression or silencing, reagents to perform similar changes in Pdx1 expression were also used. To maintain the focus of the discussion on Nkx6.1 function, the Pdx1-related data was often omitted. Therefore, in this appendix I have included the Pdx1 expression that was done in parallel with the Nkx6.1 expression studies.

Nkx6.1 and Pdx1 have redundant functions such as the regulation of glucagon expression, discussed in detail in Chapter 2, although through apparently different mechanisms. Additionally, overexpression of either Nkx6.1 or Pdx1 increased cell proliferation and cyclin B1 mRNA in primary rat islets with no detriment in beta cell function. However, there were some distinctions between Nkx6.1 and Pdx1 expression and function in the various models used throughout this dissertation: 1) Silencing Nkx6.1, but not Pdx1, in beta cell lines decreased proliferation and cyclin B1 expression; 2) Pdx1 overexpression had a much smaller effect on increasing proliferation compared to Nkx6.1 overexpression in beta cell lines cultured in serum-free conditions or exposed to cytokines; 3) Consistent with a distinct role for Nkx6.1 in the cytokine resistant model (discussed in Chapter 4), exposure to IL-1β and γ-IFN resulted in a rapid decrease in Nkx6.1 mRNA and protein levels only in cytokine-sensitive cell lines, whereas Pdx1 levels remain unchanged.

The microarray analysis presented in Chapter 3 omitted data demonstrating the effects of Pdx1 silencing on changes in gene expression in robustly glucose responsive cells. Therefore

this appendix includes the full analysis of that microarray, including the genes knocked down with Nkx6.1 silencing (as shown in Appendix A) and Pdx1 silencing. Additionally, in each list I have included the expression level of both treatment conditions, revealing whether the effect on a given gene is similar or different with Nkx6.1 or Pdx1 silencing.

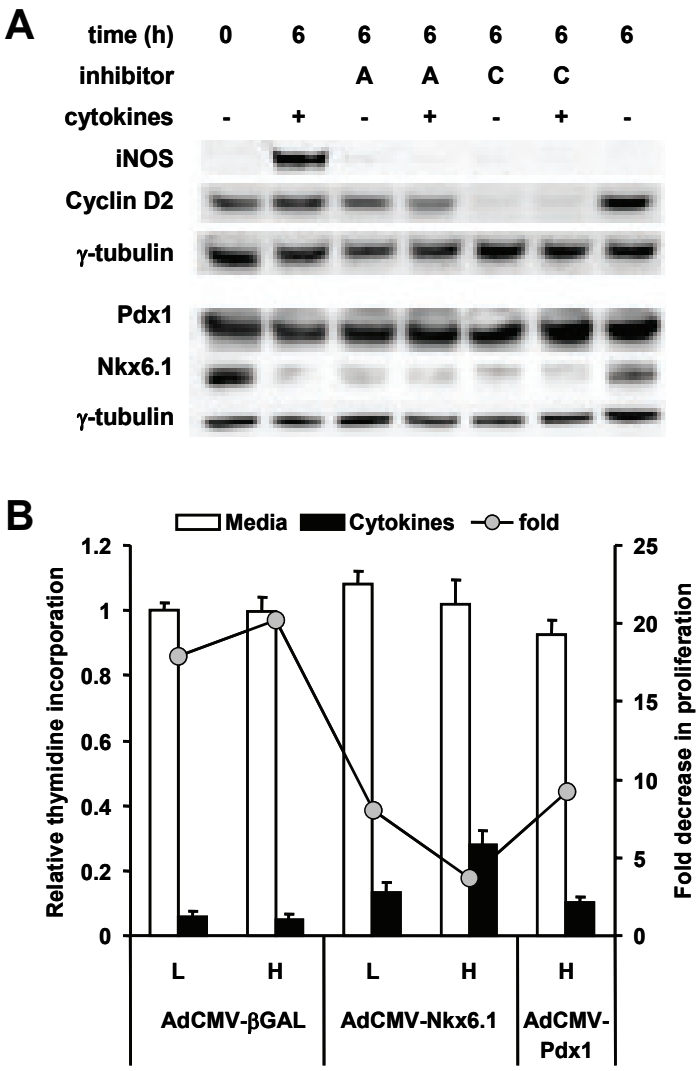
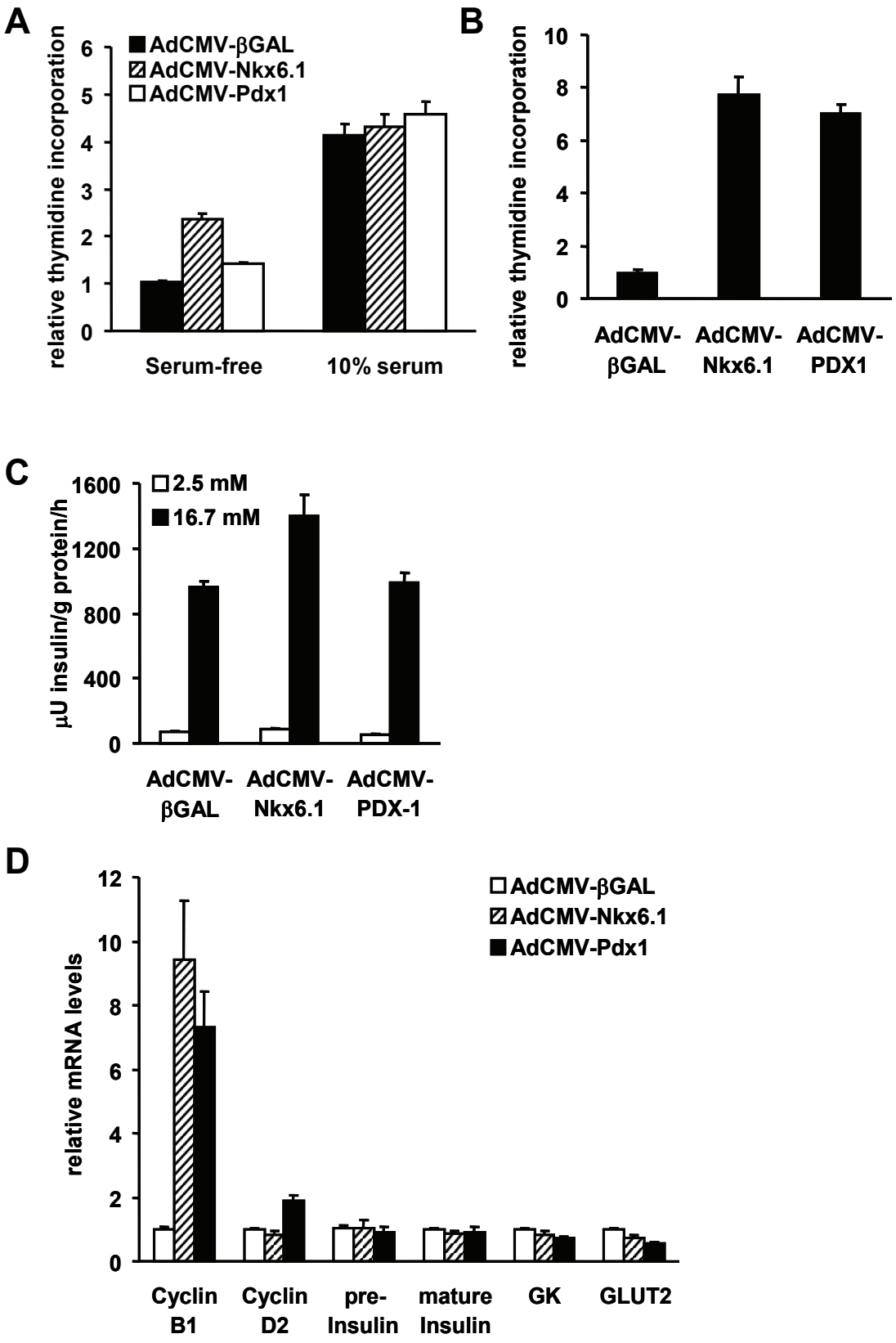


Figure F-1. The effect of cytokines on endogenous Nkx6.1 and Pdx1 protein levels and the improvement of beta cell proliferation in response to cytokines with Nkx6.1 or Pdx1 overexpression.

Panel A shows representative immunoblots from cytosolic or nuclear fractions from Class 3 cells (832/3) cultured in normal media with or without the pro-inflammatory cytokines IL-1β and γ-IFN for 6 h. In addition, cells were concurrently treated with the transcriptional or translational inhibitors *actinomycin D* (**A**) or *cycloheximide* (**C**). Protein levels of iNOS and cyclin D2 were measured from cytosolic fractions whereas Pdx1 and Nkx6.1 protein levels were measured from nuclear fractions. To ensure proper loading, membranes were re-probed for γ-tubulin to ensure equal protein loading. After 6 h of cytokine exposure, iNOS expression was detectable, a process dependent on both transcription and translation. Cyclin D2 protein has a short half-life (compare no inhibitor to cycloheximide treatment) and has been identified as being regulated post-transcriptionally. Nkx6.1 levels decrease after cytokine exposure. Likewise, inhibiting transcription or translation resulted in a similar decrease in Nkx6.1 protein, suggesting that Nkx6.1 mRNA and protein have a short half-life. Pdx1 protein levels are not affected in any condition. **Panel B** shows the effect of proliferation of Class 3 cells (832/3) treated with

adenovirus overexpressing β-galactosidase (AdCMV-βGAL), Nkx6.1 (AdCMV-Nkx6.1), or Pdx1 (AdCMV-Pdx1) for 24 h followed by culturing in normal media with or without IL-1β and γ-IFN for 18 h. There was no significant effect on proliferation in cells grown in normal media (containing 10% serum) in any virus condition. Cytokine exposure in cells treated with AdCMV-βGAL resulted in a 20-fold decrease in proliferation. Overexpression of Nkx6.1 or Pdx1 increased proliferation in cytokine-treated cells compared to βGAL-overexpressing cells. The cytokine-induced decrease in beta cell proliferation improved from a 20-fold (βGAL) to 4- to 10-fold (Nkx6.1 and Pdx1, respectively).


A
relative thymidine incorporation
AdCMV-βGAL
AdCMV-Nkx6.1
AdCMV-Pdx1
Serum-free
10% serum
B
relative thymidine incorporation
AdCMV-βGAL
AdCMV-Nkx6.1
AdCMV-PDX1
C
μU insulin/g protein/h
2.5 mM
16.7 mM
AdCMV-βGAL
AdCMV-Nkx6.1
AdCMV-PDX-1
D
relative mRNA levels
AdCMV-βGAL
AdCMV-Nkx6.1
AdCMV-Pdx1
Cyclin B1
Cyclin D2
pre-Insulin
mature Insulin
GK
GLUT2

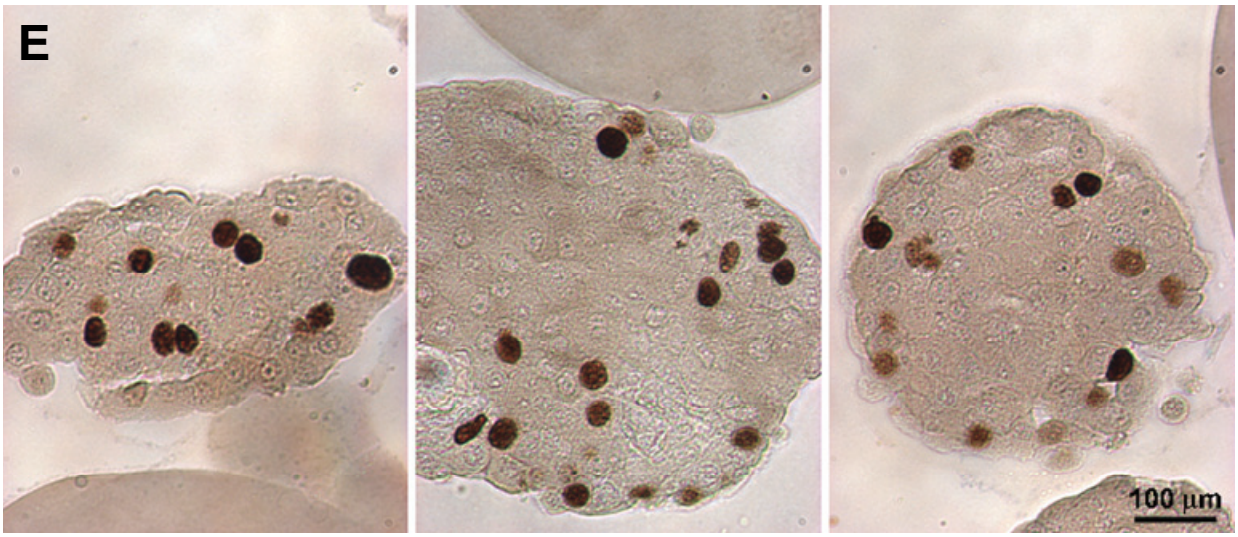


Figure F-2. The effects of Pdx1 versus Nkx6.1 on beta cell proliferation.
Panel A shows the proliferation response of Class 1 cells (834/105) treated with adenovirus overexpressing β-galactosidase (AdCMV-βGAL), Nkx6.1 (AdCMV-Nkx6.1), or Pdx1 (AdCMV-Pdx1) in either serum-free or 10% serum conditions. In the presence of serum, there was no significant change in proliferation with any virus treatment. In serum-free conditions, Nkx6.1 overexpression results in a 2.5-fold increase in proliferation, whereas Pdx1 overexpression had a smaller, 50%, but significant effect on proliferation compared to βGAL-overexpressing cells. **Panel B** shows a similar increase in islet proliferation in response to Nkx6.1 or Pdx1 overexpression compared to βGAL overexpression. **Panel C** shows that overexpression of Nkx6.1 or Pdx1 does not impair GSIS in primary rat islets relative to βGAL-overexpressing islets. **Panel D** shows the effect of Nkx6.1 and Pdx1 overexpression on mRNA levels of various transcripts in primary rat islets relative mRNA levels in βGAL-overexpressing islets. **Panel E** shows representative micrographs of sections from primary rat islets overexpressing Pdx1 that were cultured with BrdU to determine the number of cells undergoing DNA synthesis. BrdU incorporation was detected via immunohistochemistry and BrdU-positive nuclei are stained brown.

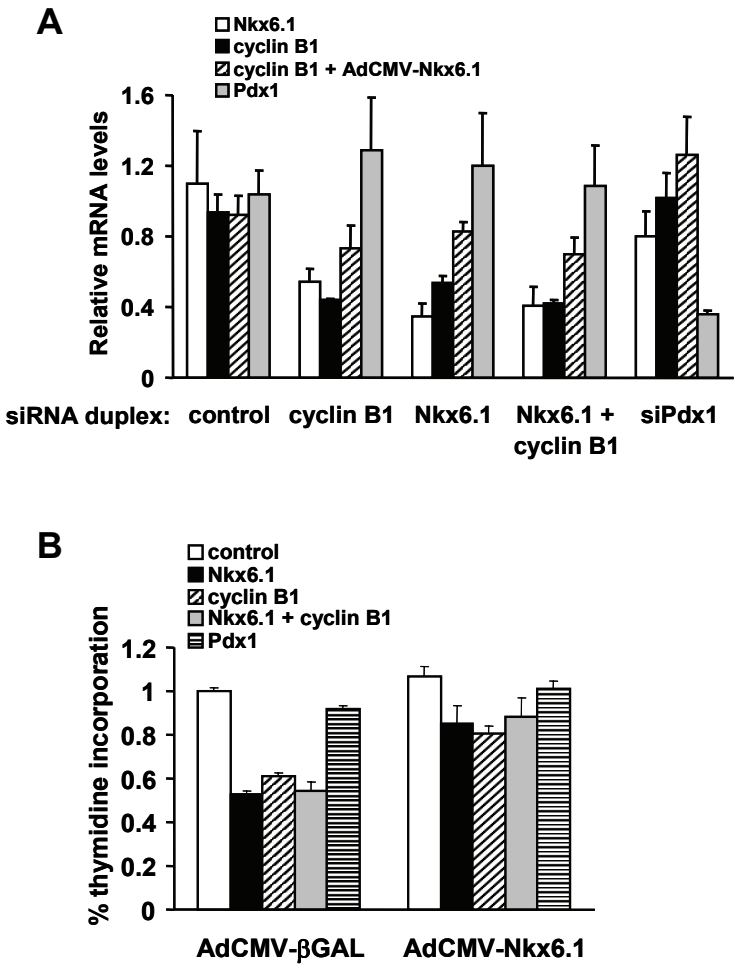


Figure F-3. Effect of Pdx1 silencing in 832/3 cells.
Panel A shows the relative mRNA levels of Nkx6.1, cyclin B1, and Pdx1 in 832/3 cells that have been transfected with either siNkx6.1, siCyclin B1, a combination of siNkx6.1 and siCyclin B1, or siPdx1, and compared to cells transfected with siRNAcontrol. The effect of overexpressing Nkx6.1 (with AdCMV-Nkx6.1 treatment) on cyclin B1 transcript levels is also shown. Data represented are from 3 independent experiments measured from duplicate RNA samples. Note the cyclin B1 levels are not changed despite a 60% decrease in Pdx1 expression. **Panel B** shows the effect of these transfections on ^{3}H-thymidine incorporation into genomic DNA after treatment with either AdCMV-βGAL or AdCMV-Nkx6.1. Data are from 4 independent experiments, each performed in duplicate. Pdx1 silencing had no significant effect on proliferation in βGAL- or Nkx6.1-overexpressing cells.

The following three tables summarize the microarray analysis of Nkx6.1 silencing in a robustly glucose responsive cells line initially described in Chapter 3 and has been expanded to include analysis of Pdx1 silencing that was performed in parallel experiments. To summarize, 832/13 cells were seeded into 6 cm plates and cultured overnight. Subsequently cells were treated with either Ad-siRNAcontrol, Ad-siNkx6.1, or Ad-siPdx1 at an MOI of 20 for 16 h. Total RNA was harvested 72 h later using the RNeasy micro kit (Qiagen) which included DNase treatment to eliminate genomic contamination. Parallel experiments were performed to validate the GSIS phenotype in cells treated with Ad-siNkx6.1 and Ad-siPdx1 (Schisler *et al.* 2005) as well as to prepare protein samples. Duplicate RNA samples (1 μg each) per condition from 3 independent experiments were used for one round of amplification and labeled with Cy5. These samples were hybridized with rat reference RNA labeled with Cy3 to a DNA chip containing the oligonucleotides from the rat operon v1.1 microarray (5600 rat genes, Operon) and the rat 10K OciChip (9715 rat genes, Ocimum) that was scanned on a Gene Pix 5000 scanner. Analysis of the data was performed using the Genespring v7.2 software (Silicon Genetics). Data was normalized using per chip and per spot intensity-dependent LOWESS normalization. Statistical analysis was performed using the software's cross-gene error model using 6 biological replicates per condition. Results of one-way ANOVA (parametric test, variances not assumed equal) were filtered for fold change (greater that 2-fold) and p-values less than 0.05 (Welch t-test).

Table F-1 and Table F-2 lists the fold change of filtered genes found with Nkx6.1 silencing or Pdx1 silencing, respectively, compared to *control* samples. To provide easy comparison between Nkx6.1 and Pdx1 and their common (or differential) effects on gene expression, Table F-1 and Table F-2 also indicates the fold change of these genes from the Pdx1 and Nkx6.1 silencing conditions, respectively. In addition, Table F-3 lists the fold change of

filtered genes that were found to be changed with Nkx6.1 compared to *Pdx1* silencing. For reference, the effect of Nkx6.1 silencing on the fold change of genes on this list relative to *control* samples is listed in the second column. Table F-3, therefore, provides a list of genes that are differentially affected by Nkx6.1 and Pdx1 silencing. **Fold Change** (FC) represents the relative level of expression from two conditions (*e.g.* cells treated with Ad-siNkx6.1 versus Ad-siRNAcontrol; therefore, a negative value or positive value indicates that the relative expression of that gene was lower (-FC) or higher (+FC), respectively, in cells treated with Ad-siNkx6.1 compared to cells treated with Ad-siRNAcontrol). Also provided is the common gene name, GenBank identifier, and a description of the gene product.

Table F-1. Changes in gene expression with Nkx6.1 silencing

Fold change siNkx6.1	Fold change siPdx1	Common	Genbank	Description
-4.42	**-7.71**	Gast	NM_012849	Gastrin
-4.16	**-1.81**	Bnip3	NM_053420	bcl2/adenovirus e1b 19 kda-interacting protein 3, nuclear gene bnip3; 3
-3.93	**-1.08**	Bcap29	NP_031556	B-cell receptor-associated protein 29 (BCR-associated protein Bap29)
-3.60	**-1.80**	Bnip3	AF243515	BCL2/adenovirus E1B 19 kDa-interacting protein 3, nuclear gene for mitochondrial product
-3.42	**0.95**	Idh1	NM_031510	cytosolic nadp-dependent isocitrate dehydrogenase; 1, soluble idh1
-3.31	**-1.82**	Pak3	NM_019210	P21 (CDKN1A)-activated kinase 3
-3.29	**-1.58**	Neurodap1	D32249	Rotein carrying the RING-H2 sequence motif
-3.17	**0.89**	Ptma	NM_021740	alpha-prothymosin myc-regulated gene; prothymosin precursor; prothymosin-alpha; alpha ptma
-3.00	**1.00**	Gucy1b3	NM_012769	Guanylate cyclase 1, soluble, beta 3
-2.90	**-1.36**	CHPT1	NP_064629	choline phosphotransferase 1
-2.88	**0.92**	Ptma	NM_021740	alpha-prothymosin myc-regulated gene; prothymosin precursor; prothymosin-alpha; alpha ptma
-2.58	**-1.11**	Pir	AW918049	ESTs, Highly similar to PIR_MOUSE Pirin [M.musulus]
-2.56	**-1.20**	Soat1	NM_031118	acyl-coenzyme a:cholesterol acyltransferase soat1
-2.49	**-2.45**	Scd2	U67995	stearyl-coa desaturase 2
-2.44	**-2.57**	P4ha1	X78949	prolyl 4-hydroxylase, alpha subunit p4halpha
-2.43	**-1.53**	Hmmr	AF133037	Hyaluronan mediated motility receptor (RHAMM)
-2.42	**-1.04**	Nkx6a	NM_031737	NK homeobox (Drosophila), family 6, A
-2.35	**-1.05**	Rpl4	NM_022510	Ribosomal protein L4
-2.33	**-1.04**	Pir	NP_081429	pirin
-2.33	**0.79**	Zcchc12	AK012833	Zinc finger CCHC domain containing protein 12 (Smad-interacting zinc finger protein)
-2.30	**-1.50**	Rab12	M83676	RAB12, member RAS oncogene family
-2.30	**-2.21**	Scd2	NM_031841	Stearoyl-Coenzyme A desaturase 2
-2.27	**-1.34**	Pak3	NM_019210	p21 cdkn1a -activated kinase 3 pak3; beta-pak
-2.27	**0.97**	PFDN4	NM_002623	ESTs, Highly similar to prefoldin 4 [Homo sapiens]
-2.16	**-1.42**	Cstn2	NM34377	calsyntenin 2
-2.14	**0.94**	Rpl4	NM_022510	ribosomal protein l4 rpl4
-2.13	**-1.35**	Cdc10	NM_022616	CDC10 (cell division cycle 10, S.cerevisiae, homolog)
-2.13	**-1.85**	P4ha1	X78949	Prolyl 4-hydroxylase alpha subunit
-2.12	**-1.34**	Atp2a2	J04022	non-muscle atpase
-2.08	**-1.88**	Cacna2d1	AF400662	Rattus norvegicus L-type calcium channel alpha2
-2.07	**-1.11**	Pdcl	NM_022247	Phosducin-like protein
-2.06	**-1.20**	kiaa1458	BAA95982.1	kiaa1458 protein (fragment) - homo sapiens
-2.05	**0.84**	Ccnb1	X64589	Cyclin B1
-2.04	**-1.40**	Senp2	NM_023989	axin-associating molecule loc78973
-2.03	**0.86**	Pawr	U05989	PRKC, apoptosis, WT1, regulator
-2.03	**0.89**	PFDN4	AL133335	prefoldin 4 (c-1) - homo sapiens
-2.02	**-1.35**	Cd47	NM_019195	integrin-associated protein form 4 iap; cd47
-2.01	**-1.30**	Itgb1	NM_017022	integrin beta-1 subunit integrin beta-1; integrin, beta 1

siNkx6.1	siPdx1	Common	Genbank	Description
2.01	**-1.28**	Nbl1	NM_031609	neuroblastoma, suppression of tumorigenicity 1 nbl1; tumor-suppressive gene; no3
2.01	**1.11**	Serf1	BF522362	ESTs, Highly similar to small EDRK-rich factor 1
2.02	**1.29**	Hpgd	NM_024390	NAD-dependent 15-hydroxyprostaglandin dehydrogenase
2.02	**1.42**	Fabp5	U13253	Fatty acid binding protein 5, epidermal
2.03	**1.29**	Nme3	NM_053507	expressed in non-metastatic cells 3, protein nucleoside diphosp nme3; diphosphate kinase dr-nm23
2.05	**1.95**	Slfn4	Y17327	CDK107
2.05	**1.22**	Txnip	NP_076208	similar to thioredoxin interacting factor - mus musculus
2.05	**1.38**	Slc25a10	AJ223355	Solute carrier family 25 (mitochondrial carrier; dicarboxylate transporter), member 10
2.06	**2.16**	Vsp45a	U81160	Vesicular transport protein rvps45
2.07	**-2.43**	Ggtp	M33821	Gamma-glutamyl transpeptidase
2.08	**-1.37**	Sh3kbp1	AF255888	SH3-domain kinase binding protein 1
2.09	**2.29**	Vsp45a	U81160	rvps45
2.09	**1.94**	Zfp106	NP_035873	SH3-domain binding protein 3 - zinc finger protein 106 - mus musculus
2.09	**1.53**	Bk	U30831	b/k protein
2.10	**1.32**	Pde9a	NM38543	phosphodiesterase 9A
2.11	**-1.05**	C1galt1	NM_022950	core1 udp-galactose:n-acetylgalactosamine-alpha-r beta 1,3-galactosyltransferase c1galt1 c1galt1
2.13	**1.88**	Asah	NM_053407	n-acylsphingosine amidohydrolase acid ceramidase asah
2.13	**1.45**	Mrpl36	BC009166	mitochondrial ribosomal protein l36 - mus musculus
2.14	**-1.17**	S100a4	NM_012618	S100 calcium-binding protein A4
2.15	**-1.04**	Acac	X53003	Rat mRNA for acetyl-coenzyme A carboxylase (EC 6.4.1.2.) 3' untranslated region
2.15	**-1.04**	Acac	NM_022193	Acetyl-coenzyme A carboxylase
2.15	**1.58**	Bdnf	NM_012513	brain-derived neurotrophic factor bdnf gene; brain derived neurothrophic
2.16	**1.96**	Lin7c	NM_021851	lin-7-c lin7c
2.16	**2.04**	Asah	AF214647	N-acylsphingosine amidohydrolase (acid ceramidase)
2.16	**1.97**	Atp5f1	M35052	ATP synthase, H+ transporting, mitochondrial F0 complex, subunit b, isoform 1
2.20	**2.23**	Gsto1	AB008807	glutathione-dependent dehydroascorbate reductase
2.21	**1.36**	Ift57	AF274590	huntingtin-interacting protein-1 protein interactor - mus musculus
2.22	**-1.48**	Jundp2	NM_053894	jun dimerization protein 2 jundp2; jdp-2
2.26	**1.56**	75564	AK006440	Protein C6orf206 homolog - mus musculus
2.31	**2.14**	Hsbp1	AF522937	Rattus norvegicus heat shock factor binding protein 1 mRNA, complete cds
2.32	**2.17**	Hsbp1	BC002153	similar to heat shock factor binding protein 1 - mus musculus
2.33	**-1.09**	Slc12a4	NM_019229	Solute carrier family 12, member 4
2.36	**-1.51**	Sh3kbp1	AF255884	sh3 domain-containing adapter protein isoform seta-1x23 seta; ruk-s ruk; ruk-h; ruk-m1; ruk-m3; ruk-l
2.36	**1.40**	Nelf	NM_057190	jacob protein, alternatively spliced isoform delta1 jac; protein; delta2; nasal embryonic lhrh factor nelf
2.38	**-1.16**	Cyp4f14	NM_019623	Cytochrome P450, subfamily IVF, polypeptide 14 (leukotriene B4 omega hydroxylase)
2.39	**1.88**	Atp6v0e2	NP_598525	similar to ATPase, H+ transporting, V0 subunit - mus musculus

siNkx6.1	siPdx1	Common	Genbank	Description
2.39	**0.99**	Lklf	AF181251	ESTs, Moderately similar to KLF6_RAT Core promoter element-binding protein (Kruppel-like factor 6) (
2.39	**1.55**	Vdup1	U30789	upregulated by 1,25-dihydroxyvitamin D-3
2.40	**0.72**	S100a4	NM_012618	protein 9 ka homologous to calcium-binding protein s100a4; s-100-related; put. p9ka
2.40	**2.15**	Hsbp1	BC002153	similar to heat shock factor binding protein 1 - mus musculus
2.41	**2.10**	AF146738	AF146738	testis specific protein
2.43	**1.84**	Ache; Hache	S50879	acetylcholinesterase t subunit acetylcholinesterase t subunit, ache
2.44	**1.01**	Lcat	NM_017024	Lecithin-cholesterol acyltransferase
2.44	**1.29**	Guca2b	NM_022284	uroguanylin guca2b; preprouroguanylin; prepro-uroguanylin
2.44	**0.50**	S100a3	AF140231	S100 calcium binding protein A3
2.47	**2.78**	BRD1	AW525127	ESTs, Highly similar to bromodomain containing protein 1; BR140-like gene
2.49	**1.58**	Cspg5	NM_019284	Chondroitin sulfate proteoglycan 5
2.49	**2.24**	AF146738	AF146738	Testis specific protein
2.49	**1.81**	Ocm; OM	X15836	Oncomodulin
2.51	**0.47**	LOC94168	U19485	Spp-24 precursor
2.52	**1.19**	Pcp4	NM_013002	neuron specific protein pep-19 purkinje cell protein 4 pcp4; neuron-specific
2.54	**2.18**	Ldhb	NM_012595	lactate dehydrogenease b; ldhb
2.55	**0.78**	Camk4	NM_012727	ca2+/calmodulin-dependent protein kinase iv beta polypeptide
2.56	**1.33**	Matk	NM_021859	Megakaryocyte-associated tyrosine kinase
2.68	**0.72**	Fgb	U05675	Fibrinogen, B beta polypeptide
2.70	**1.15**	Slc2a4	NM_012751	Glucose transporter 4, insuline-responsive
2.73	**0.91**	Klf2; Lklf	AF181251	lung kruppel-like factor lklf
2.73	**1.94**	Inexa	NM_019128	alpha-internexin alpha-1.6; internexin, alpha inexa
2.78	**1.71**	Nelf	AJ293697	Nasal embryonic LHRH factor
2.79	**0.52**	S100a3	NM_053681	s100 calcium binding protein a3 s100a3
2.83	**1.34**	Matk	NM_021859	non-receptor protein kinase protein batk
2.87	**3.05**	Gipc2	NP_058563	similar to semaf cytoplasmic domain associated protein 2 - mus musculus
2.87	**3.39**	Rgs19	AF068136	ESTs, Moderately similar to regulator of G-protein signaling 19
2.91	**0.60**	Fgb	M27220	fibrinogen b-beta-chain; b beta chain
2.93	**0.71**	Bv8	NM38852	homolog of mouse Bv8 (Bombina variegata 8 kDa); prokineticin 2 precursor
2.93	**2.05**	Prkaa2	NM_023991	AMP-activated protein kinase
3.03	**1.97**	Tac1	NM_012666	Tachykinin (substance P, neurokinin A, neuropep K)
3.07	**2.31**	Ldhb	NM_012595	lactate dehydrogenease b ldhb; dehydrogenase-b
3.27	**0.66**	Crybb3	NM_031690	betab3-crystallin aa 1 - 211; beta-b3-2-crystallin; crystallin, beta b3 crybb3
3.35	**0.72**	Crybb3	NM_031690	Crystallin, beta B3
3.79	**3.63**	Ube2m	NP_663553	similar to ubiquitin-conjugating enzyme e2m (ubc12 homolog, yeast)
3.80	**0.56**	Dmgdh	X55995	Dimethylglycine dehydrogenase precursor
3.90	**0.64**	Ptpre	Y07834	protein tyrosine phosphatase epsilon-like 2; epsilon c

siNkx6.1	siPdx1	Common	Genbank	Description
4.08	**4.11**	Ube2m	NP_663553	similar to ubiquitin-conjugating enzyme e2m (ubc12 homolog, yeast)
4.58	**-2.16**	Ptpre	D78610	protein tyrosine phosphatase epsilon c
5.17	**1.42**	Slc22a1	NM_012697	organic cation transporter slc22a1; oct1a

Table F-1. Changes in gene expression with Pdx1 silencing

Fold change siPdx1	Fold change siNkx6.1	Common	Genbank	Description
-7.71	**-4.42**	Gast	NM_012849	Gastrin
-4.63	**-1.50**	Pdx1	NM_022852	Pancreatic and duodenal homeobox gene 1
-4.40	**1.07**	Pdx1	NM_022852	somatostatin transactivating factor-1 stf-1; pancreatic and duodenal homeobox gene 1 pdx1; idx-1
-4.22	**2.80**	SPP2	U19485	spp-24 precursor
-3.85	**1.07**	Nnat	NM_053601	neuronatin alpha; nnat
-3.81	**-1.44**	Hspa5	M14050	preimmunoglobulin heavy chain binding protein
-3.75	**-1.44**	Ripk3	AF036537	Homocysteine respondent protein HCYP2
-3.69	**-1.75**	Igf1r	NM_052807	insulin-like growth factor i receptor precursor; 1 igf1r
-3.65	**-1.06**	SDF2L1	NM_022044	similar to pir nf00515193 stromal cell-derived factor 2-like protein 1 precursor (sdf2 like protein 1)
-3.44	**-1.52**	Nts	M21187	preproneurotensin/neuromedin n
-3.37	**-1.45**	Dnajb9	NM_012699	dnaj hsp40 homolog, subfamily b, member 9 mdg1
-3.35	**-1.19**	Cdh23	NM_053644	cadherin related 23; cdh23
-3.24	**-1.49**	Nnat	NM_053601	neuronatin; nnat
-3.18	**-1.79**	Nexn	AF056034	Nexilin
-3.14	**-1.58**	Ins2	NM_019130	preproinsulin ii; i; insulin 1 ins1; 2 ins2
-3.07	**-1.69**	Dnajc3	NM_022232	Protein kinase inhibitor p58
-3.05	**-1.44**	Dnajb9	NM_012699	DnaJ homolog, subfamily b, member 9
-3.03	**-1.27**	Cck	NM_012829	cck precursor aa -28 to 87; preprocholecystokinin; cholecystokinin
-3.03	**-1.57**	Lpl	NM_012598	lipoprotein lipase lpl; prelipoprotein
-3.00	**-1.10**	Idax	AF272158	Inhibitor of the Dvl and Axin complex
-2.94	**-1.38**	Fgf15	NM30753	fibroblast growth factor 15
-2.92	**-1.84**	Dlk1	D84336	Delta-like homolog (Drosophila)
-2.92	**-1.67**	Grp94	S69315	endoplasmin grp94
-2.90	**-1.33**	Npy	NM_012614	neuropeptide y precursor; npy
-2.89	**1.69**	Ptger3	NM_012704	rat kidney prostaglandin ep3 receptor alternative splicing results in two different receptors ep3a and ep3b
-2.89	**-1.61**	Ins2	NM_019130	preproinsulin ii; i; insulin 1 ins1; 2 ins2
-2.83	**1.15**	Rdc1	AF118816	Chemokine orphan receptor 1
-2.81	**-2.46**	Mmp13	M60616	collagenase umrcase
-2.76	**-1.66**	Fn1	NM_019143	c-terminal nucleotide-bonding segment; fibronectin 2; 3; 1; fn1; precursor polypeptide aa -32 to 2445
-2.75	**-1.50**	Chga	NM_021655	chromogranin a precursor loc59263; chga; prechromogranin
-2.73	**-1.64**	Ins1	NM_019129	Insulin
-2.73	**-1.92**	Edn1	NM_012548	Endothelin 1
-2.70	**-1.55**	Il18	NM_019165	interferon-gamma inducing factor precursor igif; interleukin-18 il-18; interleukin 18 il18
-2.69	**-1.96**	Octn1	NM_022270	Organic cation transporter OCTN1
-2.68	**-1.64**	Pcsk2	NM_012746	proprotein convertase subtilisin/kexin type 2 pcsk2; hormone prohormone; rpc2
-2.66	**1.54**	F3	NM_013057	Coagulation factor III (thromboplastin, tissue factor)

siPdx1	siNkx6.1	Common	Genbank	Description
-2.59	**-1.19**	Wif1	AY030278	Wnt inhibitory factor 1
-2.59	**-1.60**	Spn	Y00090	Sialophorin (gpL115, leukosianin, CD43)
-2.58	**-1.43**	Ambp	NM_012901	polyprotein 1-microglobulin/bikunin; alpha-1 microglobulin/bikunin ambp
-2.57	**-2.44**	P4ha1	X78949	prolyl 4-hydroxylase, alpha subunit p4halpha
-2.57	**-1.52**	Iapp	NM_012586	islet amyloid polypeptide iapp; amylin precursor
-2.56	**-1.75**	Nsg1	V01543	reading frame 1
-2.55	**-1.48**	Klk6	NM_019175	myelencephalon specific protease; protease, serine, 9 prss9
-2.49	**-1.33**	Erp70	M86870	Protein disulfide isomerase related protein (calcium-binding protein, intestinal-related)
-2.47	**-1.51**	Ins2	NM_019130	Insulin 2
-2.47	**-1.99**	Tgfb2	NM_031131	transforming growth factor beta 2 precursor; tgf-beta long form tgf-beta2; short; protein; tgf tgfb2; tgf-b2
-2.47	**-1.75**	Scg3	NM_053856	secretogranin iii scg3
-2.47	**1.37**	Bace2	BF523687	ESTs, Moderately similar to beta-site APP-cleaving enzyme 2 [Mus musculus] [M.musulus]
-2.45	**-2.49**	Scd2	U67995	stearyl-coa desaturase 2
-2.45	**-1.65**	Slc6a13	M95762	gaba transporter
-2.43	**2.07**	Ggtp	M33821	Gamma-glutamyl transpeptidase
-2.42	**-1.13**	Ptprz1	NM_013080	protein tyrosine phosphatase, receptor-type, zeta polypeptide ptprz1
-2.42	**-1.92**	Sgne1	NM_013175	secretory granule neuroendocrine, protein 1 7b2 protein sgne1; neuroendocrine
-2.42	**-1.48**	Olr1078	M64376	Rat olfactory protein mRNA, complete cds
-2.41	**-1.28**	Pou2f1	U17013	POU domain, class 2, transcription factor 1
-2.40	**-1.19**	P5	X79328	R.norvegicus (Wistar) CaBP1 mRNA
-2.40	**-1.43**	Shank2	AJ249562	Proline rich synapse associated protein 1
-2.40	**-1.38**	Fabp4	NM_053365	adipocyte lipid-binding protein loc84378; albp
-2.39	**-1.53**	Chga	NM_021655	Chromogranin A, parathyroid secretory protein 1
-2.37	**-1.10**	Nnat	U08290	Neuronatin
-2.37	**-1.47**	Igf1r	L29232	Insulin-like growth factor 1 receptor
-2.37	**-1.41**	Armet	AK014338	similar to gbp|ak014338|ak014338_1 riken full-length enriched library, clone:3230402m22 - mus musculus
-2.36	**-1.18**	Gsta1	NM_031509	Glutathione-S-transferase, alpha type (Ya)
-2.36	**-1.53**	Slc8a1	X68191	sodium/calcium exchanger 1 splice variant naca10 ncx1; na-ca isoform naca7; naca1; naca5;
-2.35	**-1.02**	Cck	NM_012829	Cholecystokinin
-2.34	**-1.73**	Nsg1	NM_024128	brain specific mrna b; clone p1a75; bsmrb
-2.33	**-1.58**	Resp18	NM_019278	regulated endocrine-specific protein 18 resp18; neuroendocrine-specific
-2.33	**-1.10**	Pzp	M77183	alpha-1-macroglobulin alpha-1-macrogloculin; alpha1 m
-2.32	**-1.59**	Muc13	U89744	Putative cell surface antigen
-2.31	**-1.60**	Oat	NM_022521	ornithine aminotransferase precursor ec 2.6.1.13; roat; oat
-2.30	**-1.57**	Tff3	NM_013042	intestinal trefoil factor intestinal trefoil factor, itf; precursor; protein; tff3; polypeptide p1.b; trefoil-factor ritf
-2.30	**-1.35**	Dgkb	NM_019304	diacylglycerol kinase 90kda dagk;

siPdx1	siNkx6.1	Common	Genbank	Description
-2.30	**-1.35**	Prph1	NM_012633	peripherin prph
-2.30	**1.59**	Trpv1	NM_031982	Transient receptor potential cation channel, subfamily V, member 1
-2.30	**-1.53**	Chgb	NM_012526	chromogranin b, parathyroid secretory protein chgb; b
-2.28	**-1.44**	Lpl	NM_012598	Lipoprotein lipase
-2.27	**-1.25**	Erp70	NM_053849	protein disulfide isomerase related protein calcium-binding protein, intestinal-related erp70
-2.27	**-1.34**	Calr	NM_022399	d-beta-hydroxybutyrate dehydogenase; calreticulin; calr; precursor aa -17 to 399
-2.27	**-1.43**	Gucy1b2	NM_012770	guanylate cyclase, soluble, beta 2; gtp pyrophosphate -lyase; gucy1b2
-2.26	**1.10**	Cmkor1	NM_053352	chemokine orphan receptor 1 rdc1; rdc-1 protein
-2.26	**-1.25**	Txndc7	X79328	cabp1
-2.26	**-1.37**	Pf4	M15254	platelet factor 4 precursor
-2.24	**-1.95**	Tmem27	NM_020976	kidney-specific membrane protein nx-17
-2.23	**-1.35**	Fn1	NM_019143	precursor polypeptide aa -32 to 2445; fibronectin 2; 1; fn1
-2.22	**-1.39**	Gpx3	NM_022525	plasma glutathione peroxidase precursor gpxp
-2.21	**-2.30**	Scd2	NM_031841	Stearoyl-Coenzyme A desaturase 2
-2.20	**-1.18**	Slc8a1	X68191	na+/ca2+-exchanging protein precursor; na+/ca2+ exchanger; na-ca isoform naca7; naca1; naca5
-2.20	**-1.49**	Cd164	NM_031812	endolyn cd164
-2.18	**-1.28**	Iapp	NM_012586	Islet amyloid polypeptide
-2.18	**-1.76**	Ckmt1	X59737	ubiquitous mitochondrial creatine kinase
-2.17	**-1.02**	Enpep	AF146518	Rattus norvegicus aminopeptidase A short variant mRNA, partial cds
-2.16	**4.58**	Ptpre	D78610	protein tyrosine phosphatase epsilon c
-2.16	**-1.08**	MGC109519	L00381	skeletal muscle beta-tropomyosin
-2.15	**-1.38**	Slc24a2	AF021923	Solute carrier family 24 (sodium/potassium/calcium exchanger), member 2
-2.13	**-1.40**	Slc16a7	U62316	monocarboxylate transporter 2 mct2
-2.12	**1.36**	Nrp	AF016296	neuropilin
-2.12	**-1.26**	Gdf8	NM_019151	myostatin mstn
-2.11	**2.51**	LOC94168	U19485	Spp-24 precursor
-2.10	**-2.06**	Grm4	U47331	metabotropic glutamate receptor 4b
-2.10	**-1.10**	Calr	NM_022399	Calreticulin
-2.07	**1.18**	Arntl	AF317669	bmal1g'; bmal1b
-2.07	**-1.35**	P4hb	NM_012998	Protein disulfide isomerase (Prolyl 4-hydroxylase, beta polypeptide)
-2.06	**-1.28**	Bcan	X86406	gpi-anchored brevican isoform
-2.06	**-1.24**	Fn1	NM_019143	precursor polypeptide aa -32 to 2445; fibronectin; 1 fn1; fn-1
-2.06	**-1.06**	Ambp	NM_012901	polyprotein 1-microglobulin/bikunin; alpha-1-microglobulin; alpha-1 microglobulin/bikunin ambp
-2.06	**-1.22**	Runx1	NM_017325	acute myeloid leukemia 1 aml1
-2.05	**-1.52**	Uggt	AF200359	UDP-glucose glycoprotein: glucosyltransferase UGGT
-2.05	**-1.19**	Erp99	NM_011631	similar to gbp|j03297|j03297_1 erp99 endoplasmic reticulum transmembrane protein precursor
-2.05	**-1.48**	Eif2s3y	NM_012011	similar to gbp|aj006584|aj006584_1 eif2 gamma y eif2 gamma - mus musculus

siPdx1	siNkx6.1	Common	Genbank	Description
-2.03	**-1.83**	RB13-6	NM_019370	Alkaline phosphodiesterase
-2.03	**-1.45**	Il18	NM_019165	interferon-gamma inducing factor isoform alpha precursor igif; interleukin-18 il-18; interleukin 18 il18
-2.03	**-1.33**	Clast4	BG665088	ESTs, Highly similar to Clast4 protein [Mus musculus] [M.musulus]
-2.02	**-1.32**	Dnajc3	NM_022232	protein kinase inhibitor p58 loc63880
-2.02	**2.44**	S100a3	AF140231	S100 calcium binding protein A3
-2.02	**-1.31**	Pon3	NM_001004086	similar to gbp|l76193|l76193_1 pon3 paraoxonase-3 - mus musculus
-2.02	**-1.21**	NCAML1	NM_017345	Neural cell adhesion molecule L1
-2.01	**-1.20**	Ppp1r1a	NM_022676	protein phosphatase 1, regulatory inhibitor subunit 1a ppp1r1a; inhibitor-1 i-1
-2.00	**1.58**	G0S2-like	XM_847018	similar to gbp|x95280|x95280_1 g0s2-like protein - mus musculus
-2.00	**-1.26**	Grp58	NM_031580	glucose regulated protein, 58 kda grp58; er-60 protease; er60; pi-plc i aa 1-504
2.00	**1.45**	Tro	NM_016157	similar to pir nf00522853 mage-d3 - mus musculus
2.01	**1.48**	Ppid	NM_026352	similar to gbp|bc011499|bc011499_1 riken cdna 4930564j03 gene - mus musculus
2.01	**1.89**	Nell2	NM_031070	Nel-like 2 homolog (chicken)
2.02	**1.35**	Lgals1	NM_019904	Lectin, galactose binding, soluble 1
2.02	**1.26**	Uch-l5	XM_892228	similar to pir nf00534456 ubiquitin c-terminal hydrolase uch-l5 - mus musculus
2.03	**1.85**	Sms	NM_009214	similar to pir nf00138999 spermine synthase (ec 2.5.1.22) - homo sapiens
2.03	**1.45**	Pla2g1b	NM_031585	phospholipase a2, group ib, pancreas pla2g1b; pancreatic a-2
2.04	**2.16**	Asah	AF214647	N-acylsphingosine amidohydrolase (acid ceramidase)
2.04	**1.19**	LOC286991	AF057564	Rattus norvegicus putative retrovirus-related gag protein mRNA, complete cds
2.05	**1.52**	Ankrd11	XM_134514	similar to gbp|ak019393|ak019393_1 riken full-length enriched library, clone:3010027a04
2.05	**2.93**	Prkaa2	NM_023991	AMP-activated protein kinase
2.09	**1.25**	RragB	NM_053972	ragb ras-related, alternatively spliced gtpase b; gtp-binding protein
2.09	**1.53**	Cadps	NM_013219	ca2+-dependent activator protein caps
2.09	**1.40**	LOC59303	NM_021671	db83 loc59303
2.10	**2.41**	AF146738	AF146738	testis specific protein
2.10	**1.03**	Nap1l3	AB067678	nucleosome assembly protein 1-like 3; nap1l3
2.11	**1.66**	Ser	BU671646	ESTs, Highly similar to Ser
2.12	**-1.07**	Snca	NM_019169	Synuclein, alpha
2.14	**2.31**	Hsbp1	AF522937	Rattus norvegicus heat shock factor binding protein 1 mRNA, complete cds
2.15	**1.17**	Tmeff1	NM_023020	Transmembrane protein with EGF-like and two follistatin-like domains 1
2.15	**2.40**	Hsbp1	NM_173119	similar to gbp|bc002153|bc002153_1 similar to heat shock factor binding protein 1
2.16	**-1.05**	Ggh	NM_012960	Gamma-glutamyl hydrolase
2.16	**2.06**	Vsp45a	U81160	Vesicular transport protein rvps45
2.16	**1.35**	Impa2	NM_053261	similar to gbp|bc011093|bc011093_1 unknown (protein for mgc:18988) - mus musculus

siPdx1	siNkx6.1	Common	Genbank	Description
2.17	**2.32**	Hsbp1	NM_173119	similar to gbp|bc002153|bc002153_1 similar to heat shock factor binding protein 1 - mus musculus
2.18	**2.54**	Ldhb	NM_012595	lactate dehydrogenease b; ldhb
2.19	**1.96**	PSMD13	NM_002817	similar to pir nf00503408 (26s proteasome regulatory subunit s11, p40.5)
2.20	**1.23**	Mox2	NM_031518	cell surface protein thymocyte, antigen identified by monoclonal antibody mrc-ox2 mox2; mrc ox-2
2.20	**1.27**	TSPAN1	NM_005727	similar to gbp|bc003448|bc003448_1 similar to tetraspan 1 - mus musculus
2.21	**1.24**	Pet1	U91679	ETS domain transcription factor Pet-1
2.22	**2.20**	Nrxn1	M96374	neurexin i-alpha
2.22	**1.29**	Pthlh	NM_012636	Parathyroid-like peptide
2.23	**2.20**	Gsto1	AB008807	glutathione-dependent dehydroascorbate reductase
2.24	**1.79**	Cyp4a12	NM_031605	Cytochrome P450, 4a10
2.24	**2.49**	AF146738	AF146738	Testis specific protein
2.26	**1.73**	Kif1a	NM_008440	similar to gbp|x90840|x90840_1 atsv axonal transporter of synaptic vesicles - homo sapiens
2.26	**-1.34**	Mlb1	NM_012599	mannose binding protein a, serum mbpa; mannose-binding; a mbp
2.26	**1.47**	F13b	NM_031164	similar to gbp|d10071|d10071_1 b subunit of factor xiii - mus musculus
2.27	**1.80**	Atp2b2	J03754	ATPase isoform 2, Na+K+ transporting, beta polypeptide 2
2.28	**-1.73**	Camk2d	NM_012519	ca2+/calmodulin-dependent protein kinase neuronal isoform delta a
2.28	**2.39**	Camk2b	NM_021739	Ca++/calmodulin-dependent protein kinase II, beta subunit
2.29	**2.09**	Vsp45a	U81160	rvps45
2.31	**1.88**	Ddx25	NM_031630	dead aspartate-glutamate-alanine-aspartate box polypeptide 25 ddx25; gonadotropin-regulated rna helicase
2.31	**3.07**	Ldhb	NM_012595	lactate dehydrogenease b ldhb; dehydrogenase-b ldh-b
2.40	**1.62**	Rab3c	U54807	RAB3C, member RAS oncogene family
2.49	**1.65**	Maf1	NM_026859	similar to gbp|ak004406|ak004406_1 riken full-length enriched library, clone:1110068e11 - mus musculus
2.52	**1.51**	Lphn2	AF081151	calcium-independent alpha-latrotoxin receptor homolog 2 cirl-2; cl2aa; cl2ac; cl2ba; cl2bc
2.54	**3.75**	Hpgd	NM_024390	nad-dependent 15-hydroxyprostaglandin dehydrogenase hpgd
2.54	**1.62**	Tmem14a	NM_029398	similar to gbp|ak017734|ak017734_1 riken full-length enriched library, clone:5730496e24 - mus musculus
2.60	**1.47**	RragB	NM_053972	ragb ras-related, alternatively spliced gtpase b; gtp-binding protein
2.68	**1.89**	Cfh	AJ320522	complement inhibitory factor h; fh
2.78	**2.47**	Brd1	AW525127	ESTs, Highly similar to bromodomain containing protein 1; BR140-like gene [Homo sapiens] [H.sapiens]
2.89	**2.43**	Recc1	AF059678	vip-receptor-gene repressor protein
3.02	**-1.33**	Slc6a2	NM_031343	Solute carrier family 6 (neurotransmitter transporter,noradrenalin), member 2
3.04	**-1.32**	Fgf13	AF271786	Fibroblast growth factor 13

siPdx1	siNkx6.1	Common	Genbank	Description
3.05	**2.87**	Gipc2	NM_001037210	similar to pir nf00536101 semaf cytoplasmic domain associated protein 2 (2200002n01rik protein)
3.14	**1.40**	Slc9a2	NM_012653	Solute carrier family 9 (sodium/hydrogen exchanger 2), antiporter 2, Na+/H+ (Na+/H+ exchanger 2)
3.17	**3.24**	Ieda	AY030276	Orphan seven transmembrane receptor
3.39	**2.87**	Rgs19	AW918850	ESTs, Moderately similar to regulator of G-protein signaling 19 [Rattus norvegicus]
3.53	**2.14**	Galr1	U30290	Galanin receptor 1
3.63	**3.79**	Ube2m	XM_341790	similar to pir nf00902025 ubiquitin-conjugating enzyme e2m (ubc12 homolog, yeast)
3.69	**-1.09**	Ntt73	L22022	Rat orphan transporter v7-3 (NTT73) mRNA, complete cds
3.82	**-1.15**	Serpina1	NM_022519	precursor of alpha-1-protease inhibitor; alpha-1-antitrypsin; spi1
3.87	**1.08**	Serpina1	NM_022519	Serine (or cysteine) proteinase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 1
4.11	**4.08**	Ube2m	XM_341790	similar to pir nf00902025 ubiquitin-conjugating enzyme e2m (ubc12 homolog, yeast)
4.50	**1.05**	Mafb	U56241	V-maf musculoaponeurotic fibrosarcoma oncogene family, protein B (avian)

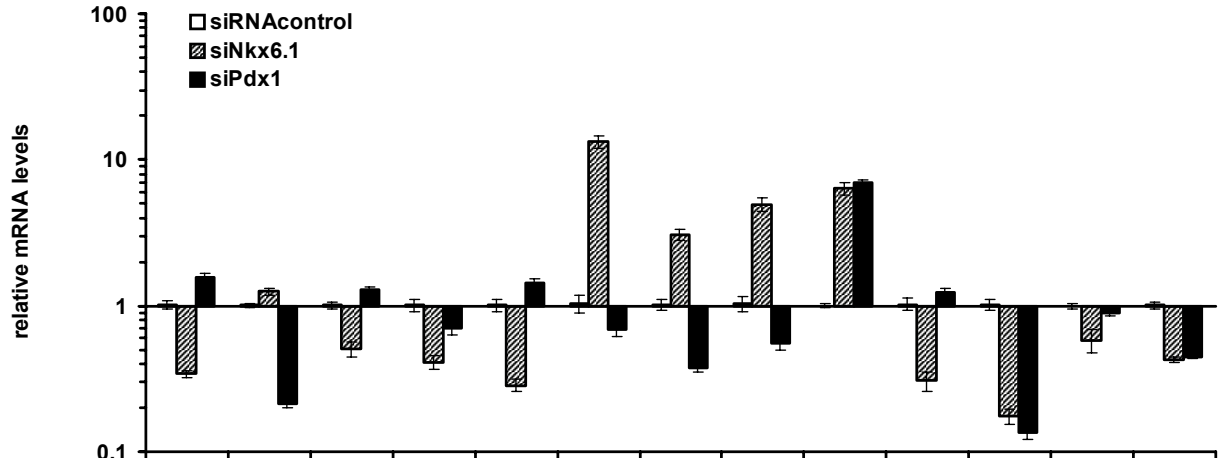
Table F-3. Differential changes gene expression with Nkx6.1 versus Pdx1 silencing

Fold change Nkx6.1 vs Pdx1	Nkx6.1 vs Cntrl	Common	Genbank	Description
-5.25	**1.18**	Plcg1	NM_013187	Phospholipase C, gamma 1
-4.38	**-1.15**	Serpina1	NM_022519	precursor of alpha-1-protease inhibitor; alpha-1-antitrypsin; spi1
-4.30	**1.05**	Mafb	U56241	V-maf musculoaponeurotic fibrosarcoma oncogene family, protein B (avian)
-4.01	**-1.09**	Ntt73	L22022	Rat orphan transporter v7-3 (NTT73) mRNA, complete cds
-4.01	**-1.33**	Slc6a2	NM_031343	Solute carrier family 6 (neurotransmitter transporter,noradrenalin), member 2
-4.01	**-1.32**	Fgf13	AF271786	Fibroblast growth factor 13
-3.96	**-1.73**	Camk2d	NM_012519	ca2+/calmodulin-dependent protein kinase neuronal isoform delta a, neuronal delta a-cam kinase;
-3.64	**-3.93**	Bcap29	NP_031556	B-cell receptor-associated protein 29 (BCR-associated protein Bap29)
-3.59	**-3.42**	Idh1	NM_031510	cytosolic nadp-dependent isocitrate dehydrogenase; 1, soluble idh1
-3.57	**-3.17**	Ptma	NM_021740	alpha-prothymosin myc-regulated gene; prothymosin precursor; prothymosin-alpha; alpha ptma
-3.57	**1.08**	Serpina1	NM_022519	Serine (or cysteine) proteinase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 1
-3.15	**-2.88**	Ptma	NM_021740	alpha-prothymosin myc-regulated gene; prothymosin precursor; prothymosin-alpha;
-3.04	**-1.34**	Mlb1	NM_012599	mannose binding protein a, serum mbpa; mannose-binding; a mbp
-2.99	**-3.00**	Gucy1b3	NM_012769	Guanylate cyclase 1, soluble, beta 3
-2.93	**-2.33**	Zcchc12	NM_028325	similar to gbp|ak012833|ak012833_1 zinc finger, CCHC domain containing 12
-2.55	**-1.69**	Arc	NM_019361	Activity regulated cytoskeletal-associated protein
-2.53	**-2.02**	Ret	AJ298999	Ret proto-oncogene (multiple endocrine neoplasia MEN2A, MEN2B and medullary thyroid carcinoma 1, Hir
-2.53	**-1.41**	NHP2L1	NM_001003796	similar to gbp|af155235|af155235_1 15.5 kd rna binding protein - homo sapiens
-2.46	**-1.31**	Ptprr	D38292	Protein tyrosine phosphatase, receptor type, R
-2.43	**-2.05**	Ccnb1	X64589	Cyclin B1
-2.37	**-2.03**	Pawr	U05989	PRKC, apoptosis, WT1, regulator
-2.34	**-1.35**	Msg1	AF104399	Melanocyte-specific gene 1 protein
-2.34	**-2.27**	PFDN4	AL133335	prefoldin 4 (c-1) - homo sapiens
-2.34	**-1.71**	Alcam	NM_031753	Activated leukocyte cell adhesion molecule
-2.32	**-1.53**	Gpcr3	L32829	g protein-coupled receptor r4
-2.32	**-2.58**	Pir	AW918049	ESTs, Highly similar to PIR_MOUSE Pirin
-2.30	**-1.88**	Srp14	NM_009273	similar to gbp|m29264|m29264_1 signal recognition particle subunit (srp14) - mus musculus
-2.29	**-4.16**	Bnip3	NM_053420	bcl2/adenovirus e1b 19 kda-interacting protein 3, nuclear gene bnip3; 3

Nkx6.1 vs Pdx1	Nkx6.1 vs Cntrl	Common	Genbank	Description
-2.28	**-2.03**	PFDN4	AL133335	prefoldin 4 (c-1) - homo sapiens
-2.27	**-1.94**	Ube2d2	U13176	Rattus norvegicus clone ubc2e ubiquitin conjugating enzyme (E217kB) mRNA, complete cds
-2.27	**-1.86**	mrps16	NM_025440	similar to gbp|ab049949|ab049949_1 mrps16 mitochondrial ribosomal protein s16 - mus musculus
-2.27	**-2.14**	Rpl4	NM_022510	ribosomal protein l4 rpl4
-2.26	**-1.05**	Ggh	NM_012960	Gamma-glutamyl hydrolase
-2.25	**-1.97**	Idh1	NM_031510	Isocitrate dehydrogenase 1, soluble
-2.25	**-2.33**	Pir	NM_027153	similar to gbp|ak009757|ak009757_1 pirin iron-binding nuclear protein
-2.24	**-2.35**	Rpl4	NM_022510	Ribosomal protein L4
-2.20	**-1.12**	Ggh	NM_012960	gamma-glutamyl hydrolase precursor; ggh
-2.19	**-1.17**	Mlb1	NM_012599	Mannose binding protein A, serum
-2.16	**-1.60**	Pcca	M22631	alpha-propionyl-coa carboxylase ec 6.4.1.3
-2.14	**-2.56**	Soat1	NM_031118	acyl-coenzyme a:cholesterol acyltransferase
-2.13	**-1.83**	Cbr4	NM_145595	similar to gbp|bc009118|bc009118_1 carbonic reductase 4
-2.13	**-1.99**	Nup98	NM_031074	Nucleoporin 98
-2.08	**-3.29**	Neurodap1	D32249	Rotein carrying the RING-H2 sequence motif
-2.05	**-1.65**	Actr3	AF307852	Actin-related protein 3 homolog (yeast)
-2.02	**-1.35**	Qdpr	NM_022390	quinoid dihydropteridine reductase qdpr; ec 1.6.99.7
-2.01	**-1.36**	Gabra4	NM_080587	gamma-aminobutyric acid gaba-a receptor, subunit alpha 4 gabra4; receptor alpha-4; gabaa
-2.01	**-3.60**	Bnip3	AF243515	BCL2/adenovirus E1B 19 kDa-interacting protein 3, nuclear gene for mitochondrial product
2.01	**-1.19**	P5	X79328	R.norvegicus (Wistar) CaBP1 mRNA
2.02	**1.66**	Ckmt2	X59736	sarcomeric mitochondrial creatine kinase
2.02	**1.41**	Cat	NM_012520	Catalase
2.02	**1.59**	Ddit3	NM_024134	dna-damage inducible transcript 3 ddit3; gadd153
2.03	**1.66**	Zcchc9	NM_145453	similar to gbp|bc010687|bc010687_1 inc finger, CCHC domain containing 9
2.04	**1.56**	Slco3a1	AF239219	prostaglandin transporter subtype 2 pgt2
2.04	**1.17**	Slc2a2	NM_012879	solute carrier family 2 a2 gkucose transporter, type 2 slc2a2; glucose transporter
2.04	**1.48**	Ugt1a6	S70355	udp-glucuronosyltransferase, udpgt; udp glucuronosyltransferase ugt1;
2.05	**1.14**	Ramp2	NM_031646	receptor activity modifying protein 2 ramp2; receptor-activity
2.06	**1.42**	Cacna1a	NM_012918	class a calcium channel variant ria-ii bcca1; ria-i; alpha-1 subunit rba-i; alpha 1a cacna1a
2.06	**1.53**	Ggt1	NM_053840	gamma-glutamyl transpeptidase ec 2.3.2.2; m33821;
2.07	**1.42**	Cat	NM_012520	catalase ec 1.11.1.6; cat
2.08	**1.82**	Kcnab3	NM_031652	potassium voltage gated channel, shaker related subfamily, beta member 3 kcnab3; rckbeta3
2.11	**2.52**	Pcp4	NM_013002	neuron specific protein pep-19 purkinje cell protein 4 pcp4; neuron-specific
2.12	**2.83**	Matk	NM_021859	non-receptor protein kinase protein batk
2.12	**-1.75**	Igf1r	NM_052807	insulin-like growth factor i receptor precursor; 1 igf1r

Nkx6.1 vs Pdx1	Nkx6.1 vs Cntrl	Common	Genbank	Description
2.12	**-1.44**	Dnajb9	NM_012699	DnaJ homolog, subfamily b, member 9
2.12	**-1.38**	Fgf15	NM30753	fibroblast growth factor 15
2.12	**-1.10**	Pzp	M77183	alpha-1-macroglobulin alpha-1-macrogloculin; alpha1 m
2.13	**-1.02**	Enpep	AF146518	Rattus norvegicus aminopeptidase A short variant mRNA, partial cds
2.15	**-1.13**	Ptprz1	NM_013080	protein tyrosine phosphatase, receptor-type, zeta polypeptide ptprz1; receptor-type phosphatase zeta/beta
2.18	**-1.19**	Wif1	AY030278	Wnt inhibitory factor 1
2.22	**1.34**	C1galt1	NM_022950	Core1 UDP-galactose:N-acetylgalactosamine-alpha-R beta 1,3-galactosyltransferase (C1galt1)
2.22	**2.11**	C1galt1	NM_022950	core1 udp-galactose:n-acetylgalactosamine-alpha-r beta 1,3-galactosyltransferase c1galt1
2.24	**2.15**	Acac	X53003	Rat mRNA for acetyl-coenzyme A carboxylase (EC 6.4.1.2.) 3' untranslated region
2.24	**2.15**	Acac	NM_022193	Acetyl-coenzyme A carboxylase
2.24	**1.17**	Herpud1	AB033771	Homocysteine-inducible, endoplasmic reticulum stress-inducible, ubiquitin-like domain member 1
2.25	**1.15**	Pace4	NM_012999	Subtilisin - like endoprotease
2.26	**-1.52**	Nts	M21187	preproneurotensin/neuromedin n
2.27	**1.93**	Lgals9	NM_012977	Lectin, galactose binding, soluble 9 (Galectin-9)
2.32	**1.88**	Cyp4f2	NM_019623	cytochrome p450 4f1 cyp4f1
2.32	**-1.45**	Dnajb9	NM_012699	dnaj hsp40 homolog, subfamily b, member 9 mdg1
2.35	**2.70**	Slc2a4	NM_012751	Glucose transporter 4, insuline-responsive
2.36	**1.48**	Wipi1	NM_145940	similar to pir nf00960386 WD-repeat domain phosphoinositide-interacting protein 1
2.39	**0.79**	Cck	NM_012829	cck precursor aa -28 to 87; preprocholecystokinin; cholecystokinin
2.40	**2.44**	Lcat	NM_017024	Lecithin-cholesterol acyltransferase
2.41	**2.39**	Lklf	AF181251	ESTs, Moderately similar to KLF6_RAT Core promoter element-binding protein (Kruppel-like factor 6)
2.41	**1.58**	Lrrn3	NM_030856	Leucine rich repeat protein 3, neuronal
2.44	**1.11**	Fgg	NM_012559	fibrinogen, gamma polypeptide fgg; gamma-fibrinogen
2.46	**1.18**	Arntl	AF317669	bmal1g'; bmal1b
2.48	**1.10**	Cmkor1	NM_053352	chemokine orphan receptor 1 rdc1; rdc-1 protein
2.51	**1.56**	aldolase B	X02284	Aldolase B, fructose-biphosphate
2.51	**2.14**	S100a4	NM_012618	S100 calcium-binding protein A4
2.55	**2.33**	Slc12a4	NM_019229	Solute carrier family 12, member 4
2.57	**1.31**	Ramp2	NM_031646	Receptor (calcitonin) activity modifying protein 2
2.58	**2.01**	Nbl1	NM_031609	neuroblastoma, suppression of tumorigenicity 1 nbl1; tumor-suppressive gene; no3
2.64	**1.48**	Col5a3	NM_021760	Collagen, type V, alpha 3
2.65	**-1.44**	Hspa5	M14050	preimmunoglobulin heavy chain binding protein
2.73	**1.51**	Rgs11	U32438	rgs11
2.75	**2.38**	Cyp4f14	NM_019623	Cytochrome P450, subfamily IVF, polypeptide 14 (leukotriene B4 omega hydroxylase)

Nkx6.1 vs Pdx1	Nkx6.1 vs Cntrl	Common	Genbank	Description
2.82	**-1.19**	Cdh23	NM_053644	cadherin related 23; cdh23
2.85	**2.08**	Sh3kbp1	AF255888	SH3-domain kinase binding protein 1
2.90	**1.36**	Nrp	AF016296	neuropilin
2.99	**2.73**	Klf2; Lklf	AF181251	lung kruppel-like factor lklf
3.06	**2.18**	Gpd3	NM_022215	Glycerol 3-phosphate dehydrogenase
3.08	**-1.50**	Pdx1	NM_022852	Pancreatic and duodenal homeobox gene 1
3.15	**1.58**	G0s2	NM_008059	similar to gbp|x95280|x95280_1 g0s2-like protein - mus musculus
3.23	**2.08**	Ntrk3	NM_019248	neurotrophic tyrosine kinase, receptor, type 3 ntrk3; protein kinase trkc; receptor ki39; ki14
3.26	**1.15**	Rdc1	AF118816	Chemokine orphan receptor 1
3.27	**2.55**	Camk4	NM_012727	ca2+/calmodulin-dependent protein kinase iv beta polypeptide cam kinase iv beta; calspermin
3.28	**2.22**	Jundp2	NM_053894	jun dimerization protein 2 jundp2; jdp-2
3.34	**2.40**	S100a4	NM_012618	protein 9 ka homologous to calcium-binding protein s100a4; s-100-related; put. p9ka
3.39	**1.37**		BF523687	ESTs, Moderately similar to E88 [Mus musculus] [M.musulus]
3.45	**-1.06**	Sdf2l1	NM_022044	similar to pir nf00515193 stromal cell-derived factor 2-like protein 1 precursor
3.56	**2.36**	Sh3kbp1	AF255884	sh3 domain-containing adapter protein isoform seta-1x23 seta; ruk-s ruk; ruk-h; ruk-m1; ruk-m3; ruk-l
3.65	**5.17**	Slc22a1	NM_012697	organic cation transporter slc22a1; oct1a
3.70	**2.68**	Fgb	U05675	Fibrinogen, B beta polypeptide
4.10	**1.54**	F3	NM_013057	Coagulation factor III (thromboplastin, tissue factor)
4.11	**2.93**	Bv8	NM38852	homolog of mouse Bv8 (Bombina variegata 8 kDa); prokineticin 2 precursor
4.14	**1.07**	Nnat	NM_053601	neuronatin alpha; nnat
4.68	**3.35**	Crybb3	NM_031690	Crystallin, beta B3
4.73	**1.07**	Pdx1	NM_022852	somatostatin transactivating factor-1, stf-1; pancreatic and duodenal homeobox gene 1 pdx1; idx-1
4.85	**2.91**	Fgb	M27220	fibrinogen b-beta-chain; b beta chain
4.90	**1.69**	Ptger3	NM_012704	rat kidney prostaglandin ep3 receptor ep3a and ep3b ptger3;
4.92	**3.27**	Crybb3	NM_031690	betab3-crystallin aa 1 - 211; beta-b3-2-crystallin; crystallin, beta b3 crybb3
4.94	**2.44**	S100a3	AF140231	S100 calcium binding protein A3
5.03	**2.07**	Ggtp	M33821	Gamma-glutamyl transpeptidase
5.29	**2.51**	LOC94168	U19485	Spp-24 precursor
5.38	**2.79**	S100a3	NM_053681	s100 calcium binding protein a3 s100a3
6.07	**3.90**	Ptpre	Y07834	protein tyrosine phosphatase epsilon-like 2; epsilon c
6.76	**3.80**	Dmgdh	X55995	Dimethylglycine dehydrogenase precursor
9.90	**4.58**	Ptpre	D78610	protein tyrosine phosphatase epsilon c
11.81	**2.80**	SPP2	U19485	spp-24 precursor



	Nkx6.1	Pdx1	Cyclin B1	p21	ProT-A	slc22a1	Crbb3	PTPCe	UBC12	BNIP3	GAST	CDC25b	CDKN1B
siRNAcontrol	1.01	1.01	1.01	1.02	1.02	1.03	1.01	1.03	1.00	1.02	1.02	1.00	1.01
siNkx6.1	0.34	1.25	0.50	0.41	0.28	13.40	3.07	4.95	6.37	0.31	0.18	0.58	0.43
siPdx1	1.56	0.21	1.30	0.70	1.45	0.68	0.38	0.55	7.03	1.24	0.14	0.90	0.45

Figure F-4. Real-time PCR confirmation of selected genes identified in microarray analysis.

Selected genes identified in the microarray described in Tables F-1, F-2, and F-3 were measured via real-time PCR on RNA samples from cells treated with either Ad-siRNAcontrol, Ad-siNkx6.1, or Ad-siPdx1. The data represented are the mean ± SEM from the three independent experiments and are normalized to mRNA levels measured in Ad-siRNAcontrol-treated cells.

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VITAE

Jonathan Cummings Schisler was born in Buffalo, New York, on November 3, 1975, the son of Kurt Jonathan Schisler and Susan Elizabeth Schisler. After graduating from Saint Francis de Sales High School in Toledo, Ohio in 1993, he entered The University of Toledo, in Toledo, Ohio, as an undergraduate biology student. During his junior year, fall 1995 to spring 1996, he studied abroad in an exchange program with The University of Salford, located in Salford, England. He received the degree of Bachelor of Science with a major in biology and a minor in chemistry and graduated cum laude and with honors in June, 1997. During he next six months he worked at Argonne National Laboratory, run by the United States Department of Energy, located in Argonne, Illinois. In the fall of 1998, he returned to The University of Toledo and entered the graduate program in bioengineering. In June, 2000, he was awarded the degree of Masters of Science in Bioengineering. Jonathan entered the Graduate School of Biomedical Sciences at the University of Texas, Southwestern Medical Center at Dallas, Texas in the summer of 2000. In 2001, he married Stacy Lynn Czubachowski of Maumee, Ohio. Their son, Matthew Cummings Schisler, was born in 2003.

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