CHAPTER I

GENERAL INTRODUCTION

Diabetes Mellitus

Diabetes mellitus is a condition marked by high levels of blood glucose resulting from defects in insulin production, insulin action, or both. Diabetes can lead to serious complications, such as heart disease and stroke, high blood pressure, blindness, kidney disease, nervous system disease and others. The latest data from the Centers for Disease Control and Prevention reported that 23.6 million children and adults in the United States, 7.8% of the population, have diabetes, while another 57 million Americans are estimated to have pre-diabetes. The total cost of diabetes in the US has reached $218 million in 2007 (2007). Diabetes mellitus, as a worldwide epidemic has gained tremendous attention in the past decade. Many types of diabetes are recognized, the major types are: type 1 diabetes mellitus (T1DM), type 2 diabetes mellitus (T2DM), maturity onset diabetes of the young (MODY) and gestational diabetes mellitus (GDM). Accounting for 5 to 10% of all diagnosed cases of diabetes in the US, T1DM develops when immune system destroys pancreatic β-cells, the only cells in the body that make the hormone insulin [some neurons in the brain expressing insulin mRNA but not the protein (Havrankova et al., 1981; Le Roith et al., 1983; Grunblatt et al., 2007)] that regulates blood glucose. T2DM results from a combination of insulin resistance and β-cell dysfunction (Meier, 2008a). 90 to 95% of all diagnosed cases of diabetes in adults are T2DM. MODY is a monogenic form of diabetes that usually first occurs during adolescence or early adulthood. However, MODY sometimes remains undiagnosed until later in life. Up to 5% of all diabetes cases may be due to MODY (Giuffrida & Reis,

2005). GDM is a form of hyperglycemia diagnosed only during pregnancy. It affects ~ 7% of the pregnancies in the US each year (Perkins et al., 2007).

Gestational Diabetes Mellitus

Definition

The term “Gestational Diabetes Mellitus (GDM)” was first applied in the 1950s to what was thought to be a transient condition that affected fetal outcomes adversely, then abated after parturition (Buchanan & Xiang, 2005). While the clinical definition of GDM has evolved over the years, the currently well-accepted definition is glucose intolerance that appears or is first recognized during pregnancy and resolves after delivery. Resolution after delivery is especially important since previously undiscovered diabetes is often mistaken for GDM (Perkins et al. 2007).

Maternal and Fetal Complications

GDM is a major public health issue due to both its prevalence and its complications. In the US, ~7% of the pregnancies each year are complicated by this illness (2000). It causes numerous maternal and fetal complications. The main maternal complication of GDM is a higher long-term risk for developing metabolic syndrome and T2DM. A recent systematic review summarizing 20 studies reported that women who have had GDM have at least a seven-fold higher risk of developing T2DM in the future compared with those who had a normoglycemic pregnancy (Bellamy et al., 2009). Other maternal complications include hypertensive disorders and preeclampsia (Joffe et al., 1998), cesarean section and birth trauma (Lindsay, 2009).

Glucose travels freely from mother to the fetus; however, maternal insulin does not cross the placenta. Thus, GDM exposes the fetus to abnormally high concentrations

of glucose, forcing the fetus to increase insulin production. Excess insulin exposure causes macrosomia (infants with an excessive birth weight), a condition strongly correlated with GDM (Reece et al., 2009). After birth, the high glucose environment resolves in infants; however, they often have life-long high risk for glucose intolerance/diabetes and obesity (Dabelea et al., 2000; Yogev & Visser, 2009), supporting the theory that maternal hyperglycemia might exert particular *in utero* programming effects to increase the risk of metabolic disturbance in the offspring (Lindsay, 2009). In addition, after delivery, infants born to GDM mothers are at increased risk for neonatal complications such as respiratory distress syndrome, cardiomyopathy, hypocalcaemia, hypomagnesaemia, polycythaemia, and hyperviscosity (Reece et al., 2009). Sever diabetic pregnancy (hyperglycemia starting during the first week of pregnancy) can lead to neural tube defects (Schaefer et al., 1997; Aberg et al., 2001). Studies on rodent models revealed that diabetic pregnancy induces oxidative stress thus inhibiting expression of *Pax3 (Paired box gene 3)*, a gene essential for neural tube closure (Phelan et al., 1997; Chang et al., 2003; Li et al., 2005a).

Diagnosis and Treatment

The “gold standard” for diagnosing GDM is the 100 gram 3 hour oral glucose tolerance test (OGTT). A patient’s blood glucose level is measured after overnight fasting and then one, two and three hours after drinking a 100 gram glucose solution. The cutoff values for acceptable blood glucose concentrations at different time points are listed in Table 1. Two or more abnormal values must be measured for the test to be considered a positive diagnostic test (Perkins et al., 2007).

Treatment options begin with diet and exercise for most patients if they are not severely hyperglycemic. If this fails, two other main options are insulin therapy and glyburide, an insulin secretagogue (Coustan, 2007; Perkins et al., 2007).

Pathophysiology

During normal pregnancy, maternal metabolism changes substantially (Table 2). Insulin sensitivity progressively decreases approximately 50% by late gestation (Di Cianni et al., 2003). The change in insulin sensitivity during pregnancy is largely due to a combination of increased maternal adiposity and the insulin desensitizing effects of some hormones and cytokines associated with pregnancy. Potential factors include human chorionic somatomammotropin (HCS), leptin, progesterone, cortisol, adiponectin and tumor necrosis factor-α (TNF-α). The effects of these factors on insulin action are evident by: first, progressive insulin resistance during pregnancy is paralleled by elevated level/activity of placental hormones due to growth of the fetal-placental unit. Second, administration of HCS, progesterone, or glucocorticoids to non-pregnant individuals is able to induce hyperinsulinemia but not hypoglycemia, indicating blunted insulin action. Third, *in vitro* exposure of insulin target cells such as muscle cells and adipocytes to pregnancy hormones leads to impaired glucose uptake in those cells. Finally, TNF-α down-regulates insulin receptor signaling in cultured adipocytes and skeletal muscle cells through inhibiting insulin receptor autophosphorylation and association of insulin receptor substrate-1 (IRS-1) with insulin receptor.

To compensate for the reduction in insulin sensitivity, insulin secretion increases in normal pregnancy. The two contributors to increased insulin secretion are increased β-cell mass and enhanced β-cell function. β-cell mass expands two to three fold by mid-gestation due to a combination of hyperplasia and hypertrophy (Lapolla et al., 2005); however, augmentation in β-cell mass alone is not sufficient to explain the increase of insulin secretion in pregnancy. To clear glucose in a more efficient manner, the threshold for insulin release in response to glucose is lowered in β-cells (from 5.75 mM to 3.25

Table 1. Diagnosis of GDM (100 g, 3h OGTT)

[Based on recommendations of the American Diabetes Association (2003)]

Time of Measurement	Glucose Concentration (mg/dl)	
	T1DM or T2DM	GDM*
Random	≥ 200	-
After Overnight Fast	≥126	95
1 hour postchallenge		180
2 hour postchallenge		155
3 hour postchallenge		140

*: Two or more value meeting or exceeding the cut points is required for diagnosis of GDM.

Table 2. Changes in measures of metabolism in normal pregnancy [Adapted from (Lain & Catalano, 2007), used with permission]

	Early Pregnancy	Late Pregnancy
Fasting Glucose	Unchanged	Decreased (0.9X)
First phase insulin secretion	Increased (2X)	Increased (3X)
Second phase insulin secretion	Increased (1.5X)	Increased (3X)
Insulin sensitivity	Decreased (0.7X)	Decreased (0.4X)

mM) and cell-cell contact is enhanced in pancreatic islets in pregnancy (Sorenson & Brelje, 1997). In conclusion, robust plasticity of β-cell function in the face of progressive insulin resistance is the hallmark of normal glucose regulation during pregnancy.

In GDM patients, insulin sensitivity drops up to 40% compared to people with normal pregnancy, presumably due to defects in insulin receptor signal transduction and lower levels of insulin-sensitizing cytokines such as adiponectin (Kautzky-Willer et al., 1997; Lain & Catalano, 2007). Indeed, one human study supported the association of insulin resistance in GDM women with decreased IRS-1 phosphorylation, primarily due to decreased IRS-1 expression (Friedman et al., 1999). In addition, the majority of women who develop GDM have chronic insulin reistance evidenced by several studies in which whole-body insulin sensitivity has been measured directly (Kautzky-Willer et al., 1997; Homko et al., 2001). Most patients also demonstrate defective insulin secretion due to long-term deterioration of β-cell function. (Ryan et al., 1995; Homko et al., 2001), Other than the impaired islet function, defective increase in β-cell mass could also account for development of GDM given the fact that expansion of β-cell mass is one of the major avenues to cope with increased metabolic demand in pregnancy. However, up to now, no detailed study has been done in human patients with respect to this issue.

In summary, accumulating evidence suggests that pregnancy triggers a series of metabolic imbalances leading to a diabetic state in some women who are genetically predisposed to develop diabetes. Therefore, GDM may represent a unique window to study the early pathogenesis of diabetes.

Genetics

Tremendous effort has been made to identify candidate genes associated with T1DM and T2DM. However, there has been relatively little research on the genetics of GDM. Accumulating evidence supports the idea that GDM and T2DM may share a

Table 3. Genes associated with GDM based on human association studies [Adapted from (Robitaille & Grant, 2008), used with permission]

Gene	Function	Polymorphisms	Defects Associated with polymorphism	Reference
Insulin Secretion				
ABCC8	Essential subunit of the β-cell K_{ATP} channel which plays a central role in insulin secretion	Exon 16 -3T>C splice variant	beta-cell dysfunction reflected by reduced second-phase insulin secretion	(Hart et al., 2000)
KCNJ11	Essential subunit of the β-cell K_{ATP} channel which plays a central role in insulin secretion.	Glu23Lys	Increased threshold ATP concentration inducing overactivity of pancreatic ß-cell K_{ATP} channels and inhibiting insulin secretion.	(Schwanstecher et al., 2002)
ND1	Part of the electron transport chain involved in glucose metabolism	Met31Thr (3398T>C) 3316G>A 3394T>C 3399A>T	Not assessed	(Chen et al., 2000)
TCF7L2	A transcription factor downstream of the Wnt signaling pathway	IVS3C>T (rs7903146) rs12255372 rs7901695	Not assessed	(Shaat et al., 2007; Watanabe et al., 2007)
Insulin and Insulin Signaling				
INS	Hormone secreted by pancreatic β-cells involved in glucose metabolism.	Variable number of tandem repeats (VNTR) located 0.5kb upstream of the insulin gene	Class III VNTR showed lower expression of the *INS* gene	(Lucassen et al., 1995; Shaat et al., 2004; Litou et al., 2007)
IGF-2	Mediation of growth hormone action, stimulation of the action of insulin, pancreatic ß-cell growth and development	BamH I	Not assessed	(Ober et al., 1989)
INSR	Binds insulin stimulating glucose uptake	Kpn I	Not assessed	(Ober et al., 1989)
IRS-1	Downstream component of Insulin signaling pathway	Gly972Arg	Reduced tyrosine phosphorylation allowing *IRS1* to act as an inhibitor of the insulin receptor kinase	(McGettrick et al., 2005; Shaat et al., 2005)
MODY				
HNF4α	Transcription factor involved in the regulation of genes related to the glucose homeostasis and lipid metabolism	Rs2144908 Rs2425637 Rs1885088	Not assessed	(Shaat et al., 2006)
GCK	Phosphorylates glucose to glucose-6-phosphate and is the rate-determining step for glucose metabolism	-30G>A*	Reduces the activity of the wild-type promoter construct in cell line	(Chiu et al., 1994; Stone et al., 1996; Shaat et al., 2006)
HNF1α	Transcription factor involved in the regulation of genes related to glucose homeostasis and lipid metabolism	Ile27Leu Ala98Val	The Leu27 allele is associated with decreased transcriptional activity in HeLa and INS-1 cells Not assessed for Ala98Val	(Lauenborg et al., 2004; Shaat et al., 2006; Zurawek et al., 2007)
Other				
MBL2	Important component of the innate immune system and influences inflammatory response by inhibiting TNF-α release	Gly54Asp Arg52Cys	Both mutations are associated with a great lowering effect on plasma MBL levels.	(Sumiya et al., 1991; Garred et al., 1992; Madsen et al., 1995)
CAPN10	Regulation of a variety of cellular functions, including intracellular signalling, proliferation, adipocyte differentiation and glucose homeostasis	SNP-43 SNP-43, -19, -63 Haplotype: haplotype combination 121/221	SNP-43 regulates calpain-10 expression	(Horikawa et al., 2000; Leipold et al., 2004)
PAI-1	Regulates antifibrinolytic activity of plasma	-675 4G/5G*	Lowest PAI-1 activity in 5G/5G carriers.	(Leipold et al., 2006)

ABCC8: ATP-binding cassette transporter sub-family C member 8
KCNJ11: Potassium inwardly-rectifying channel, subfamily J, member 11
ND1: Mitochondrially encoded NADH dehydrogenase 1
TCF7L2: T-cell factor 7-like 2
INS: Insulin
IGF-II: Insulin like growth factor II
INSR: Insulin receptor
IRS1: insulin receptor substrate 1
HNF4α: Hepatocyte nuclear factor 4α
GCK: glucokinase
HNF1α: Hepatocyte nuclear factor 1α
MBL2: Mannose-binding lectin (protein C) 2
CAPN10: Calpain 10
PAI-1: Plasminogen activator inhibitor type 1

*: mutations reside in the promoter region

common genetic basis as revealed by the discovery of common candidate genes in human genetic association studies (Table 3) (Robitaille & Grant, 2008). In genetic association studies, if genetic variations are more frequent in people with certain disease, the varianions are thought to be “associated” with this disease. The candidate genes commonly associated with T2DM and GDM fall into four categories:

(1) Genes involved in insulin secretion such as *KCNJ11* and *TCF7L2*.

The β-cell ATP-sensitive potassium (K_{ATP}) channel is composed of two subunits: the regulatory subunit sulfonylurea receptor 1 (ATP-binding cassette, sub-family C, member 8; ABCC8) and the pore-forming subunit, potassium inward rectifying channel, subfamily J, member 11 (KCNJ11). Upon glucose stimulation, ATP generated via glycolysis inhibits the activity of the K_{ATP} channel, leading to depolarization of the β-cell membrane, which opens voltage-gated calcium channels, and stimulates insulin vesicle fusion and insulin release (Figure 1). An *in vitro* study performed in COS-1 cells revealed that the missense mutation Glu23Lys in KCNJ11 induced overactivity of the K_{ATP} channel thus reducing insulin release (Schwanstecher et al., 2002).

A number of meta-analyses also suggested that polymorphisms in KCNJ11, were significantly associated with T2DM (Hani et al., 1998; Gloyn et al., 2001; Ruchat et al., 2009), and two studies reported the impact of polymorphisms in KCNJ11 on GDM. Shaat et al. found an increased frequency of the Glu23Lys allele among 588 Scandinavian women with GDM compared with 1189 non-diabetic pregnant controls (Shaat et al., 2005). In line with Shaat’s finding, Cho et al. reported a strong association of reference SNP (rs) 5219 (Glu23Lys) and rs5125 (Ile337Val) in KCNJ11 with GDM in 869 Korean women patients compared with 345 non-diabetic controls (Cho et al., 2009).

The transcription factor 7-like 2 (TCF7L2, also referred to as transcription factor 4

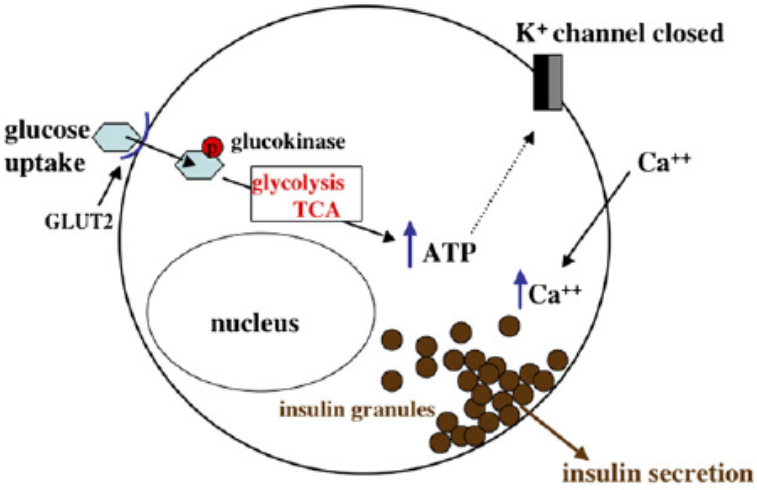


Figure 1. Glucose stimulated insulin secretion (GSIS). Glucose is taken up by the β-cell through the membrane-bound GLUT2 transporter. In the cytoplasm, glucose is phosphorylated by glucokinase to initiate glycolysis. ATP produced during glucose metabolism binds to and inactivates the plasma membrane potassium channel, thus allowing an inward flow of Ca^{2+} ions. This influx of Ca^{2+} causes the insulin-containing vesicles to fuse with the plasma membrane, releasing insulin into the bloodstream (Gannon, 2007, used with permission).

or TCF4) is a distal effector of the canonical Wnt signaling pathway. There is a consistent and strong association between variants of TCF7L2 and T2DM in multiple ethnic groups (Munoz et al., 2006; Loos et al., 2007; Palmer et al., 2008). With a per-allele relative risk of 1.4, this association is the strongest association among the common T2DM genes (Frayling, 2007). There are multiple potential mechanisms whereby TCF7L2 variants may cause T2DM. (a) Decreased β-cell mass: siRNA knockdown of *TCF7L2* decreased β-cell proliferation/apoptosis and GSIS in human islets. In contrast, overexpression of *Tcf7l2* protected islets from glucose and cytokine-induced apoptosis and proliferation defect (Shu et al., 2008). Knockdown of *Tcf7l2* inhibited β-cell mass expansion due to deficient β-cell proliferation in normal neonatal rats. In diabetic rats, reduced Tcf7l2 hindered β-cell regeneration (Figeac et al., 2009). (b) Impaired insulin processing or release: there is a strong association between variants of TCF7L2 and impaired insulin synthesis and/or processing of insulin precursors (Loos et al., 2007). Expression of genes important for late events of insulin release were altered in mouse islets with *Tcf7l2* knockdown, including Munc18-1 (member of Sec1/Munc18-related protein family, involved in membrane fusion reactions), ZnT8 (zinc transporter 8, a β-cell specific zinc transporter) and Syntaxin 1A (a key player in the control of insulin exocytosis) (da Silva Xavier et al., 2009). (c) Impaired incretin signaling in β-cells: GLP-1 (glucagon-like peptide-1) infusion combined with a hyperglycaemic clamp showed a significant reduction in GLP-1-induced insulin secretion in human carriers of the TCF7L2 variants. In addition, TCF7L2 variants seem to have no impact on plasma GLP-1 concentrations during an oral glucose tolerance test, suggesting a defect in GLP-1 signaling rather than GLP-1 production (Schafer et al., 2007). Consistent with this, Villareal et al. reported that the TCF7L2 variant rs7903146 decreased sensitivity of β-cells to GLP-1 and GIP (gastric inhibitory polypeptide) (Villareal et al., 2009).

The association between TCF7L2 variants and GDM has been studied in three ethic groups. Genetic association studies done in Scandinavian (Shaat et al., 2007), Mexican-American (Nikaidou et al., 1992), and Korean women (Cho et al., 2009) all supported the association between *TCF7L2* and GDM. Functional studies about TCF7L2 variants in glucose homeostasis during pregnancy are needed in the future.

(2) Insulin and insulin signaling genes.

IRS-1 is a substrate of the insulin receptor tyrosine kinase. Upon insulin binding its receptor, IRS-1 is phosphorylated by the insulin receptor kinase and then propagates the insulin signaling cascade by recruiting and activating signal transduction molecules (Boura-Halfon & Zick, 2009). Consistent with its vital role in insulin signaling, there is strong association between the Gly972Arg polymorphism of IRS-1 and T2DM (Jellema et al., 2003). However, association of this polymorphism with GDM remains controversial, presumably due to the differences in ethic groups examined in different studies (Shaat et al., 2005; Fallucca et al., 2006).

(3) MODY genes

MODY typically refers to any form of diabetes caused by a single autosomal dominant mutation. MODY is a monogenic form of T2DM characterized by early onset and a specific defect in the β-cell (Holmkvist et al., 2008). Nine MODY genes have been identified: *Hepatocyte nuclear factor 4α* (*HNF4α*, MODY1) (Bell et al., 1991) (Yamagata, Furuta et al., 1996), *Glucokinase* (*GCK*, MODY2) (Hattersley et al., 1992) (Froguel et al., 1993), *Hepatocyte nuclear factor 1α* (*HNF1α*, MODY3) (Yamagata, Oda et al., 1996), *Pancreatic and duodenal homeobox 1* (*PDX1*, MODY4) (Stoffers et al., 1997), *Hepatocyte nuclear facor 1β* (*HNF1β*, MODY5) (Horikawa et al., 1997), *Neurogenic differentiation 1* (*NEUROD1*, MODY6) (Malecki et al., 1999), *Kruppel-like factor 11*

(*KLF11*, MODY7) (Neve et al., 2005), *Bile salt dependent lipase* (or *BSDL*), also known as *Carboxyl ester lipase* (or *CEL*) (MODY8) (Hardt et al., 2003; Raeder et al., 2006) and *Paired box gene 4* (*PAX4*, MODY9) (Plengvidhya et al., 2007). Women with mutations in MODY genes, such as *GCK* (Stoffel et al., 1993), *HNF1α* (Weng et al., 2002) and *PDX1* (Gragnoli et al., 2005), often present with GDM. Therefore, the impact of MODY gene mutations on GDM is of great interest. *GCK* variants have been associated with GDM (Stoffel et al., 1993). HNF4α is a member of the nuclear hormone receptor family and regulates the coordinated expression of multiple genes necessary for normal liver and pancreatic function (Love-Gregory & Permutt, 2007). Despite its association with T2DM and MODY, human association studies have not observed significantly high frequency of HNF4a variants in GDM patients (Shaat et al., 2006), presumably due to the limitations of this type of study including possible biases resulting from case and control selection, genotyping errors, insensitivity to rare variants and structural variants (Pearson & Manolio, 2008).

(4) Other genes such as *Mannose-binding lectin (protein C) 2* (*MBL2*) and *Calpain 10 (CAPN10).*

MBL2, a member of the collectin protein family, is a plasma protein synthesized in the liver. It is an important component of the innate immune system. Two variants with the *MBL2* gene are associated with decreased plasma MBL2 levels: Arg52Cys and Gly54Asp (Sumiya et al., 1991; Garred et al., 1992; Madsen et al., 1995). MBL2 deficiency predisposes individuals to recurrent infections and chronic inflammatory diseases (Kilpatrick, 2002). Since insulin resistance might be induced by the direct action of inflammatory cytokines on insulin signaling postreceptor molecules (Hotamisligil et al., 1996), the association of MBL2 variants with GDM was investigated in a case-control study of 105 GDM women and 173 controls (Megia et al., 2004). Their

pregnant women bearing the Gly54Asp *MBL* allele have a greater risk for developing GDM and having heavier infants.

Calpains are processing proteases that cleave specific substrates at a limited number of sites, leading to activation or inactivation of protein function (Saido et al., 1994). They have been implicated in regulation of a variety of cellular functions, including intracellular signaling, especially insulin-induced down-regulation of IRS-1 (Smith et al., 1996). Several SNPs have been identified within the *CAPN10* gene. Among them, the SNP-43 regulates *CAPN10* expression. This SNP, as well as SNP-19 and SNP-63 in allelic combination, has been previously associated with T2DM (Horikawa et al., 2000). However, only SNP-63 is linked to GDM (Leipold et al., 2004).

In summary, we are beginning to understand the genetics of GDM. It seems that many T2DM susceptibility genes are also associated with the pathogenesis of GDM, However, some diabetes candidates, such as peroxisome proliferative activated receptor, gamma (PPARG) and adiponectin, despite their established role in T2DM pathogenesis (Deeb et al., 1998; Altshuler et al., 2000; Ukkola et al., 2005; Zacharova et al., 2005; Schwarz et al., 2006), showed no association with GDM based upon current knowledge (Shaat et al., 2004; Shaat et al., 2007). More association studies with larger sample size on multiple populations are needed to explain this discrepancy. In addition, some well-studied complications of GDM, such as macrosomia, neural tube and heart defects in infants, should also be evaluated in human association studies. This could help to more accurately define the association with GDM. More importantly, functional analyses of candidate gene variants will provide valuable information to understand the mechanism whereby these genetic components lead to GDM, and benefit the discovery of novel preventive, screening or therapeutic options for GDM.

Animal Models of GDM

To date, there are only four published potential animal models of gestational diabetes. The first model is a heterozygous mutation in the leptin receptor (*Lepr*$^{db/+}$). *Lepr*$^{db/+}$ female mice develop spontaneous GDM (Kaufmann et al., 1981) and produce macrosomic fetuses compared with wild-type (WT) mothers (Kaufmann et al., 1987). The newborn pups also exhibit abnormal carbohydrate metabolism, with increased liver glycogen, decreased blood glucose, and increased plasma insulin levels (Ishizuka et al., 1999). Leptin normally reduces appetite, increases energy expenditure, inhibits insulin secretion and increases glucose utilization and lipolysis (Anubhuti & Arora, 2008). Consistent with the role of leptin, impaired insulin signaling in skeletal muscle, increased maternal fat mass and energy intake were observed in *Lepr*$^{db/+}$ mice (Ishizuka et al., 1999). Therefore, the GDM phenotype in *Lepr*$^{db/+}$ may result from multiple defects in metabolic homeostasis.

β-cell specific deletion of *HNF4α* (*HNF4α*$^{loxP/loxP}$*; Ins-Cre*) leads to mild glucose intolerance during pregnancy due to decreased β-cell proliferation, β-cell mass, pancreatic insulin content and islet size (Gupta et al., 2007). The requirement of HNF4α for normal β-cell proliferation/mass expansion could be explained by its vital role in maintaining Ras/ERK signaling activity through its direct target *Suppression of tumorigenicity 5* (*ST5). HNF4α*$^{loxP/loxP}$*; Ins-Cre* mice are an imperfect model of GDM because their glucose concentrations in IPGTTs (Intraperitoneal glucose tolerance test) are not high enough to be diagonised as GDM. It is also unknown whether the glucose intolerance resolves after pregnancy. In addition, a molecular link between pregnancy and *HNF4α* activation is lacking.

Overexpression of Menin in β-cells (*RIP-rtTA; TRE-Men1*) causes GDM in mice due to impaired β-cell /islet expansion in pregnancy (Karnik et al., 2007). Menin is encoded by *Multiple endocrine neoplasia type 1 (Men1).* It functions in a histone

methyltransferase protein complex promoting the trimethylation of histone H3 at lysine 4 (H3K4me3), an epigenetic marker associated with transcriptionally active chromatin (Karnik et al., 2005). Menin expression is decreased in response to prolactin signaling during pregnancy. Menin normally inhibits β-cell proliferation through its direct transcriptional targets, p18 and p27 in both mouse and human islets (Karnik et al., 2007). Although the molecular mechanism by which Menin functions to inhibit β-cell proliferation in pregnancy has been characterized, this Menin overexpression mouse model is not known to be an ideal model for GDM since OGTT results showed that control mice in this study were also diabetic during pregnancy (Karnik et al., 2007), possibly due to the genetic background or diets used.

Our laboratory recently reported *Foxm1*$^{fl/fl}$*; Pdx1-Cre* (*Foxm1*$^{\Delta panc}$) mice as a model for GDM (Zhang et al., 2010). The phenotype of *Foxm1*$^{\Delta panc}$ meets all the diagnostic criteria for human GDM. Despite being euglycemic as virgins, *Foxm1*$^{\Delta panc}$ female mice develop glucose intolerance by gestational day (GD) 12.5 and overt gestational diabetes at GD15.5. The hyperglycemia resolves after pregnancy at P8. The role of Foxm1 in the β-cell will be better described in a later section.

In three out of four published GDM mouse models, defective β-cell mass expansion is thought to be the primary reason for GDM phenotype, highlighting the significance of normal growth in maternal β-cell mass to maintaining euglycemia during pregnancy.

Regulation of Postnatal Pancreatic β-cell Mass

The total population of β-cells in the pancreas is termed “β-cell mass” (Bouwens & Rooman, 2005). β-cell mass, at any given time, is governed by cell proliferation, neogenesis (β-cell newly differentiated from progenitors), increased or decreased cell size (hypertrophy or atrophy), and cell death (apoptosis) (Figure 2A). In adults, the

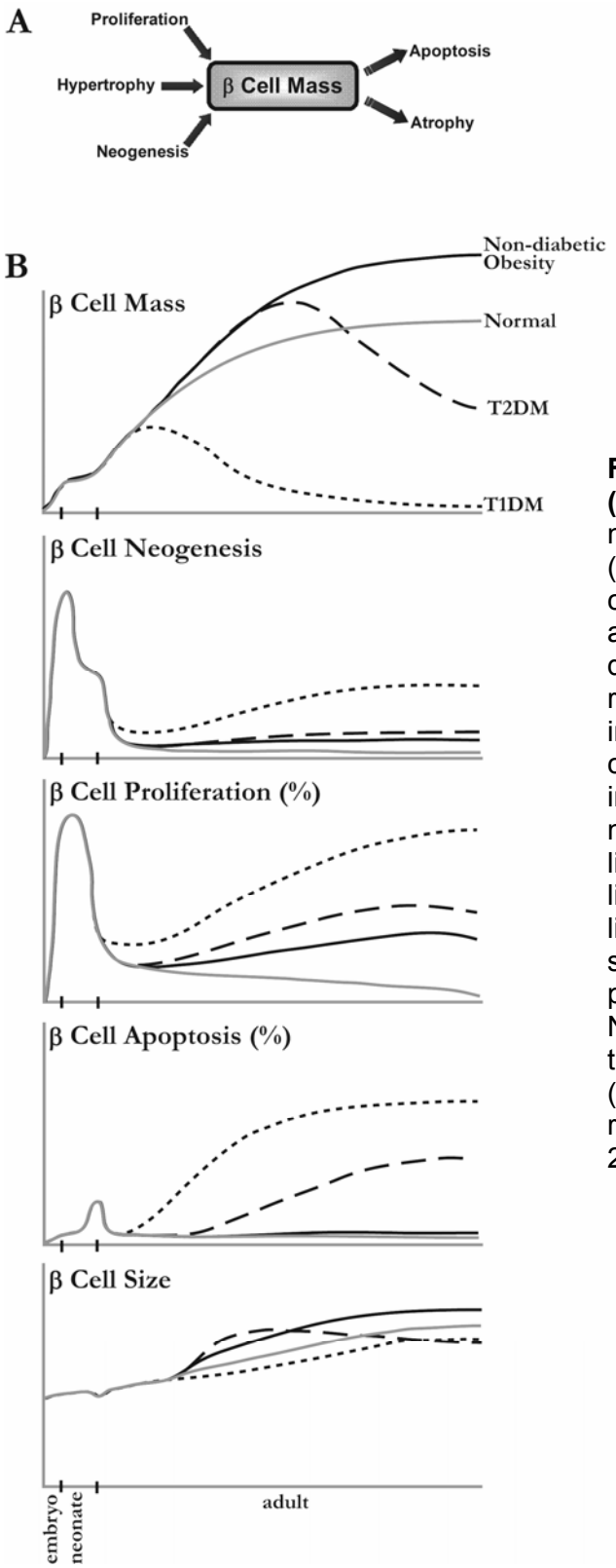


Figure 2. β cell mass dynamics. **(A)** β-cell proliferation, neogenesis, and hypertrophy (enlarged cell size) increase β-cell mass, while β-cell apoptosis and atrophy (reduced cell size) decrease β-cell mass. **(B)** Graphs represent approximate changes in these processes over the course of a lifetime in normal individuals (gray solid line), in non-diabetic obesity (black solid line), in T2DM (black dashed line), and in T1DM (black dotted line), based on rodent and human studies. Embryo denotes the period of time prior to birth. Neonate denotes the period of time between birth and weaning (approximately 3 weeks in the rodents). (Ackermann & Gannon, 2007, used with permission)

majority of new β-cells come from the replication of pre-existing β-cells rather than neogenesis in both rodents and humans (Dor et al., 2004; Meier et al., 2008). Both T1DM and T2DM are due to an either absolute or relative loss of β-cell mass (Figure 2B). Therefore, understanding how β-cell mass, especially postnatal mass, is regulated and maintained has been of great importance for prioritizing strategies for ameliorating or treating diabetes.

Neonatal β-cell Mass Expansion

Fetal β-cell proliferation occurs at late gestation, with an approximate doubling of β-cell population each day starting from 16 dpc (days post coitum) in rats (McEvoy & Madson, 1980). In early neonatal period, growth of β-cell mass is achieved through both β-cell replication and ongoing neogenesis. A transient increase of β-cell apoptosis is observed around weaning and is associated with a transient decrease in islet growth in both rodents (Scaglia et al., 1997) and humans (Kassem et al., 2000).

Adult β-cell Mass Expansion

β-cell mass is capable of undergoing dynamic changes throughout life. In adulthood, β-cells proliferate at a low rate and this declines with age. Approximately 1-4% β-cells proliferate per day in rats between 30 and 100 days old (Finegood et al., 1995), while <1% of β-cells proliferate per day in one year old mice (Teta et al., 2005) . Physiological conditions such as pregnancy and obesity lead to β-cell mass expansion, while parturition and prolonged fasting cause decreased β-cell mass.

Maternal β-cell Mass Expansion During Pregnancy

To meet the increased metabolic demand in gestation, β-cell mass increases more than 50% in rodents, resulting from an approximate three-fold increase in β-cell

proliferation by mid-gestation in addition to substantial individual cell hypertrophy (Scaglia et al., 1995). Gestational changes are transient and the β-cell mass eventually returns to the non-pregnant state after delivery through increased β-cell apoptosis and cell atrophy (Sorenson et al., 1993). The dynamic changes of maternal β-cell mass during pregnancy are illustrated in Figure 3. Pregnancy also induces a two to three-fold β-cell mass expansion in humans. However, the growth of β-cell mass in human pregnancy is thought to be mainly achieved via β-cell neogeneis rather than proliferation, evident by significantly more small islets and insulin positive cells in pancreatic ducts in pregnant human islets (Minh, 2008).

The lactogen hormones, prolactin (PRL) and placental lactogens (PLs), are the principle stimulators of β-cell proliferation/mass expansion in pregnancy (Brelje et al., 1993). Their common receptor, prolaction receptor (PRLR) is expressed in β-cellsand its expression is up-regulated during pregnancy (Galsgaard et al., 1999). β-cell proliferation reaches the peak at approximately GD14.5 and then gradually decline (Figure 4) due to the arising activity of PL-counteracting hormones such as progesterone and glucocorticoids during the late pregnancy (Kawai & Kishi, 1999; Nasir et al., 2005). β-cells also adopt to pregnancy through other avenues including hypertrophy, decreased apoptosis, enhanced cell function. At around GD14.5, other compensatory events maybe sufficient to maintain gestational glucose homoestasis, therefore β-cell proliferation slows down after this time point.

In vivo, the overexpression of PL in β-cells results in increased islet mass and β-cell proliferation (Vasavada, Garcia-Ocaña et al., 2000). Moreover, global PRLR-deficent mice display islet and β-cell hypoplasia. The requirement of PRLR for β-cell compensation in pregnancy was demonstrated using pregnant mice heterozygous for the PRLR null mutation. These mice exhibited reduced β-cell proliferation, decreased β-cell size and mass, and impaired glucose tolerance (Huang et al., 2009). The

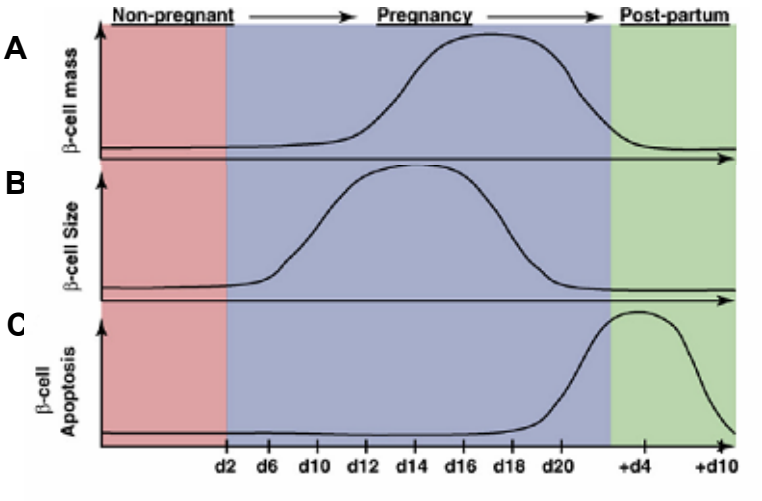


Figure 3. β-cell mass dynamics during pregnancy in mice. (A) β-cell mass is increased by both β-cell replication and **(B)** β-cell hypertrophy during the first two thirds of gestation. After parturition, maternal β-cell mass returns to non-pregnant levels by **(C)** β-cell apoptosis, which increases through the end of pregnancy and is still detected 4–6 days after birth. The graphs represent approximate changes in these processes before pregnancy (red, non-pregnant), over the course of pregnancy (light purple) and post-partum (green), and show what is believed to occur during human pregnancy based on rodent studies (Rieck & Kaestner, 2009, used with permission)

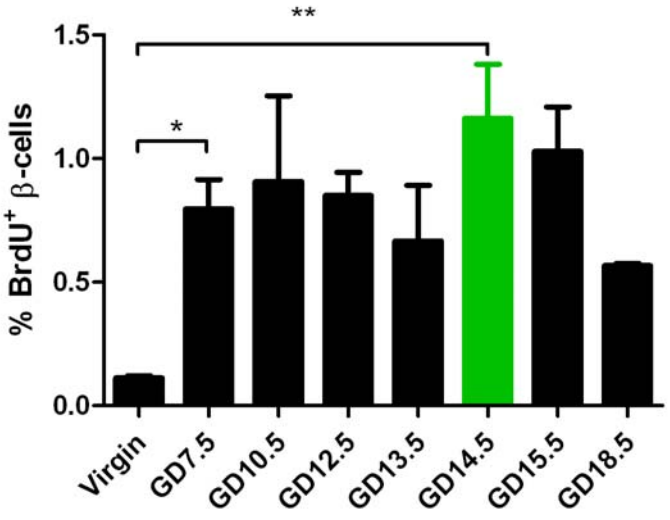


Figure 4. Maternal β-cell proliferation during gestation in B6D2 mice. β-cell proliferation increases as pregnancy progresses and reaches a peak around GD14.5. Unpaired, two tailed student's t-test was used to measure significance. Error bars represent SD. n= 3, * P<0.05, ** P< 0.01. (Zhang et al., 2010, used with permission)

mechanisms by which lactogen signaling promotes increased β-cell mass are becoming more clear. *In vivo* evidence suggested that in β-cells during pregnancy, PL activates its receptor PRLR and downstream Janus-activated-kinase-2/Signal transducer and activator of transcription 5 (JAK2/STAT5) signaling pathway, resulting in rapid nuclear translocation of phospho-Stat5. The activated Stat5 transcription factor inhibits Menin production via its direct target Bcl-6. Attenuated Menin levels and function reduces p27 expression, thereby promoting the increase of maternal β-cell proliferation (Karnik et al., 2007). Activation of other signaling pathways such as mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K) and IRS1/2 have been shown to augment β-cell mass downstream of the PRLR *in vitro* (Amaral et al., 2003; Amaral et al., 2004). However, it remains unclear whether PL and/or PRL signal through these pathways *in vivo*.

To achieve proper β-cell mass expansion in gestation, the proliferating β-cells also need to initiate programs ensuring their survival. Other than their cell proliferative role, PL and PRL also function to protect β-cells against dexamethasone (DEX)-induced death via Jak2/Stat5 and subsequent elevation of the anti-apoptotic protein Bcl-X_L (B-cell Lymphoma-extra Large) in both rat insulinoma (INS-1) cells and mouse islets (Fujinaka et al., 2007).

Mammalian Cell Cycle Progression

The cell cycle of mammalian cells can be divided into four successive phases: M phase (mitosis), in which the nucleus and the cytoplasm divide; S phase (DNA synthesis) and two gap phases, G1 and G2. The mammalian cell cycle is governed by Cyclin-dependent kinase (Cdk) complexes comprised of two components: Cdk and its activating cyclin subunit. Transition from G1 to S-phase is regulated by the early activity of the cyclin D-dependent Cdk4 and Cdk6 and the later activity of cyclin E/A-dependent

Cdk2 (Sherr & Roberts, 2004). Phosphorylation of the pocket protein retinoblastoma (Rb) by these Cdk complexes releases bound E2F transcription factor and allows it to stimulate the expression of genes required for DNA synthesis (Harbour & Dean, 2000). The cyclin E/Cdk2 complex also phosporylates non-Rb substrates to further promote G1 exit and S-phase initiation. Cdk2 is subsequently activated by cyclin A2 during late stages of DNA replication to drive the transition from S through G2 phase. Subsequently, Cdk1 is activated by A-type cyclins at the end of the G2 phase to facilitate the onset of mitosis. Following nuclear envelop breakdown, A-type cyclins are degraded, allowing the formation of the cyclin B/Cdk1 complex that is responsible for driving cells through mitosis (Malumbres & Barbacid, 2009). Finally, Aurora B, Polo-like kinases (Plks) and nuclear protein centromere-associated protein A (Cenp-A) and Cenp-B function to complete cytokinesis (Mellone & Allshire, 2003; Barr et al., 2004; Meraldi et al., 2004). A illustrative summary of cell cycle regulation in mammalian cells is shown in Figure 5.

Cdk activity requires dephosphorylaton of the Cdk catalytic subunit by the cell-division cycle (Cdc) 25A, Cdc25B and Cdc25C phosphatase proteins (Nilsson & Hoffmann, 2000). Cdc25A de-phosphorylates and thus activates cyclin E/Cdk2, promoting the G1 to S transition and also allowing S-phase progression. Cdc25B activates cyclin A/Cdk2 at the late G2 and cyclin A/Cdk1 at the onset of M-phase. Cdc25C functions primarily in mitosis and catalyzes mitotic progression by activating cyclin B/Cdk1(Lyon et al., 2002).

The activities of cyclin/Cdk enzymes can be blocked by cyclin-dependent kinaseinhibitors (CDKIs). CDKIs are comprised of seven members classified into two groups: INK4 and Cip/Kip. The INK4 group has four members ($p16^{INK4a}$, $p15^{INK4b}$, $p18^{INK4c}$, and $p19^{INK4d}$) which specifically inhibit Cdk4 and Cdk6. The Cip/Kip group consists of $p21^{CIP1}$, $p27^{KIP1}$, and $p57^{KIP2}$ which selectively inhibit Cdk2 during cell cycle

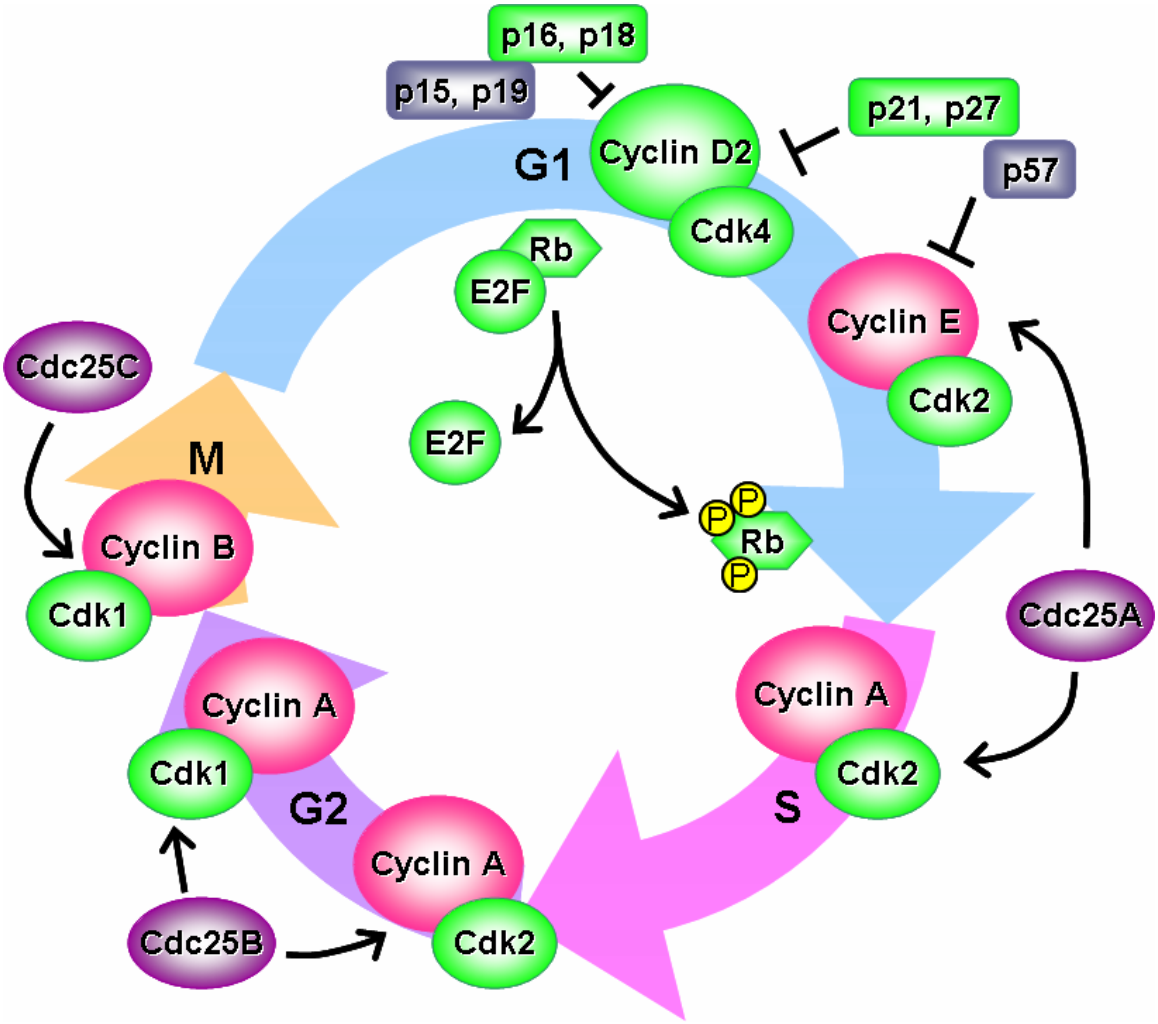


Figure 5. The simplified schematic representation of the mammalian cell cycle. P, phosphate; Rb, retinoblastoma protein. Proteins labled in green are critical for β-cell cell cycle based on knockout studies as reviewed in Ackermann and Gannon 2007 and Heit *et al.* 2006.

arrest. However, during cell cycle progression, Cip/Kip proteins actually increase Cdk4/6 function by promoting cyclin/Cdk complex stabilization, increasing nuclear import and Cdk kinase activity (Sherr & Roberts, 1999).

Regulation of β-cell Proliferation by Cyclins, CDKs and CDKIs.

β-cell cell cycle machinery, similar to other cell types is comprised of three major classes of proteins, cyclins, Cdks and CDKIs. However, emerging evidence has shed light on several distinctive features of the β-cell cell cycle. First, cell cycle regulators are differentially expressed in the β-cell (Table 4). In islets, cyclin D2 is the most abundant D-type cyclin. *Ccnd2 (cyclin D2)*$^{-/-}$ mice have decreased β-cell mass; reduced serum insulin levels and in some strains, impaired glucose tolerance (Georgia & Bhushan, 2004; Kushner et al., 2005). In contrast, cyclin D1 is not required for β-cell development since *Ccnd1*(*cyclin D1)*$^{-/-}$ mice are viable, and display normal islet number, size and morphology (Martin et al., 2003). Interestingly, *Ccnd2*$^{-/-}$ mice harboring one null allele of *Ccnd1* exhibit a further reduction in β-cell mass in comparison with *Ccnd2*$^{-/-}$ mice (Kushner et al., 2005). Although cyclin D1 does not appear to be necessary for normal islet growth, adenovirus-mediated ectopic expression of cyclin D1 in human and rat islets is sufficient to enhance β-cell proliferation (Cozar-Castellano et al., 2004). One possible signaling pathway regulating cyclin D1 expression could be PRL-Stat signaling, given the fact that the cyclin D1 promoter contains Stat binding sites and has been shown to be a target of PRL-induced STAT signaling in mammary carcinoma and ovarian cell lines (Brockman et al., 2002). However, whether this regulation remains true in the pancreas is unknown. The effect of overexpressing cyclin D2 on β-cell proliferation has not been determined. Balcazar et al. reported that mTORC1 modulates cyclin D2 synthesis and stability to promote β-cell proliferation/mass in mice overexpressing Akt in the β-cell (Balcazar et al., 2009). In addition, one study in pituitary cells reported that

Table 4. Expression of cell cycle regulators in the β-cell [Adapted from ((Heit et al., 2006) used with permission].

Protein	Expression Level	References
cyclins		
cyclin A	+	(Cozar-Castellano et al., 2006)
cyclin D1	++	(Georgia & Bhushan, 2004; Cozar-Castellano et al., 2006)
cyclin D2	+++	(Georgia & Bhushan, 2004; Cozar-Castellano et al., 2006)
cyclin D3	+	(Georgia & Bhushan, 2004; Cozar-Castellano et al., 2006)
cyclin E1/2	++	(Cozar-Castellano et al., 2006)
CDKs		
CDK2	+	(Cozar-Castellano et al., 2006)
CDK1	+	
CDK4	+++	(Rane et al., 1999; Martin et al., 2003; Marzo et al., 2004; Karnik et al., 2005)
CDK6	-	(Cozar-Castellano et al., 2006a)
CDKIs		
p15	+++	(Latres et al., 2000; Karnik et al., 2005; Cozar-Castellano et al., 2006; Cozar-Castellano et al., 2006a)
p16	+	(Cozar-Castellano et al., 2006a)
p18	++	(Franklin et al., 2000; Latres et al., 2000; Karnik et al., 2005; Cozar-Castellano et al., 2006; Cozar-Castellano et al., 2006a)
p19	++	(Karnik et al., 2005; Cozar-Castellano et al., 2006a; Dhawan et al., 2009; Chen et al., 2009a)
p21	+++	(Franklin et al., 2000; Latres et al., 2000; Karnik et al., 2005; Cozar-Castellano et al., 2006; Cozar-Castellano et al., 2006a)
p27	++	(Franklin et al., 2000; Latres et al., 2000; Karnik et al., 2005; Cozar-Castellano et al., 2006; Cozar-Castellano et al., 2006a)
p57	+	(Cozar-Castellano et al., 2006a)
Others		
E2F1	+++	(Fajas et al., 2004; Iglesias et al., 2004; Cozar-Castellano et al., 2006a)
E2F2	+	(Iglesias et al., 2004; Cozar-Castellano et al., 2006a)
E2F3	+	(Cozar-Castellano et al., 2006a)
E2F4	+++	(Cozar-Castellano et al., 2006a)
E2F5	++	(Iglesias et al., 2004; Cozar-Castellano et al., 2006a)
E2F6	+++	(Iglesias et al., 2004; Cozar-Castellano et al., 2006a)
E2F7	+	(Fallucca et al., 2006)
Rb	+++	(Cozar-Castellano et al., 2006a; Harb et al., 2009)

cyclin D2 expression is regulated by Wnt signaling via the transcription factor Pitx (Kioussi et al., 2002). It would be interesting to examine this relationship in the pancreas.

Second, compared to other cell types, β-cells are particularly vulnerable to disturbances in cell cycle progression due to less redundancy among related cell cycleregulators. For instance, only Cdk4 but not the closely-related protein Cdk6 is expressed in adult mouse β-cells, precluding compensatory effect in CDK4 mutants (Martin et al., 2003). *Cdk4*$^{-/-}$ mice develop normal islets but fail to undergo postnatal islet expansion, resulting in β-cell hypoplasia, hyperglycemia, and diabetes (Rane et al., 1999). Expression of *Cdk4* alleles insensitive to regulation by CDKI proteins (*Cdk4*$^{R/R}$) in *Cdk4*$^{-/-}$ mice restores the defects in *Cdk4*$^{-/-}$ animals through inducing β-cell hyperplasia (Rane et al., 1999). Recently, indirect evidence from mice after partial pancreatecomy showed that Cdk4 may function to recruit not only quiescent β-cells but also ductal progenitors into active cell cycle (Lee et al., 2010).

Third, different factors govern β-cell proliferation at different stages. Both cyclin D2 and CDK4 global mutations lead to normal β-cell development and mass at birth, but during postnatal growth, the mice experienced impaired postnatal β-cell mass expansion, impaired glucose tolerance and even diabetes (Rane et al., 1999; Georgia & Bhushan, 2004). p16 is upregulated in islets from older adult mice. Global deletion of p16 causes increased β-cell proliferation only in older adult mice (Krishnamurthy et al., 2006). In contrast, *p27*$^{-/-}$ mice displayed increased β-cell proliferation during embryonic and adult stages but not neonatal stage (Georgia & Bhushan, 2006). However, β-cell specific overexpression of p27 inhibits proliferation of embryonic and neonatal, but not adult, β-cells (Rachdi et al., 2006), indicating that p27 is critical for the establishment, instead of the maintenance, of β-cell mass. In the endocrine pancreas, connective tissue growth factor (CTGF) expression can be only detected during embryogenesis. Loss of CTGF in mice causes a dramatic decrease in β-cell proliferation at 18.5 dpc (Crawford et

al., 2009). Similarly, PEPK (PKR-like ER kinase) is specifically required for β-cell proliferation during the embryonic and early neonatal period as a prerequisite for postnatal glucose homeostasis evident by severely defective fetal/neonatal β-cell proliferation, and low β-cell mass in the global null mice (Zhang et al., 2006a).

Studies on INK4 CDKI proteins, especially p16, opened the door to a newly emerging field: β-cell aging. β-cell proliferation gradually declines with aging in rats to a steady state by seven months of age (Montanya et al., 2000). Furthermore, long-term bromodeoxyuridine (BrdU) labeling in one-year-old mice also revealed the decline of β-cell replication with age (Teta et al., 2005). Not only β-cell proliferation under normal conditions, but also the adaptive β-cell growth is affected as animals age. Tschen et al. showed that the capacity to expand β-cell mass in response to the GLP-1 analog exendin-4 or streptozotocin (STZ, a chemical specifically toxic to the β-cell) treatment was greatly compromised in eight-month old mice compared to 1.5-month old mice (Tschen et al., 2009). Given its age-dependent expression pattern, p16 has been proposed as a biomarker of aging. p16 is the only CDKIs whose expression (mRNA) increases significantly with aging in pancreatic islets among CDKIs examined including p15, p18, p19, p21 and p27 (Krishnamurthy et al., 2006). Recent studies on the regulation of p16 have shed light on the molecular mechanisms underlying the age-dependent decline of β-cell proliferation. Bhushan's group discovered that in aged islets, decreased Bmi-1 [B cell-specific Mo-MLV integration site 1, a polycomb group (PcG) protein] expression level results in loss of H2A ubiquitylation, increased MLL1 [mixed-lineage leukaemia 1, a trithorax group (TxG) member] recruitment and a concomitant increase in H3K4me3, ultimately leading to derepression of the *INK4a/Arf* locus (Dhawan et al., 2009). In STZ-treated animals, this histone modification is reversed to allow β-cell regeneration (Dhawan et al., 2009). Chen et al. identified another component of a PcG protein complex, enhancer of zeste homolog 2 (Ezh2), a histone

methyltransferase, as a repressor of the *INK4a/Arf* locus. Similar to Bmi-1, Ezh2 expression level also declines in aging islet β-cells and is induced in response to STZ treatment. Mice with conditional deletion of *Ezh2* in β-cells showed reduced β-cell proliferation and mass, and mild diabetes due to precocious increases of p16 and p19 (Chen et al., 2009). The upstream signaling pathways bridging external stimuli and these epigenetic modifications still remain to be determined.

Regulation of β-cell Proliferation by Growth Factors

Insulin-like growth factor-1 (IGF-1) belongs to the IGF peptide family which is comprised of over 10 different proteins including insulin, IGF-2 and relaxin. IGF-1 is well known as a major mediator of both embryonic and postnatal growth (Cohen, 2006). IGF-1 is produced locally within the tissues where it acts. In addition, IGF-1 is also available as a circulating hormone bound a number of binding proteins. While postnatal IGF-2 production increases gradually in humans, IGF-1 is the major form of IGFs in adult rodents (van Haeften & Twickler, 2004). Both IGF-1 and its receptor IGF-1R are expressed in islets. IGF-1 is a potent stimulator of β-cell proliferation *in vitro* as demonstrated in rat islets and insulinoma cell lines (Sieradzki et al., 1988; Hogg et al., 1993). Moreover, overexpression of IGF-1 in transgenic mice results in a three-fold increase of both β-cell proliferation and β-cell mass (George et al., 2002). However, the β-cell specific deletion of either the insulin receptor (INSR) or IGF-1R alone fails to cause changes in β-cell mass (Kulkarni et al., 1999; Kulkarni et al., 2002). This suggested that insulin and IGF-1 signaling pathways may be compensatroy. To address this issue, mice with global double inactivation of INSR and IGF1R have been derived by Accili's group (Kido et al., 2002). These mice showed a 90% reduction in exocrine tissue size due to decreased cell proliferation, consistent with the function of IGF signaling in promoting the growth of exocrine tissue (Williams et al., 1984; O'Brien & Granner, 1996;

Ludwig et al., 1999). In contrast, endocrine cell populations in these double null mice were surprisingly unperturbed at 18.5 dpc (Kido et al., 2002). This result indicated that IGFR and INSR are not required for endocrine cell development. However, since this study did not examine the double mutant animal beyond 18.5 dpc, it does not rule out the possibility that in adulthood, these receptors could be implicated in regulation of β-cell replication.

Some evidence suggested that IGF-1 acts synergistically with glucose to promote β-cell proliferation, in the physiologically relevant glucose concentration range. This proliferative effect appears to be mediated through the sustained (>24 h) activation of downstream signaling pathways including ERK (Extracellular signal-regulated kinase) 1/2 and PI3 kinase (PI3K) induction and subsequent activation of mTOR/p70 (S6K) likely via IRS-2 (Hugl et al., 1998; Dickson et al., 2001; Lingohr et al., 2002).

Several clinical trails with systemic admistration of IGF-1 were conducted to study patients with severe insulin-resistant states (Quin et al., 1994; Moses et al., 1996; Vestergaard et al., 1997) and/or T2DM (Schalch et al., 1993; Jabri et al., 1994). In these studies, IGF-1 improves the glucose control effectively, however, the underlying molecular mechanism remain unclear. The clinical studies were brought to an abrupt halt in late 90s because of the association between high levels of IGF-1 and cancer risk (Burroughs et al., 1999). Therefore, to explore its therapeutic potential in combating diabetes, it is crucial to obtain a throught understanding of the molecular mechanism of IGF-1 promoted cell proliferation, especially within the β-cell.

Hepatocyte growth factor (HGF) was originally identified as a circulating factor in liver regeneration (Nakamura et al., 1986). HGF also exhibits its mitogenic and morphogenic activities in a wide variety of cells (Zarnegar & Michalopoulos, 1995). In the pancreas, both HGF and its receptor c-MET (mesenchymal-epithelial transition factor) are expressed in islets (Otonkoski et al., 1996). HGF is a potent mitogen for both fetal

and adult human β-cells both *in vitro* (Beattie et al., 1997; Beattie et al., 2002) and *in vivo* (Garcia-Ocana et al., 2000), and the islet-associated ductal cells, where endocrine precursors are thought to reside (Otonkoski et al., 1996). Mice with ubiquitious exogenous overexpression of HGF are resistant to diabetogenic factors such as STZ treatment (Dai et al., 2003; Park et al., 2003). Thus, HGF is a very promising therapeutic tool not only to generate more functional β-cells *in vitro* but also to improve β-cell function/proliferation *in vivo*. However, in *c-met* knockout mice, β-cell mass and β-cell proliferation are normal, suggesting that HGF signaling is not essential for normal β-cell growth, but it is essential for normal glucose-dependent insulin secretion in mice (Roccisana et al., 2005). Analysis of the signaling pathways indicated that PI3K and atypical PKCs, but not ERK1/ERK2, play an important role in the HGF-induced increase in INS-1 cell replication (Gahr et al., 2002). Unlike IGF-1, the mitogenic effect of HGF is non-additive with glucose (Gahr et al., 2002).

GLP-1 is an incretin hormone encoded by the *Glucagon (Gcg)* gene. It is generated from the proglucagon precursor and then has secreted from L cells of the small and large intestine, brain and pancreatic islets (Drucker, 2001). GLP-1 activity requires binding to its specific receptor, GLP-1R, a 7-transmembrane G-protein coupled receptor. GLP-1 regulates blood glucose levels by stimulating insulin secretion, insulin biosynthesis, β-cell proliferation, islet neogenesis and reducing β-cell apoptosis and toxicity (Egan, 2007). The proliferative effect of GLP-1 on β-cells is largely mediated by Pdx1 (Li et al., 2005b) which is both necessary and sufficient for β-cell replication in other contexts (Kulkarni, 2005; Gannon et al., 2008). However, serum GLP-1 is rapidly degraded by an enzyme named dipeptidylpeptidase IV (DPP-IV) (Hansen et al., 1999). Therefore, many modifications have been made to GLP-1 to increase its biological half-life and consequently its efficacy *in vivo*. Exendin-4, a long-lasting analog of GLP-1 (Egan et al., 2002; Egan et al., 2003) is a currently available treatment for T2DM. *In vitro*

studies show GLP-1 and its analog exendin 4 are capable of inducing β-cell proliferation in rodent islets and insulinoma cell lines in a non-glucose dependent manner (Perfetti & Hui, 2004; Li et al., 2005b). Administration of exendin-4 for several days results in stimulated expansion of β-cell mass and increased β-cell proliferation in several adult diabetic mouse models in adulthood (Farilla et al., 2002; Wang & Brubaker, 2002). *GLP-1R*$^{-/-}$ mice display normal β-cell mass, suggesting that GLP-1R activation is not essential for basal β-cell proliferation or that loss of GLP-1 is compensated for by other factors (Scrocchi et al., 1996; Ling et al., 2001). However, β-cell regeneration is impaired in GLP-1R null mice after pancreatectomy (De Leon et al., 2003). *In vitro* evidence from INS cells and islets supported that GLP-1 exerts its proliferative function through PI3K-PKB/AKT (Buteau et al., 1999; Wang et al., 2004), PKCζ (Buteau et al., 2001) or canonical Wnt signaling pathways (Liu & Habener, 2008)

Despite their well-established mitogenic role, the application of growth factors to diabetes treatments is halt by numerous concerns, such as sever side-effects and rapid degradation in the body which attenuates the proliferative effects. To obtain more functional β-cells in an efficient and controllable manner, it is crucial to determine the intrinc mechanism by which growth factors direct β-cell cell cycle. Transcription factors governing β-cell proliferation just begun to be understood. The current study focuses on understanding whether and how PL, HGF, and IGF-1 function through Foxm1 to mediate β-cell proliferation. Figure 6 illustrates a simplified summary of currently known signaling pathways involved in β-cell proliferation downstream of these three growth factors.

Forkhead Box m1 (Foxm1)

Forkhead Transcription Factor Family

The forkhead box or Fox gene family of transcription factors is an evolutionarily ancient gene family named after the *Drosophila melanogaster* gene *fork head* (*fkh*). *Fkh* mutants display perturbed head involution in embryogenesis, resulting in a characteristic spiked head in adulthood (Weigel et al., 1989). All Fox proteins are characterized by a 100-amino acid, monomeric DNA-binding domain. This DNA-binding domain folds into a variant of the helix-turn-helix motif and is made of three α-helices and two large loops called “wings”. Therefore, the DNA-binding motif is named the winged helix DNA-binding domain. Other portions of the Fox proteins, for example, the trans-activation or trans-repression domains, are highly divergent. Fox proteins are assigned to individual subclasses based on the phylogenetic analysis (Kaestner et al., 2000). Up to now, more than 80 Fox proteins have been identified with diverse functions in many different cellular processes, such as cell proliferation, apoptosis, differentiation, transformation, longevity, and metabolic homeostasis.

Only a few Fox proteins are implicated in maintenance of normal islet function in adulthood. Both *Foxa1* and *Foxa2* single deletion in mice results in marked hypoglycemia accompanied by abnormal insulin secretion. However, further study from the Kaestner group revealed distinctive function of these two Foxa proteins in controlling glucose homeostasis. Other than its developmental requirement for *Gcg* expression, which protects animals from hypoglycemia, Foxa1 is required for efficient uncoupling of oxidative phosphorylation, allowing the increase of intracelluclar cAMP levels in response to glucose challenge thus efficient insulin secretion (Vatamaniuk et al., 2006). Foxa2 controls vesicle docking and insulin secretion in mature mouse β-cells presumably through its direct targets involved in membrane targeting and fusion (Vatamaniuk et al., 2006; Gao et al., 2007) as well as in K_{ATP} channel activation (Lantz et al., 2004). Foxo1 is perhaps the best studied Fox protein regarding its role in governing whole body energy metabolism. Foxo1 is important for multiple aspects of energy

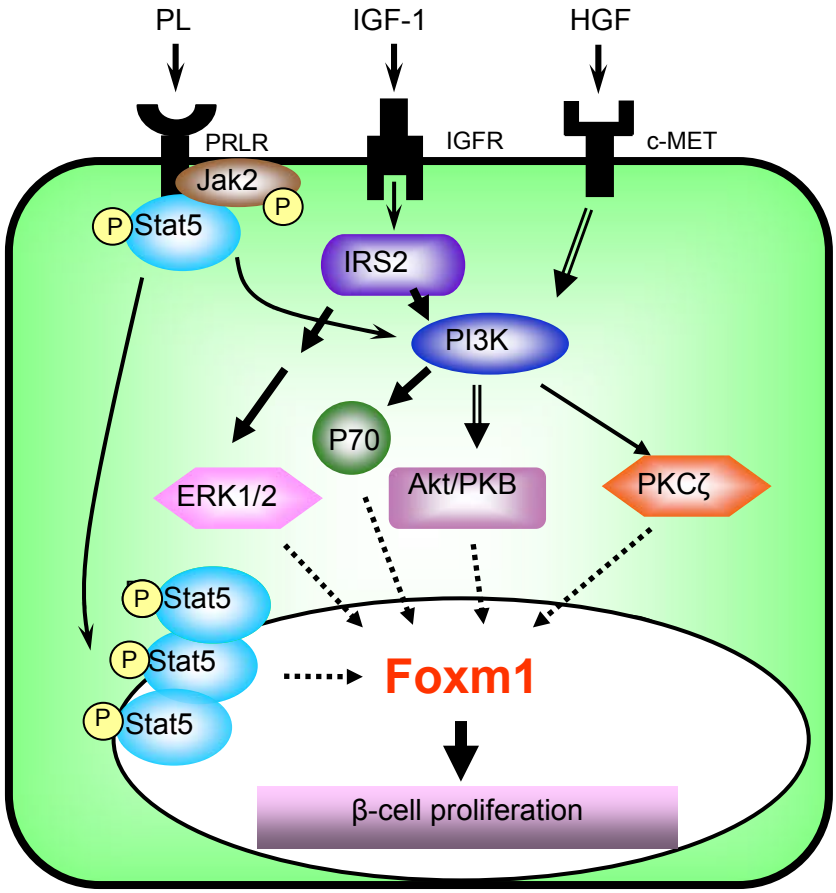


Figure 6. Growth factor-stimulated β-cell proliferation. Foxm1 transcription factor may operate downstream of PL, IGF-1 and/HGF to promote adult β-cell proliferation.

homeostasis, such as promoting adaptation to fasting and loss of muscle mass, altering insulin sensitivity, protecting adipocyte from oxidative stress-induced damages, regulating osteocalcin thus modulating the responsiveness of skeleton muscle to glucose (Gross et al., 2008; Rached et al., 2010). Foxo1 is also a key regulator of β-cell proliferation/mass. Kitamura and colleagues' research supported the idea that IGFs/Insulin signaling regulates β-cell proliferation/mass by relieving *Foxo1's* inhibition of *Pdx1* expression (Kitamura et al., 2002).

In search for new candidates to augment adult β-cell mass, we are determined to identify noval positive regulators of β-cell cell cycle. Before our study, Foxm1 has been recognized as a potent cell cycle accelerator in many cell types. The Costa group demonstrated that Foxm1 is crucial for development and regeneration of liver, an organ sharing close orgins with the pancreas. Therefore, we became interested in characterizing Foxm1 function in the pancreas.

FoxM1 Transcription Factor: Gene and Protein

The Foxm1b protein (or Foxm1 in mice), previously known as Trident (in mouse) (Korver et al., 1997c), WIN (Winged helix from INS-1 cells) (Yao et al., 1997), FKHL16 (Forkhead, homolog-like 16), MPP2 (M-phase phosphoprotein 2) (Westendorf et al., 1994; Matsumoto-Taniura et al., 1996; Luscher-Firzlaff et al., 1999), and HFH-11[HNF-3 (Hepatocyte nuclear factor 3)/forkhead homolog 11] (Ye et al., 1997), is a proliferation-specific member of the Fox family of transcription factors. The Foxm1 gene exhibits 45% identity in the DNA-binding domain with five of its closest related Forkhead members: Foxa3 (HNF-3r), Foxc1 (fkh-2), Foxf2 (FREAC-2), Foxk1 (ILF) and Foxn2 (Korver et al., 1997a). Foxm1 binds DNA *in vitro* through the consensus site TAAACA (Korver et al. 1997b . The human *FOXM1* gene contains 10 exons spanning approximately 25kb. Different splicing of exons Va (A1) and VIIa (A2) gives rise to 3 classes of transcripts,

encoding 3 different protein isoforms: FOXM1A, containing both alternative exons; FOXM1B, containing none of the alternative exons and FOXM1C, containing only exon Va (Figure 7). Only one form of murine Foxm1 protein has been recognized. Murine Foxm1 is most homologous to FOXM1B as these two share 80% secondary structure identity and 82% DNA sequence identity. Both FOXM1B and FOXM1C are transcriptional activators with the same DNA binding specificity (Korver et al. 1997a; Ye et al. 1997). In contrast, FOXM1A, which binds the same DNA consensus, is trancriptionally inactive, due to the insertion of exon VIIa in the C-terminal transactivation domain (Ye et al. 1997). The transcriptional inactivation property of FOXM1A may cause this isoform of FOXM1 to act as a dominant-negative variant, functioning as a regulatory molecule to attenuate overall FOXM1 transactivity under certain circumstances.

In mouse embryos, Foxm1 is expressed in all proliferating tissues (Korver et al., 1997c), whereas expression decreases after differentiation. In the adult, expression is largely restricted to tissues with high rates of cell turnover, such as the testis, thymus, lung, and intestinal crypts (Ye et al., 1997). In addition, Foxm1 is expressed in a wide variety of cell lines and cancer-derived cell lines (Ye et al., 1997; Korver et al., 1997a; Kalin et al., 2006; Liu et al., 2006). Taken together, Foxm1 expression pattern correlates with the proliferative state of a cell.

Transcriptional Regulation of Foxm1

Due to the up-regulation of Foxm1 in numerous tumor-derived cell lines and many types of human cancer, such as lung cancer, breast cancer, hepatocellular carcinoma, glioblastmas, prostate cancer, and pancreatic cancer and its contribution to transformation (Laoukili et al., 2007), most studies on transcriptional regulation of *Foxm1* have been done in the context of human cancer cell lines or tissues. Krover et al. characterized a 2.4 kb fragment of the human FOXM1 promoter, which is responsible for

the serum induced FOXM1 expression. Further analysis revealed that a 0.3kb (-296 to +60 bp) region of the FOXM1 promoter is sufficient for the serum response of the promoter. This region was activated during S-phase and harbors a putative E2F binding site at 550 bp and a Myc E-box at -49 to -44 bp (Korver et al., 1997b). Xia et al. recently reported that hypoxia (1% O2) induces FOXM1 expression through the direct and specific binding of Hypoxia-inducible Factor 1α (HIF-1α) to the HIF-1 binding sites in the FOXM1 promoter by chromatin immunoprecipitation assay and competition assay in human cancer cell lines (Xia et al., 2009a). Moreover, several studies have provided indirect evidence on the signaling pathways governing FOXM1 expression. Teh et al. identified *FOXM1* as a downstream target of Sonic Hedgehog (SHH)/Gli1 signaling and a putative Gli-binding element located at approximately 230bp upstream of *FOXM1* transcriptional start site in basal cell carcinomas (Teh et al., 2002). McGovern et al. reported that Gefitinib (Iressa), a specific and effective epidermal growth factor receptor inhibitor, also a drug for breast cancer treatment, represses *FOXM1* expression via FOXO3α in breast cancer (McGovern et al., 2009). A study from Francis and colleagues suggested that the tyrosine kinase recptor, HER2, up-regulates *FOXM1* at least partially through transcriptional activation in breast cancer (Francis et al., 2009). However, these studies mostly focused on the correlation of *FOXM1* transcription level and the expression/activity of molecule of interest in deletion or overexpression models. None of them identified the direct upstream factors that bound the *FOXM1* promoter region and actived its transcription. The elements within the *FOXM1* promoter region crucial for induction of *FOXM1* transcription in above scenarios also remain unknown. In addition, whether these upstream regulators and binding sites within *FOXM1* promoter are conserved in mouse has not been tested.

In mouse, the only direct evidence uncovering a transcriptional activator of *Foxm1* came from the Trumpp group. They showed that in a liver hyperplasia mouse

model, c-Myc, induced by an agonist of the constitutive androstane receptor (CAR), promotes hepatocyte proliferation through its direct binding to a conserved E-box in the *Foxm1* promoter region thus activating *Foxm1* transcription (Blanco-Bose et al., 2008). In addition, Krupczak-Hollis et al. reported that human growth hormone (GH) administration to older mice (12 month) dramatically increased both expression of *Foxm1* and regenerating hepatocyte proliferation, indicating *Foxm1* as a transcriptional target of GH. However, the molecular linkage remains unclear. The regulation of *Foxm1* by CAR through c-Myc has yet to be assessed in humans.

In conclusion, emerging evidence linkes *Foxm1* to multiple signaling pathways governing cell proliferation. However, most current studies did not provide in-depth direct evidence to build the linkage between the signaling pathways of interest and *Foxm1* transcriptional induction. Numerous putative transcription factor binding sites can be fouond in *Foxm1* 5' promoter region through online transcription factor database. However, as stated earlier, only a few have been validated by *in vivo* or *in vitro* experiments. A systematic characterization of murine *Foxm1* promoter will serve as a great entry point toward a better understanding of the transcriptional regulation of *Foxm1.* Given the more convenient genetic manipulation in mice and high similarity of murine *Foxm1* to human FOXM1B, our knowledge of the *Foxm1* transcriptional regulation in mouse could be easily extended to human studies.

Post-translational Regulation of FoxM1

Foxm1 protein contains many phosphorylation sites, primarily within the C-terminal transactivation domain (Figure 8). During the cell cycle, in U2OS cells (a human osteosarcoma cell line), Foxm1 binds cyclin/Cdk complexes through an LXL docking sequence in the C-terminal region of Foxm1 protein (Major et al., 2004), allowing

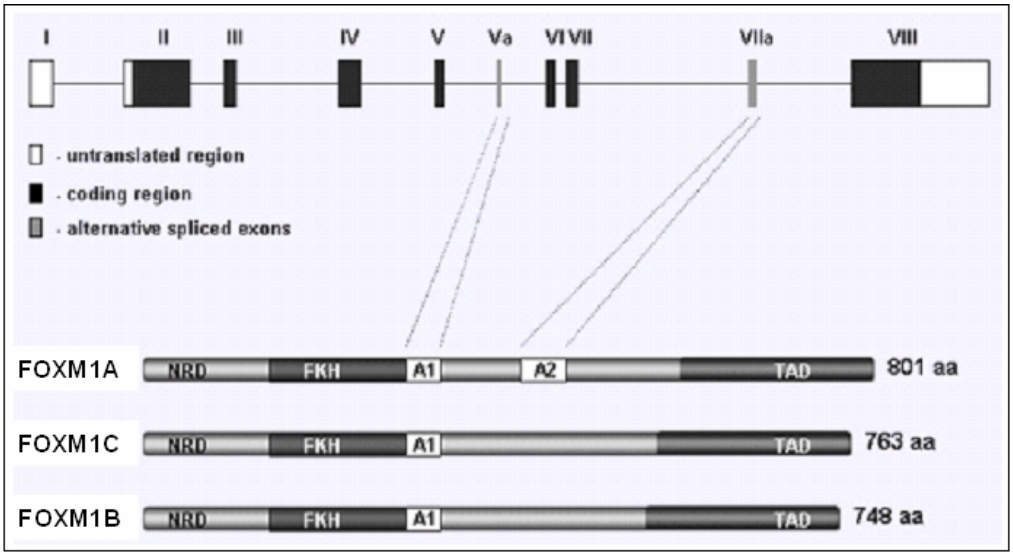


Figure 7. Splicing variants of human FOXM1 gene and structure of protein isoforms. NRD: N-terminal-repressor domain. FKH: forkhead DNA binding domain. TAD: Transcriptional activation domain. Murine FoxM1 protein is most similar to human FOXM1B.

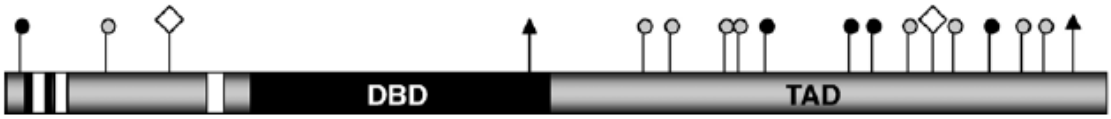


Figure 8. Post-translational modification of human FOXM1. FOXM1 comprises a conserved "Forkhead box" DNA binding domain (DBD) in its N-terminal region, and a transactivation domain (TAD) within its C-terminal region. Based on the described consensus sequences, two potential RXL cyclin binding motifs (dark boxes) are present in the N-terminal region of FOXM1. Similarly, several conserved putative phosphorylation sites for cyclin/Cdk2 complexes (S/T-P-X-K/R consensus, black-rounded pins), for other cyclin/Cdk complexes (S/T-P sites, grey-rounded pins), for Plk1 mitotic kinase (D/E-X-S/T-F consensus, squared pins), and for MAPK (P-X-S/T-P consensus, triangular pins) can be identified. Finally, putative proteasome-dependent protein degradation boxes are present in the N-terminus (white boxes), namely two D-boxes (consensus RXXL), and a more C-terminal KEN box (Laoukili et al., 2007, used with permission)

efficient phosphorylaton of Foxm1 by cyclin/Cdk (Major et al., 2004). This cyclin/Cdk dependent phosphorylation is particularly important for Foxm1 activation as it serves to recruit a transcriptional co-activator, the histone deacetylase p300/CREB (cAMP response element-binding) binding protein (p300/CBP) (Major et al., 2004). Foxm1 displayed increased association with cyclin E/Cdk2 complexes in G1 and S phases, whereas Foxm1 preferentially binds the cylcin B/Cdk1 in G2 phase (Major et al., 2004).These stage-dependent interactions may function to maintain proper oscillation Foxm1 activity which is critical for the progression from G1 through G2 phases. In addition, Foxm1 also interact with other players in the cell cycle, pRb and Cdc25B in G1 and in G1/S, respectively (Major et al., 2004). This association may indirectly influence Foxm1 transcriptional activity via affecting Cdk activity.

Putative consensus phosphorylation sites predicted that kinases such as MAPK and ERK1/2 may be involved in phosphorylation of Foxm1. Indeed, Raf/MEK/MAPK signaling stimulates nuclear translocation of FOXM1B through phosphorylation in late S-phase (Ma et al., 2005). An ERK1/2 serine phosphorylation site is located within the C-terminus of FOXM1 near a cyclin/Cdk complex interaction domain, and mutation of this site impairs FOXM1 activity (Ma et al., 2005). In addition, indirect evidence from the Costa group supported that GH may regulate Foxm1 at the post-translational level. Administration of GH to mice increases nuclear levels of Foxm1 protein in regenerating hepatocyte, suggesting that GH signaling promotes nuclear localization of Foxm1, presumably through altering its phosphorylation status.

Post-translational modifications can also inhibit Foxm1 transcriptional activity. The Cdk inhibitor p27 can be found in the Cdk/cyclin/Foxm1 complex. Such interaction might prevent phosphorylation of the C-terminal transactivation domain of Foxm1 thus repressing Foxm1 activity. p19/ARF, through its residues 26 to 44, interacts with the Foxm1 C-terminal transactivation domain, to inhibit its transcriptional activity

(Kalinichenko et al., 2004). Indeed, this peptide alone can inhibit Foxm1 activity *in vitro* and *in vivo*, through targeting Foxm1 to the nucleolus, presumably precluding the interaction of Foxm1 with its target genes (Gusarova et al., 2007).

Similar to Foxm1, Fox proteins such as Foxo1 and Foxa2 are also tightly controlled by phosphorylation. However, for both proteins, phosphorylation leads to nuclear export thus inhibited protein activity (Kitamura et al., 2002). Both Foxo1 and Foxa2 are regulated by Akt through phosphorylation, therefore, it is possible that Akt also modulates Foxm1 activity.

Another negative regulatory pathway for Foxm1 is proteosome-mediated degradation. During late mitosis and early G1, Foxm1 is found in a complex with the Anaphase-promoting complex/cyclosome (APC/C) E3 ubiquitin ligase and its adaptor, Cdc20 homolog1(Cdh1). Two “destruction” D-boxes and a KEN box, interacting with Cdh1, are required for Foxm1 degradation. This negative regulation of Foxm1 is necessary for prevention of re-entry into S-phase (Park et al., 2008a).

In conclusion, the transcriptional activity of Foxm1 is tightly controlled throughout the cell cycle via protein-protein interaction and subsequent phosphorylation by mitogenic kinases and cyclin/Cdk complexes. However, most of the observations summarized above were from *in vitro* studies. *In vivo*, the Foxm1 phosphorylation pattern may be very different. In particular, to our interest, the post-translational modification of Foxm1 has not been studied in the context of pancreas. In the future, *in vivo* characterization of the physiological or pathological relevant Foxm1 phosphorylation sites in the β-cell by mass spectrometry and site-mutagenesis would provide more insights into the endeavor of utilizing Foxm1 as a therapeutic target for enhancing β-cell mass.

Cell Proliferative Role of Foxm1: Lessons Learn from Foxm1 Mouse Models

To understand the role of *Foxm1*, mice with global *Foxm1* mutation were derived by two groups (Korver et al., 1998; Krupczak-Hollis et al., 2004). Korver et al. inserted a *Phosphoglycerate kinase* (*PGK*)-*Neomycin resistance* (*Neo*) cassette into the fourth exon of *Foxm1* (*Foxm1*^{Neo}), thus disrupting the sequence encoding the DNA binding domain. This *Foxm1* mutantation is postnatally lethal, probably due to circulatory failure in mutant animals. These mice displayed defective myocardium development and nuclear polyploidy in both cardiomyocytes and hepatocytes, suggesting a failure to enter mitosis (Korver et al., 1998). It was later discovered that the *Foxm1*^{Neo} allele was in fact a hypomorph instead of a true null allele. The Costa group generated mice with global *Foxm1* inactivation by using Cre recombinase (EIIA-Cre) to remove exons 4-7 encoding the winged helix DNA binding domain and the C-terminal transcriptional activation domain. Dying in utero by 18.5 dpc, these mutant mice displayed abnormal liver development (absence of intrahepatic bile ducts and reduced number of large hepatic veins) presumably due to a 75% reduction in the number of hepatoblasts, resulting from impaired DNA replication and DNA polyploidy (Krupczak-Hollis et al., 2004). The defects in hepatoblasts are partially explained by diminished protein levels of Polo-like kinase 1 and Aurora B kinase. These results suggested that FoxM1 is critical for the proliferation and differentiation of hepatoblasts. This conclusion further supported by the finding that hepatocyte-specific deletion of FoxM1 using Alpha-fetoprotein (AFP)-Cre results in the same phenotype (Krupczak-Hollis et al., 2004).

Foxm1 is also important for proliferation of adult hepatocytes, suggested by *Foxm1*$^{fl/fl}$*; Albumin-Cre* mice in which *Foxm1* is specifically inactivated after birth (Postic & Magnuson, 2000) in hepatocytes (Wang et al., 2002b). These mice develop normally but presented a significant decrease in liver regeneration due to reduced hepatocyte proliferation after partial hepatectomy (PHx). The blunted hepatocytes proliferation was

associated with increased nuclear p27 levels, decreased expression of Cdc25A, Cdc25B and cyclin B, and reduced phosphorylation of Rb protein.

Since *Foxm1* is a crucial cell cycle regulator (Figure 5) involved in G1/S and G2/M transitions as well as regulating cytokinesis in the liver (Wang et al., 2002b; Krupczak-Hollis et al., 2004). Ye et al. generated transgenic mice overexpressing FoxM1 in order to promote liver regeneration. Indeed, liver-specific overexpression of FOXM1B accelerated hepatocyte proliferation after two-thirds PHx but not under normal conditions (Ye et al., 1999). In addition, Wang et al. reported that overexpression of FOXM1B either by the -3kb transthyretin (TTR) promoter or adenoviral infection, reversed the age-dependent decrease in hepatocyte proliferation in old mice (12 month old) to a level similar to that in young animals (2-month old) (Wang et al., 2002b).

The Role of Foxm1 in the Pancreas

Our laboratory first characterized Foxm1 pancreatic function. We showed that *Foxm1* is highly expressed in embryonic endocrine cells and its expression declines but is still maintained in subpopulations of adult endocrine cells (Figure 9A-C) (Zhang et al., 2006b). To study the pancreatic function of Foxm1, we derived a mouse model with pancreas-wide inactivation of two floxed *Foxm1* alleles using *Pdx1-Cre* (*Foxm1*$^{\Delta panc}$). The *Foxm1*$^{\Delta panc}$ mice displayed a defect in postnatal β-cell mass expansion and a gradual decline in β-cell mass with age due to decreased β-cell proliferation with unchanged apoptosis (Figure 9D and E). In male mice, these defects resulted in impaired islet function by 6 weeks of age and overt diabetes by 9 weeks (Zhang et al., 2006b). These results demonstrated that *Foxm1* is required for expansion and maintenance of β-cell mass in adulthood. During embryogenesis, Foxm1 is not required for gross pancreas development (Zhang et al., 2006b) or β-cell proliferation (Figure 10) (Ackermann Misfeldt et al., 2008). Recently, our laboratory reported that Foxm1 also

played a vital role in the regenerative proliferation of β-cell in 60% partial pancreatectomy in mice (Ackermann Misfeldt et al., 2008). This study demonstrated that not only the basal maintenance but also the facultated β-cell growth requires Foxm1, This is particularly insightful since Foxm1 is potentially useful for restoring the replicating capacity of adult β-cell under stimulatory conditions in humans with metabolic disorders.

Foxm1 Target Genes

Many genes have been described as Foxm1 targets, among which a total of 28 are direct target genes of Foxm1 by ChIP assays and/or electrophoretic mobility shift assays (EMSAs) (Table 5). Foxm1 target genes are involved in multiple aspects of cell cycle progression such as control of the G1/S transition, S-phase progression, G2/M transition, M-phase progression, DNA repair, execution of mitosis and chromatin assembly. Many of Foxm1 targets are also expressed in the β-cell (Figure 5). Regulation of these genes is consistent with the established role of FoxM1 in stimulation of cell proliferation. Foxm1 positively regulates genes that promote cell cycle progress such as *cyclin B1, Cdc25A, Cdc25B phosphatases* (Wang et al., 2002b), *Aurora B, Survivin, Polo-like kinases* (Krupczak-Hollis et al., 2004; Kim et al., 2005), *Cenp-A and Cenp-B* (Wang et al., 2005; Wonsey & Follettie, 2005). In addition, Foxm1 prevents nuclear accumulation of the CDKIs, p27 and p21 by directly activating transcription of the Skp1-Cullin1-F-box (SCF) ubiquitin ligase complex that targets p27/p21 for degradation (Wang et al., 2002c; Krupczak-Hollis et al., 2003; Wang et al., 2005) or by enhancing the phosphorylation of p27 thus its nuclear export via directly stimulating expression of *Kinase-interacting stathmin (KIS)* (Petrovic et al., 2008).

Figure 11 is a schematic summary of Foxm1 targets in cell cycle regulation. Foxm1 also regulates extracellular matrix proteins, as well as genes that function in

angiogenesis/vasculogenesis, invasion, differentiation and signal transductions (Table 5). These target genes explain the role of Foxm1 in a variety of phases of carcinogenesis and also point to Foxm1 as a promising target for the prevention and/or treatment of multiple types of human cancer.

Overview and Aims of Dissertation

Previous studies in our laboratory have demonstrated that Foxm1 is highly expressed within the pancreatic endocrine compartment. Foxm1 is crucial for not only normal (Zhang et al., 2006b) but also facultative growth of β-cell mass via promotion of postnatal β-cell proliferation in a β-cell regeneration model, 60% partial pancreatectomy (Ackermann Misfeldt et al., 2008). Pancreas-wide inactivation of Foxm1 results in diabetes due to a failure in postnatal β-cell mass expansion (Zhang et al., 2006b). These findings, for the first time, suggested Foxm1 as a candidate target for *in vitro* and/or *in vivo* β-cell mass expansion, a promising avenue to cure diabetes. However, whether Foxm1 is generally required for β-cell proliferation/mass expansion in other metabolically challenging conditions, such as pregnancy, obesity or in response to external proliferative stimuli, has not been evaluated. In addition, the upstream signaling events that direct Foxm1 to initiate β-cell cell cycle machinery remain unclear.

Growth factors, such as PL, HGF and IGF-1, are known potent β-cell proliferation stimulators both *in vitro* and *in vivo* (Vasavada et al., 2006). Although emerging evidence has begun to uncover the signaling pathways downstream of growth factors, we are still limited in our knowledge of the intrinsic factors, especially transcription factors within a β-cell to mediate mitogenic effects. In the liver, GH-stimulated hepatocyte proliferation is mediated by Foxm1 induction and nuclear translocation (Krupczak-Hollis et al., 2003). Therefore, it is possible that Foxm1 may operate downstream of other growth factors to provoke β-cell proliferation in the pancreas, an organ very similar to liver in origin. With

the long term goal to investigate the potential of Foxm1 as a target for diabetes treatment, we hypothesize that **Foxm1 is the downstream effector of one or multiple growth factor-stimulated β-cell proliferation.** This dissertation describes our effort to test this hypothesis in four chapters: Chapter II introduces the methods used. Chapter III presents the data to answer the question of whether and how Foxm1 stimulates β-cell replication downstream of PL signaling both *in vitro* and *in vivo*. Chapter IV describes results regarding the role of Foxm1 in IGF-I/HGF stimulated β-cell proliferation. Chapter V presents our progress in generating a specific anti-mouse Foxm1 antibody. Conclusions, implications and future directions for this dissertation are presented in Chapter VI.

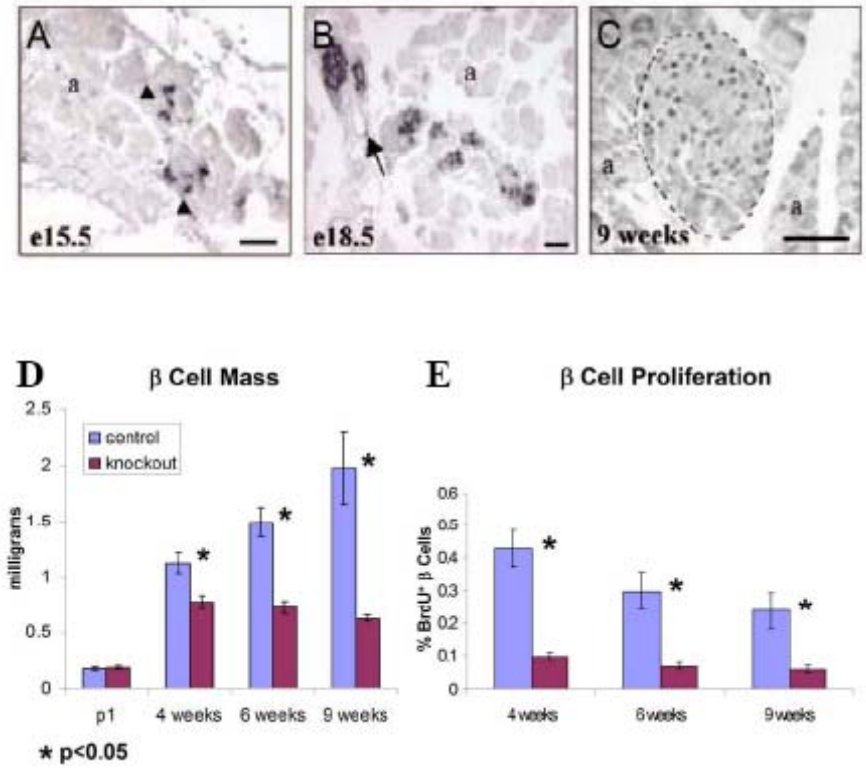


Figure 9. Foxm1 is highly expressed within the endocrine pancreas and is required for normal postnatal β-cell proliferation and growth. Foxm1 protein is highly expressed within the endocrine cords (arrowheads) at e15.5 **(A)** and within the developing islets at e18.5 **(B)**. At these time points, Foxm1 is expressed to a lesser degree in acinar (a) and ductal (arrow) tissue. At 9 weeks of age **(C)**, Foxm1 is expressed at a low level in a subset of islet (outlined) cells, and to an even lesser extent in exocrine cells. Pancreas-wide deletion of *Foxm1* in *Foxm1*$^{flox/flox}$;*Pdx1*$^{5.5kb}$*-Cre* mice resulted in reduced growth of postnatal β-cell mass **(D)** and reduced β-cell proliferation **(E)** as early as 4 weeks of age. Pancreas and β-cell mass development during embryogenesis were not impaired. [Adapted from (Zhang et al., 2006b), used with permission]

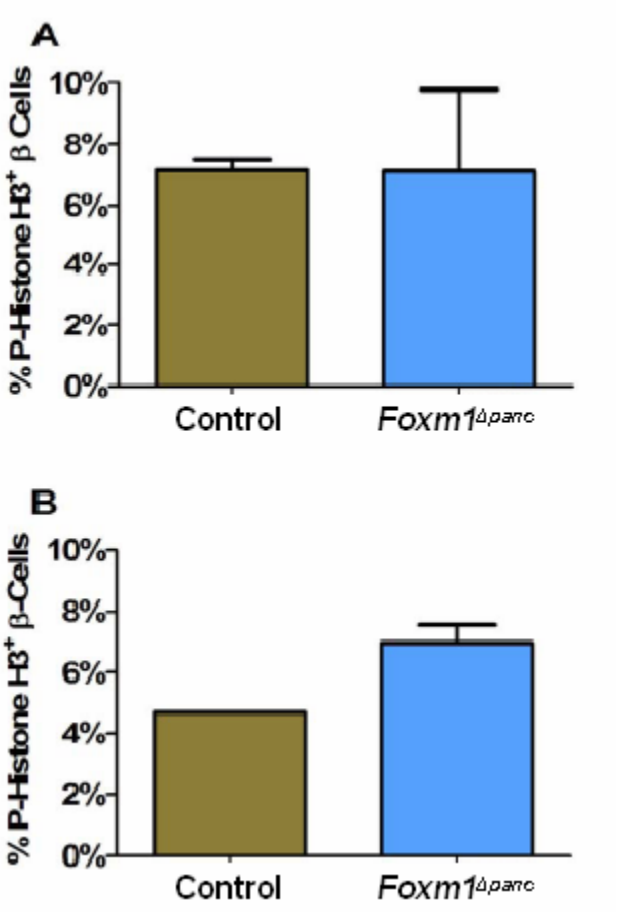


Figure 10. β-cell proliferation was unaffected in *Foxm1*$^{\Delta panc}$ embryos versus control littermates. Phospho-histone H3 labeling at e17.5 **(A)** and e18.5 **(B)** was used to measure β-cell proliferation. Error bars represent SEM. Unpaired t-test was used to measure significance. n=2-3 per group. (Ackermann Misfeldt et al., 2008, used with permission)

Table 5. Direct Foxm1 target genes and Foxm1–regulated genes. [Adapted from (Wierstra & Alves, 2007), used with permission]

Gene	FoxM1 binding to promoter		Regulation of endogenous gene expression		Regulation of promoter		References
	Method	Comment	Expression	Manipulation of FoxM1	Manipulation of binding site	Manipulation of FoxM1	
Cell cycle regulation							
cyclin D1	C		T, P	OE, siRNA, tg	wt	OE	(Ye et al., 1999; Wang et al., 2001a; Wang et al., 2001b; Liu et al., 2006; Tan et al., 2006; Zhao et al., 2006))
cyclin D2			T	siRNA			(Zhao et al., 2006)
cyclin E			T	tg			(Wang et al., 2001a; Kalinichenko et al., 2003)
cyclin A2			T, P	OE, siRNA, tg, KO			(Wang et al., 2001a; Wang et al., 2001b; Wang et al., 2002b; Kalinichenko et al., 2003; Krupczak-Hollis et al., 2004; Kim et al., 2005; Wonsey & Follettie, 2005; Kalin et al., 2006; Kim et al., 2006; Zhao et al., 2006; Yoshida et al., 2007)
cyclin B1	C		T, P	OE, siRNA, tg, KO	Wt, del	OE, del, P	(Ye et al., 1999; Leung et al., 2001; Wang et al., 2001a; Wang et al., 2001b; Wang et al., 2002b; Kalinichenko et al., 2003; Krupczak-Hollis et al., 2004; Kim et al., 2005; Laoukili et al., 2005; Ma et al., 2005; Wang et al., 2005; Kalin et al., 2006; Kim et al., 2006; Zhao et al., 2006; Yoshida et al., 2007)
cyclin B2			T, P	OE, tg, KO			(Ye et al., 1999; Wang et al., 2001b; Wang et al., 2002b; Laoukili et al., 2005)
cyclin F			T	siRNA, tg			(Wang et al., 2001a; Wang et al., 2001b; Kalinichenko et al., 2003; Zhao et al., 2006)
Cdk1			T, P	OE, siRNA, tg, KO			(Ye et al., 1999; Wang et al., 2002b; Kalinichenko et al., 2003; Krupczak-Hollis et al., 2004; Zhao et al., 2006)
Cdc25A			T, P	siRNA, tg, KO			(Wang et al., 2001a; Wang et al., 2002b; Wang et al., 2005)
Cdc25B	C		T, P	OE, siRNA, tg, KO,CI	wt	OE, del	(Wang et al., 2001a; Wang et al., 2001b; Wang et al., 2002a; Wang et al., 2002b; Kalinichenko et al., 2004; Krupczak-Hollis et al., 2004; Wang et al., 2005; Wonsey & Follettie, 2005; Madureira et al., 2006; Radhakrishnan et al., 2006; Tan et al., 2006; Ramakrishna et al., 2007; Tan et al., 2007)
Cdc25C			T, P	OE, siRNA, KO			(Kim et al., 2005; Madureira et al., 2006; Zhao et al., 2006)
Plk1			T, P	OE, siRNA, KO	wt	OE, del, KO	(Krupczak-Hollis et al., 2004; Kim et al., 2005; Laoukili et al., 2005; Wang et al., 2005; Madureira et al., 2006; Gusarova et al., 2007)
Aurora B	C		T, P	OE, siRNA, KO	wt	OE	(Krupczak-Hollis et al., 2004; Kim et al., 2005; Wang et al., 2005; Gusarova et al., 2007)
Survivin (Birc5)	C		T, P	siRNA, tg, KO, CI			(Wang et al., 2005; Radhakrishnan et al., 2006; Gusarova et al., 2007; Yoshida et al., 2007)
CENP-A	C		T, P	OE, siRNA, KO			(Wang et al., 2005; Wonsey & Follettie, 2005)
CENP-B	C		T	siRNA, CI			(Wang et al., 2005; Radhakrishnan et al., 2006)
CENP-F	C		T, P	OE, siRNA, KO	wt	KO	(Laoukili et al., 2005)
Nek2			T	OE, siRNA, KO			(Laoukili et al., 2005; Wonsey & Follettie, 2005)
ARF (p14, p19)			T	KO			(Wang et al., 2005)
p21			T, P	siRNA, tg, KO, CI			(Wang et al., 2001a; Wang et al., 2002b; Kalinichenko et al., 2003; Wang et al., 2005; Kalin et al., 2006; Kim et al., 2006; Radhakrishnan et al., 2006; Ramakrishna et al., 2007; Tan et al., 2007)
p27			P	OE, siRNA, KO			(Wang et al., 2002a; Kalinichenko et al., 2004; Wang et al., 2005; Kalin et al., 2006; Kim et al., 2006; Liu et al., 2006; Zhao et al., 2006)
Skp2	C		T, P	OE, siRNA, KO			(Wang et al., 2005; Liu et al., 2006)
Cks1	C		T, P	siRNA, KO			(Wang et al., 2005)
Gas-1			T, P	OE, KO			(Laoukili et al., 2005)
KIS	C		T, P	siRNA, KO	wt, P	KO	(Petrovic et al., 2008)

Table 5. (Continuted)

Gene	FoxM1 binding to promoter		Regulation of endogenous gene expression		Regulation of promoter		References
	Method	Comment	Expression	Manipulation of FoxM1	Manipulation of binding site	Manipulation of FoxM1	
XRCC1	C		T, P	OE, siRNA, tg, KO	wt	OE	(Ye et al., 1999; Tan et al., 2007)
BRCA2	C		T, P	OE, siRNA, KO	wt	OE	(Tan et al., 2007)
JNK1			T	siRNA, KO			(Wang et al., 2008a; Ustiyan et al., 2009)
TOPO-2α	C		T,P	siRNA, KO	wt	OE	(Wang, Meliton et al., 2009a)
Transcritpion factors							
c-Myc	E, C	IV, S, C	T	tg	wt, del ,P, H	OE, dn, del	(Ye et al., 1999; Wang et al., 2001a; Tan et al., 2007)
TCF-4			T	tg, KO			(Yoshida et al., 2007)
NFATc3	C		T	siRNA, KO			(Ramakrishna et al., 2007)
Hnf-6	C				wt, del	OE	(Tan et al., 2006)
Foxf1			T	KO			(Kim et al., 2005)
Cdx2	E	IV, C			H	OE, del	(Ye et al., 1997; Kalinichenko et al., 2004; Major et al., 2004; Tan et al., 2006)
Signal transduction							
ERα	C, D	C, P	T, P	OE, siRNA	wt, del, P	OE	(Madureira et al., 2006)
TGF-α	C				wt	OE	(Tan et al., 2006)
Flk-1			T	KO			(Kim et al., 2005)
VEGF	E	IV	T, P	OE, siRNA	wt, P	OE, siRNA	(Zhang et al., 2008)
Extracellular matrix remodeling							
Pecam1			T	KO			(Kim et al., 2005)
MMP-2	E, C	S, C	T	OE, siRNA	wt, P	OE, siRNA	(Dai et al., 2007)
Lama2			T	KO			(Kim et al., 2005)
Lama4	E	S, C	T	KO	wt	OE	(Kim et al., 2005)
Procollagen type XII α1			T	KO			(Kim et al., 2005)
Integrin β1			T	tg, KO			(Wang et al., 2001a; Kim et al., 2005)
E-cadherin	E	IV, C	T	tg			(Ye et al., 1997; Wang et al., 2001a)
Cox-2	C		T, P	tg, siRNA		OE, siRNA, del	(Wang et al., 2008b)
Differentiation							
transferrin					wt	dn, as	(Chaudhary et al., 2000)
Fabpi	E	IV, C					(Ye et al., 1997)
Sftpa	C		T	KO	wt	siRNA, OE	(Kalin et al., 2008)
Sftpb	C		T	KO	wt	siRNA, OE	(Kalin et al., 2008)

Method: E, EMSA (electrophoretic mobility shift assay); C, ChIP (Chromatin Immunoprecipitation assay with anti-Foxm1 antibody).
Comment: IV, in vitro (purified FoxM1); S, supershift experiments with anti-Foxm1 antibody; C, competition experiments; P, Foxm1 binding site was point-mutated.
Expression: T, transcript (endogenous mRNA level affected); P, protein (endogenous protein level affected).
Manipulation of Foxm1: OE, overexpression of Foxm1; dn, dominant-negative form of Foxm1; del, analyzed with deletion mutants of Foxm1; P, analyzed with point mutations of Foxm1; siRNA, siRNA-mediated knockdown of Foxm1; as, addition of antisense oligonucleotide to Foxm1; tg, *Foxm1* transgenic cells/mice; KO, *Foxm1* knockout cells/mice; CI, chemical inhibitor of Foxm1 (Siomycin A).
Manipulation of Foxm1 binding site: wt, "wild-type" promoter; del, analyzed with deletion mutants of promoter; P, Foxm1 binding sites was point-mutated; H, Foxm1 binding site in context of heterologous core promoter.
Abbreviations:
Cdk1:Cyclin-dependent kinase 1
Cdc25A: Cell division cycle 25 homolog A
Cdc25B: Cell division cycle 25 homolog B
Cdc25C: Cell division cycle 25 homolog C
Plk1:Polo-like kinase 1
CENP-A: Centromere protein A
CENP-B: Centromere protein B
CENP-F: Centromere protein F
Nek2: NIMA (never in mitosis gene a)-related expressed kinase 2
Skp2: S-phase kinase-associated protein 2 (p45)
Cks1: CDC28 protein kinase 1b
Gas-1: Growth arrest-specific gene-1
KIS: Kinase-interacting stathmin
BRCA2: Breast cancer-associated gene 2
XRCC1: X-ray cross-complementing group 1
TOPO-2α: Topoisomerase-2α
TCF-4: T-cell factor 4
NFATc3 : Nuclear factors of activated T-cell (NFAT)
Hnf6: Hepatic nuclear factor 6
Foxf1: Forkhead box f1
Cdx2: Caudal type homeobox 2
ERα: Estrogen receptor α
TGF-α:Transforming growth factor α
Flk1: VEGF receptor type II
VEGF: Vascular endothelial growth factor
JNK1: c-Jun N-terminal kinase
Pecam1: Platelet endothelial cell adhesion molecule 1
MMP-2: Matrix metalloproteinase (MMP)-2
Lama2, Laminin 2
Lama4, Laminin 4
Cox2 : Cyclooxygenase2
Fabpi: Fatty acid binding protein
Sftpa: Surfactant protein a
Sftpb: Surfactant protein b

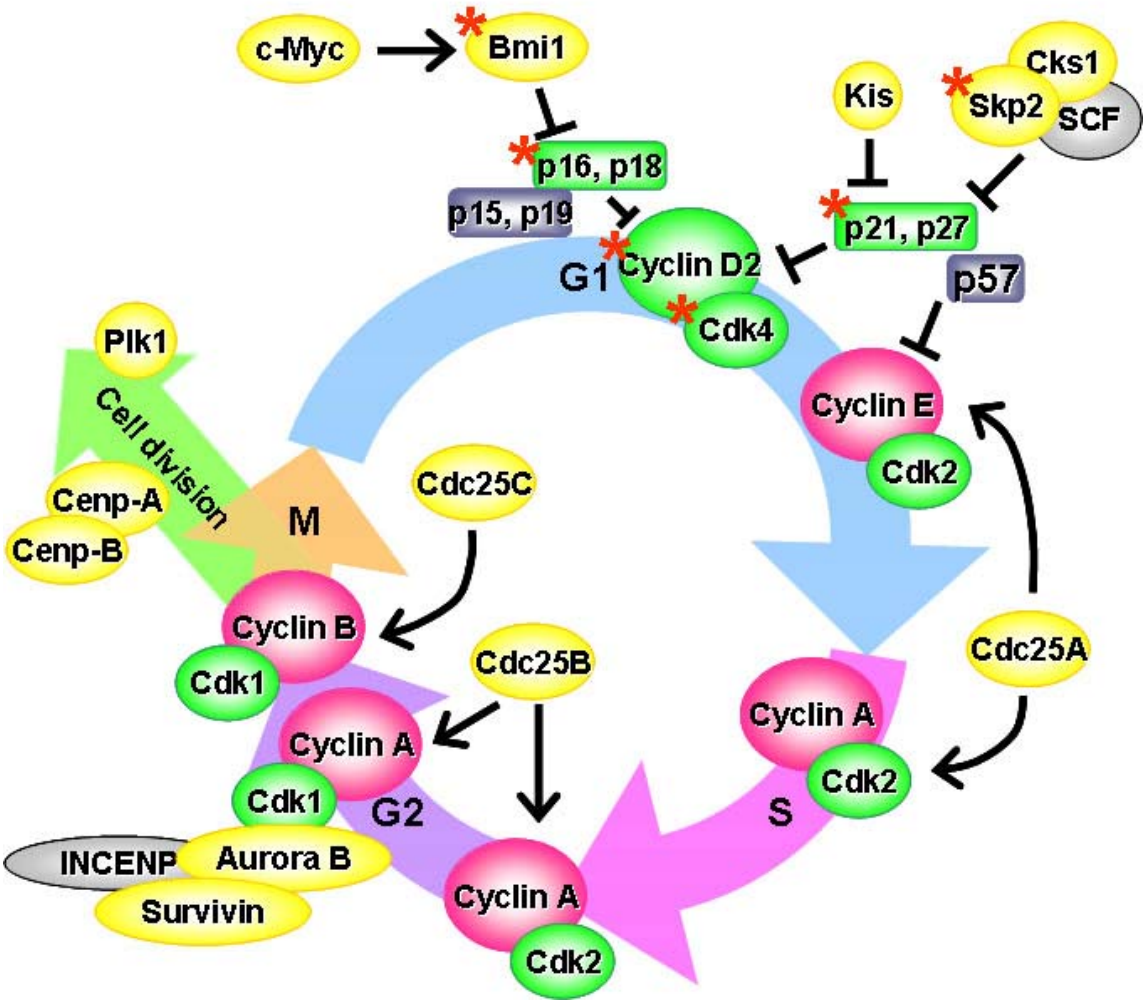


Figure 11. Foxm1 directly regulates many targets (yellow ovals) involved in multiple stages of cell cycle regulation. Proteins important for β-cell proliferation are designated with a red asterisk (*). Figure is adapted from Wang *et al.* 2005., used with permission.

CHAPTER II

MATERIALS AND METHODS

Mice

Foxm1$^{fl/fl}$

Foxm1$^{fl/fl}$ mice on a mixed genetic background (129SvJ, C57BL/6) were generously provided by Dr. Robert H. Costa (University of Illinois at Chicago) and have been characterized previously (Wang et al., 2002b). Briefly, a triple-LoxP *Foxm1b*-targeting vector was constructed to generate mice carrying a floxed *Foxm1b*-targeted locus consisting of a LoxP site inserted into the third *Foxm1b* intron prior to the winged helix DNA-binding domain and LoxP site flanking the PGK-1 neomycin (neo) selection cassette placed 3′ to the final *Foxm1b* exon 7 (Figure 12). Upon recombination mediated by Cre recombinase, exons 4-7 encoding the DNA binding and transcription activation domains will be deleted, yielding a *Foxm1* null allele since the *Foxm1* heterozygotes are normal. For some experiments, *Foxm1^{fl}* mice were backcrossed for 8 generations to the C57BL/6JBom strain (Taconic).

Pdx1$^{5.5Kb}$-Cre

Pdx1$^{5.5Kb}$-Cre mice (on a mixed ICR, CBA, C57BL/6 background) were generously provided by Dr. Guoqiang Gu (Vanderbilt University) (Gu et al., 2002). In these transgenic mice, Cre recombinase expression is driven by a 5.5 kb region of the *Pdx1* promoter (between the S*al* I and S*ma*I restriction sites). This region is sufficient to direct *Cre* expression throughout the endogenous *Pdx1* domain, including the antral stomach, rostral duodenum, and the entire pancreatic epithelium, as early as 10.5 dpc

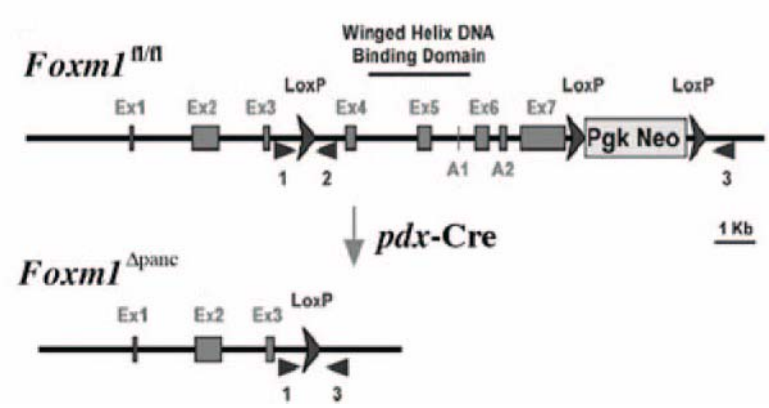


Figure 12. Schematic of *Foxm1*^{fl} gene targeting and generation of *Foxm1*$^{\Delta panc}$ (Zhang et al., 2006b used with permission)

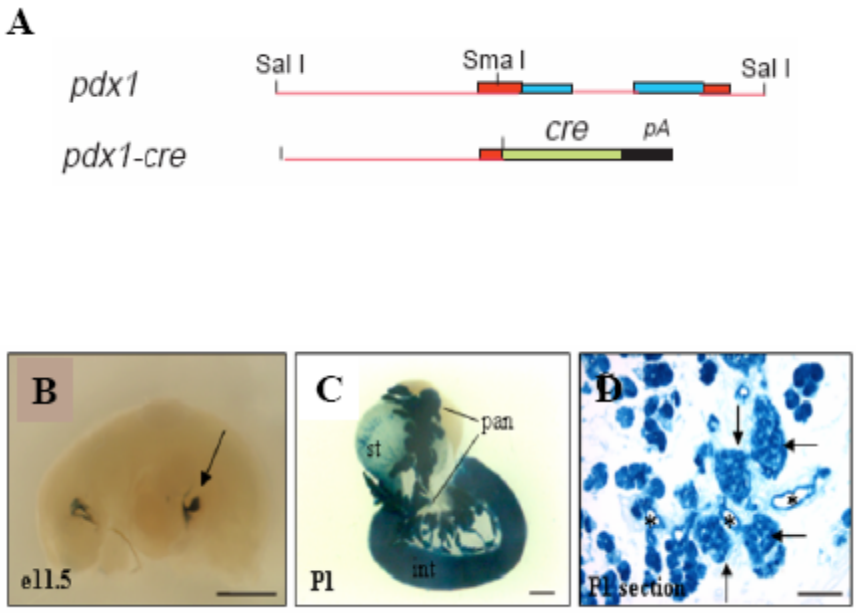


Figure 13. *Pdx1*$^{5.5kb}$*-Cre (Pdx1-Cre)* transgene. *Pdx1-Cre* mice carry a transgene consisting of a 5.5 kb region of the *Pdx1* promoter, from the *Sal*I restriction site to the *Sma*I restriction site, placed upstream of the *Cre* coding sequence. **(A)** X-gal staining reveals that the *Pdx1*$^{5.5kb}$*-Cre* transgene mediates recombination of the *Rosa26* reporter allele throughout the entire pancreatic epithelium as early as 11.5 dpc **(B)** (arrow), as well as in the remainder of the endogenous *Pdx1* expression domain, including the antral stomach and rostral duodenum **(C)** (pan = pancreas, st = stomach, int = intestine). All endodermally-derived cells within the pancreas underwent recombination **(D)** (arrows = islets, asterisks = ducts). Part A of the figure is from (Gu et al., 2002) and parts B-D of the figure are from (Zhang et al., 2006b), used with permission.

(Figure 13) (Stoffers et al., 1999; Gannon et al., 2001). From now on, *Pdx1*$^{5.5KB}$*-Cre* is referred to as *Pdx1-Cre* for short. For some experiments, *Pdx1-Cre* mice were backcrossed for 6 generations to the C57BL/6J strain (The Jackson Laboratory).

RIP-PL

RIP-PL mice (CD-1 background) were kindly provided by Dr. Rupangi C. Vasavada at the University of Pittsburgh (Vasavada, Garcia-Ocaña et al., 2000). The RIP-PL transgene consists of a 650 bp segment of the rat insulin II gene promoter, driving transcription of the 800 bp mouse placental lactogen I (mPL-I) cDNA, downstream of which lie untranslated human growth hormone sequences providing transcriptional termination, polyadenylation, and splicing signal (Vasavada et al., 1996). This transgenic line exclusively expresses mPL-I in the pancreas as revealed by immunohistochemisty (Figure 14). In addition, no mPL-1 was detected in the plasma of either normal or transgenic mice using an assay with detection limit in plasma of 4.5 ng/ml. In contrast, the plasma level of PL in GD10-12 female mice ranges from 30 to 3,600 ng/ml (Ogren et al., 1989).

RIP-HGF

RIP-HGF mice were generously provided by Dr. Adolfo Garcia-Ocaña at the University of Pittsburgh (Vasavada, Garcia-Ocaña et al., 2000). RIP-HGF mice were generated as described previously for the RIP-PL transgenic mice. Briefly, the RIP-HGF transgene was constructed by placing a 2.2 kb cDNA fragment containing the entire coding region of murine HGF downstream of the 650 bp segment of the rat insulin II gene promoter. HGF is highly expressed in the islets from transgenics compared to controls as revealed by RNase protection assay and immunohistochemisty (Figure 15 and 16). For all the experiments for this project, *RIP-HGF* mice were backcrossed for 6

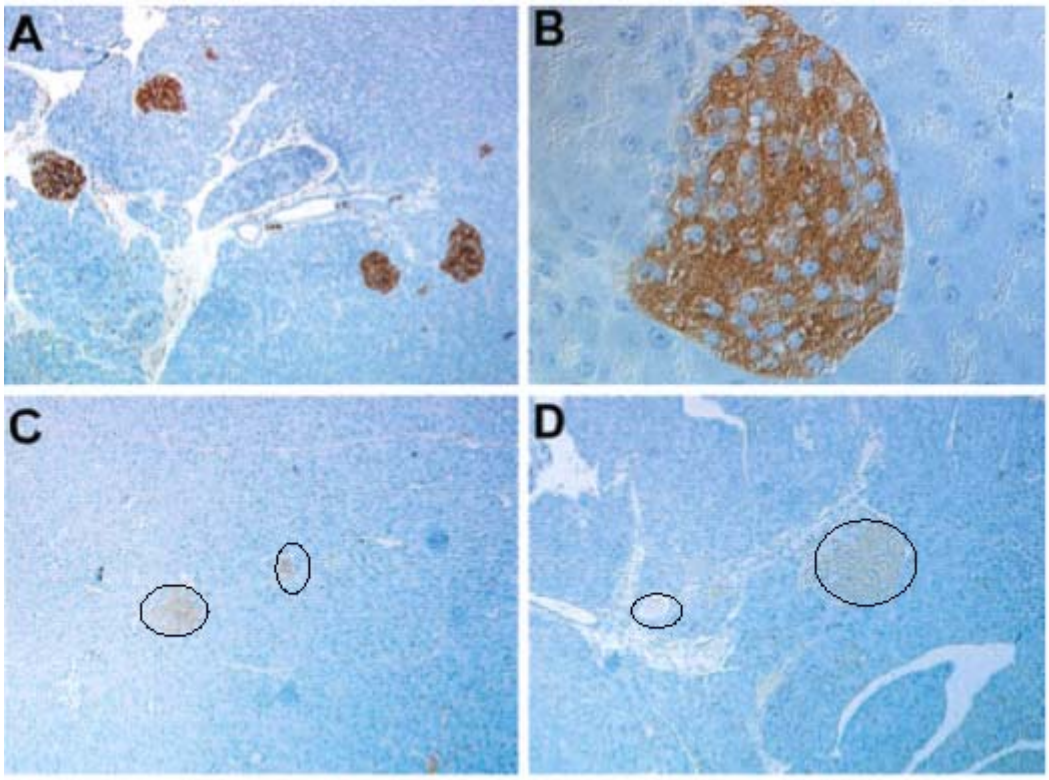


Figure 14. Immunohistochemical localization of PL in the *RIP-PL* transgenic and normal pancreas. *RIP-PL* pancreas is shown under low magnification (**A:** X100) and high magnification (**B**: X400). There is intense reactivity specifically in the islets of RIP-PL pancreas, but only faint non-specific labeling in the normal islet (**C**: X100). The immunostaining in *RIP-PL* islets is specific for PL, as evidenced by the fact that preincubation of the antibody with 10^{-6} M PL-I peptide resulted in a marked reduction in labeling (**D**: X100). Islets in (**C**) and (**D**) are outlined. [Adapted from (Vasavada, Garcia-Ocana et al., 2000), used with permission]

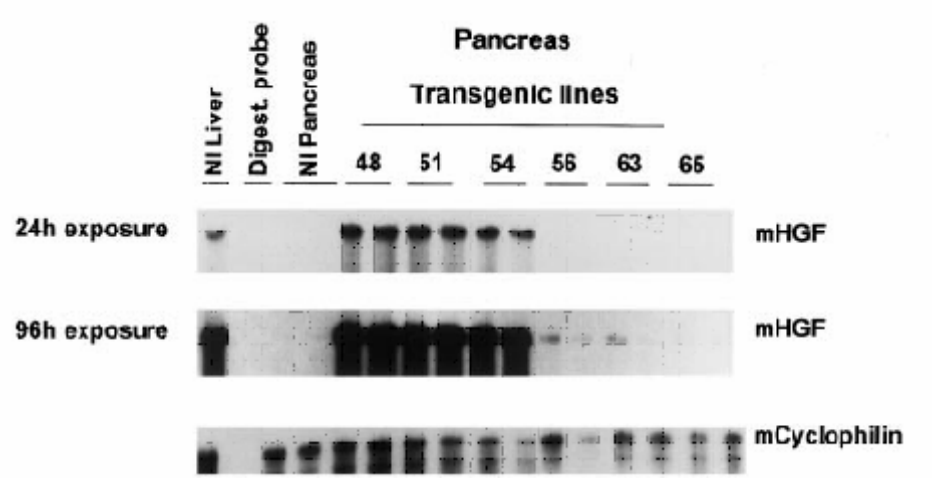


Figure 15. RNase protection analysis of total RNA isolated from pancreas from *RIP-HGF* transgenic mice (Tg) and normal littermates (NI). Total pancreatic RNA (50-100 µg) from 2 normal mice and 2 transgenic mice per line, was hybridized with mouse HGF and mouse cyclophilin (internal control) RNA probes. HGF mRNA expression in the normal mouse pancreas became only visible after exposure of the gel for 96 h (Garcia-Ocana et al., 2000, used with permission).

generations to the C57BL/6J strain (The Jackson Laboratory).

RIP-IGF-1

RIP-IGF-1 mice were generously provided by Dr. Robert Ferry at the University of Tennessee Health Science Center in Memphis. For all the experiments for this project, *RIP-IGF-1* mice were backcrossed for 9 generations to the C57BL/6J strain (The Jackson Laboratory).

For all pregnancy studies, the morning of vaginal plug was counted as GD 0.5. Mice were fed mouse diet 5015 (LabDiet). All mice received food and drink *ad libitum* and were on a 12 hour light-dark cycle. All mouse studies were carried out in accordance with the Vanderbilt Institutional Animal Care and Use Committee guidelines under the supervision of the Division of Animal Care.

DNA Extraction and Genotyping

Ear-punches of 3wk-old mice were digested overnight at 55°C in 80 µl per sample Magic Tissue Bufffer [35.2 mM Tris, 2.5 mM ethylenediaminetetraacetic acid (EDTA), 2.5 mM sodium citrate,8.8 mM ammonium sulfate, 5% (v/v) Tween20] supplemented with fresh 0.3 mg/ml Proteinase K and 0.3 mg/ml RNase A. Tissues were then digested at 37°C for 15 minutes and heat-inactivated at 95°C for 10 minutes followed by centrifugation at 13,000 rpm for 10 minutes. The supernatant was stored at 4°C. For DNA extraction from paraffinized tissue sections, sections were deparaffinized in xylene and washed with 100% enthanol prior to extraction procedure as described above.

Genotyping for the *Foxm1*^{fl} allele was done as previously described in (Zhang et al., 2006b) using PCR. The primers are: *Foxm1*^{fl} forward 5'-TAG GAG ATA CAC TGT TAT AT-3' and reverse 5'-TGT GGG AAA ATG CTT ACA AAA G-3', which amplify a 180

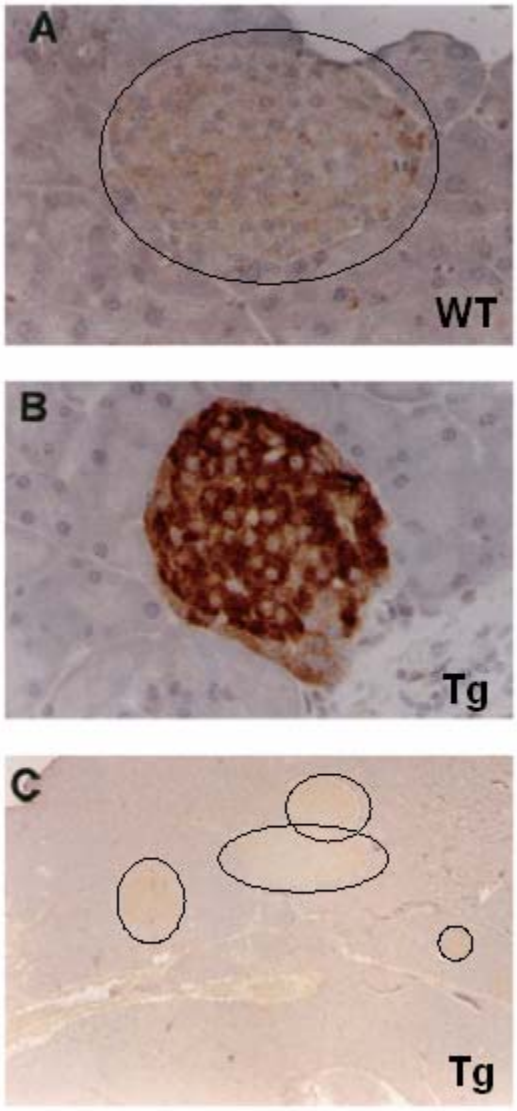


Figure 16. Immunohistochemical detection of HGF in the pancreas.
Immunohistochemistry for HGF in **(A)**, normal (WT), and **(B)**, *RIP-HGF* transgenic mouse pancreas (Tg). Normal and transgenic pancreas sections were processed identically and simultaneously. The pattern of labeling for HGF is consistent with the expression of the transgene in islet cells. **(C)** Labeling of a transgenic pancreas section with primary antibody preincubated for 24 h at 4°C with an excess of blocking peptide (Garcia-Ocana et al., 2000, used with permission). Islets in **A** and **C** are outlined.

bp product for wild-type *Foxm1* and a 230 bp product for *Foxm1*^{fl} due to insertion of a loxP site in the third intron of *Foxm1* (Figure 11). PCR reactions contained approximately 50 to 100 ng genomic DNA, 250 nM of each primer, 2X FailSafe PCR PreMix C (Epicenter Technologies), and 0.5 µl REDTaq DNA polymerase (Sigma) in a total volume of 10 µl. The following PCR program was used: 92°C for 6 minutes (hot-start); 32 cycles of 92°C for 30 seconds (denaturing), 48°C for 30 seconds (annealing), and 72°C for 1 minutes (elongation); and 72°C for 6 minutes. PCR products were examined by 2% [w/v] agarose gel electrophoresis.

Genotyping for the *Foxm1*^{null} allele, *Pdx1-Cre, RIP-HGF, RIP-PL* and *RIP-IGF1* transgenes was performed as described above except that Amptemp Mix was used instead of the 2XFailSafe PCR PreMix C and REDTaq DNA polymerase in the PCR identifications of *RIP-HGF* and *RIP-PL* transgenes. In some experiments, 125 nM of IL-2 primers were added in the PCR reaction mixture to amply *IL-2* as a positive control. Detailed information about primers and PCR programs is listed in Table 6.

Intraperitoneal Glucose Tolerance Test (IPGTT)

After a 16 hour fast, baseline blood glucose levels (in mg/dl) were measured in tail vein blood from mice using a FreeStyle glucometer (Abbott Diabetes Care). Glucose (2 gram dextrose/kg body weight) in sterile PBS was injected intraperitoneally, and blood glucose was measured 15, 30, 60, 90, and 120 minutes after injection.

Plasma and Pancreatic Insulin Content

To examine plasma insulin content, approximately 100 µl blood was collected at 0 and 30 minutes after glucose injection during an IPGTT from the saphenous vein. For saphenous venous blood collection, the mouse was restrained, the distal-lateral portion

of the leg was shaved, and the saphenous vein was punctured using a 25 5/8 G needle. Blood was drained into heparanized microvette tubes (Sarstedt) and immediately placed on ice. Blood samples were then centrifuged at 2,000 rpm for 15 minutes at 4°C, and the Supernatant was stored at -80°C. 10 µl of each sample was analyzed using insulin Ultrasensitive ELISA kit according to manufacture's instruction (Alpco). For pancreatic insulin content analysis, dissected pancreata were rinsed in PBS, blotted with filter paper, weighed, and homogenized (Polytron PT 10/35; Brinkmann Instruments) in 1 ml of acid alcohol. The homogenate was extracted with an additional 5 ml of acid alcohol for 48 h at 4°C and centrifuged at 2500 rpm for 30 minutes. The supernatant (stored at -20°C) was assayed for insulin in the Hormone Assay and Analytical Services Core at Vanderbilt University by liquid-phase radioimmunoassay (RIA) using the Rat Insulin RIA Kit (Linco/Millipore). Insulin concentrations were normalized to pancreas wet weight.

Islet Isolation

All islet isolations were performed in the Islet Procurement and Analysis Core at Vanderbilt University. Mice were anesthetized via an intraperitoneal injection of 0.15 mg/g ketamine-HCl and 0.04 mg/g xylazine (Henry Schein), the abdominal cavity was exposed, and 3-4 ml of 0.6% collagenase P (Roche) in Hank's Balanced Salt Solution (HBSS) containing calcium and magnesium (Gibco) was injected into the common bile duct. The pancreas was then harvested and digested in 6.5 ml of the collagenase P solution for 4 minutes at 37°C followed by 1.5 minutes at room temperature, with shaking (the digestion conditions vary depending on the genetic background of the animal). Islets were then hand-picked using at least 4 passages to reach 98% purity.

RNA Isolation and Quantitative Real-Time RT-PCR (qRT-PCR)

INS-1, MIN6 cells or freshly isolated islets were washed three times in 1 ml cold

1XPBS each time. Total RNA (75-125 islets/mouse or $4X10^6$ cultured INS-1 or MIN6 cells) was extracted using the RNAqueous kit (Ambion) according to the manufacturer's instructions. RNA concentration and integrity were assessed using the ND-1000 Spectrophotometer (NanoDrop) and the 2100 Electrophoresis Bioanalyzer (Agilent) at the Vanderbilt Microarray Shared Resource.

cDNA was synthesized using the Superscript III First-Strand synthesis system (Invitrogen) from 50-65 ng total islet mRNA. Reactions were carried out in technical duplicate with iQ SYBR Green supermix (Bio-Rad) according to the manufacturer's instructions at an annealing temperature of 58°C. Data were collected using an iCycler iQ Real-Time PCR Detection System (Bio-Rad) and software (Bio-Rad). Primers were optimized by melting curve and standard curve assays first before applying to qRT-PCR. Detailed sequences of primers are listed in Table 7. Expression levels were normalized against the levels of *hypoxanthine-guanine phosphoribosyltransferase* (*HPRT*), a ubiquitously expressed gene that shows little change during cellular growth or differentiation. Results were analyzed using the $2^{-\Delta\Delta Ct}$ Method (Livak & Schmittgen, 2001).

Tissue Preparation and Histology

Pancreata from adult mice were dissected in cold 1XPBS, fixed in 4% paraformaldehyde at 4°C for 4 h and stored in 70% ethanol for short term after twice washes with cold PBS. Tissues were then dehydrated, embedded in paraffin, and sectioned at 5 μm. Sections were deparaffinized using xylene, and rehydrated in a decreasing ethanol series.

Primary antibodies were diluted in 5% normal donkey serum and 1% BSA (bovine serum albumin) in PBS. Slides were incubated with primary antibody overnight in a humid chamber at 4°C. The following primary antibodies were used in this study:

Table 6. Genotyping primers and parameters of PCR programs.

Gene ID	Forward primer	Reverse Primer	Annealing (°C)	Cycles	Product
Foxm1^{null}	TAGGAGATACACTGTTATAT	CTCATGTAGCATAGAGGGCTG	56	30	510bp
RIP-IGF-I	CTGGGGAATGATGTGGAAAAATGC	GATAGAGCGGGCTGCTTTTGTAG	58	35	800bp
RIP-HGF/PL	CTACGGGCTCTACTGCTTCAGG	GGCACTGGAGTGGCAACTTCCAAG G	56	35	171bp
Pdx1-Cre	TGCCACGA CAAGTGACAGC	CCAGGTTACGGATATAGTTCATG	58	32	650bp
IL-2	CTAGGCCACAGAATTGAAAGATCT	GTAGGTGGAAATTCTAGCATCATCC	56	30	324bp
GAPDH	CGTGGAGTCTACTGGTGTCTTCACC	GATGGCATGGACTGTGGTCATGAGC	56	35	259bp

Table 7. Primers for qRT-PCR.

Gene ID	Species	Forward Primer	Reverse Primer
Foxm1	Mouse	CACTTGGATTGAGACCACTT	GTCGTTTCTGCTGTGATTCC
HPRT	Mouse	AGTCAACGGGGGACATAAAA	TGCATTGTTTTACCAGTGTCAA
Birc5/Survivin	Mouse	TGATTTGGCCCAGTGTTTTT	CAGGGGAGTGCTTTCTATGC
Cenp-a	Mouse	CAAGGAGGAGACCCTCCAG	GTCTTCTGCGCAGTGTCTGA
Plk-1	Mouse	TTGTAGTTTTGGAGCTCTGTCG	AGTGCCTTCCTCCTCTTGTG
PL	Mouse	CCGCAGATGTGTATAGGGAATTT	CTTCCTCTCGATTCTCTGGAGT
Bcl-xl	Mouse	CCTTGGATCCAGGAGAACG	CAGGAACCAGCGGTTGAA
HGF	Mouse	CACCCCTTGGGAGTATTGTG	GGGACATCAGTCTCATTCACAG
Foxm1	rat	CGAGGACCACTTCCCTTATTT	GGAGAGAAAGGTTGTGACGAA
Bcl-X	rat	TGACCACCTAGAGCCTTGGA	TTCCCGTAGAGATCCACAAAA
HPRT	rat	GACCGGTTCTGTCATGTCG	ACCTGGTTCATCATCACTAATCAC
FOXM1	Human	GGAGGAAATGCCACACTTAGCG	TAGGACTTCTTGGGTCTTGGGGTG
glucuronidase, beta	Human	ACGCAGAAAATATGTGGTTGGA	GCACTCTCGTCGGTGACTGTT

guinea pig anti-insulin (1:1,000; Linco), rabbit anti-cytokeratin (1:1,000; akoCytomation), rat anti-BrdU primary antibody (1:400; Accurate Chemical & Scientific), mouse anti-Neurogenin3 (1:100; Developmental Studies Hybridoma Bank, The University of Iowa) and rabbit anti Glut2 (1:500; Bernard Thorens). For BrdU detection, slides were treated with 1.5N HCl for 20 min at 37^{o}C, neutralized in sodium borate buffer for 1 min at room temperature, and treated with 0.005 mg/ml trypsin (Sigma-Aldrich, St. Louis, MO) and 0.005 mg/ml $CaCl_2$ (in Tris buffer; pH 7.5) for 3 min at 37°C. Vectastain ABC kit (Vector lab) was used for immunohistochemical labeling of insulin for β-cell mass measurement according to the manufacturer's instructions. The Histomouse-SP kit (Invitrogen, Carlsbad, CA) was used for immunohistochemical staining of p27. Detection of cytokeratin required pre-treatment with 20 µg/ml Proteinase-K (DakoCytomation) in PBS for 5 minutes at room temperature. Detection of Neurogenin (Neurog) 3 required slow-boil microwave antigen retrieval with Tris-EGTA (TEG) buffer, pH 9.0 [10 mM Tris, 500 µM ethylene glycol tetraacetic acid (EGTA)) and tyramide signal amplification with TSA Kit #2 (Invitrogen).

All secondary antibody incubations were performed at room temperature for 1 h with antibodies diluted in 1% BSA in PBS at a 1:500 dilution. The following secondary antibodies (Jackson Immunoresearch) were used: Cy2-conjugated donkey anti-guinea pig, Cy2-conjugated donkey rabbit, Cy3-conjugated donkey anti-guinea pig, Cy3-conjugated donkey anti-rat and peroxidase-conjugated donkey anti-guniea pig. Slides were mounted with Permount (Fisher) for immunohistochemistry, or with anti-fade mouting medium for immunofluorescence [50% glycerol (v/v) and 2% N-propylgalate (w/v) in PBS, pH 7.4 with 1.5 µg/ml nuclear fluorogen 4',6'-diamidino-2-phenylindole (DAPI)] (Molecular Probes, Eugene, OR).

Samples were viewed under bright-field illumination or appropriate optical filters (immunofluorescence) using an Olympus BX41 microscope (Tokyo, Japan) and digital camera with the Magnafire program (Optronics, Chelmsford, MA). TIFF images from each experiment were processed identically with Adobe Photoshop.

β-Cell Mass Analysis

Entire pancreata were removed, weighed and fixed as above. Five-micron longitudinal sections were prepared for insulin immunoperoxidase labeling and eosin counterstaining. Every 100th section (an average of 4-6 sections per pancreas) was used. Images of anti insulin-labeled sections were scanned using a Nikon Super Coolscan9000. Using Metamorph 6.1 software (Molecular Devices), β-cell mass was measured by obtaining the fraction of crosssectional area of pancreatic tissue positive for insulin and multiplying this by the pancreatic weight. At least three animals of each genotype at each stage were examined.

β-cell Proliferation Analysis

BrdU (100 mg/kg; Sigma-Aldrich) was injected intraperitoneally every 2 h three times before harvesting pancreata. Pancreata were isolated and processed for histology as above. BrdU-labeled β-cells were detected by double immunolabeling with BrdU and insulin antibodies, and DAPI to visualize nuclei. Using Metamorph software, all BrdU-positive and negative nuclei in insulin-positive cells were counted at 400X magnification. At least 3,000 β-cells from 4-6 pancreatic sections were counted for each animal. At least three animals were analyzed for each group. The proportion of BrdU-positive β-cell nuclei to total β-cell nuclei was calculated and represented the percentage of β-cells undergoing replication. For pregnancy experiments, the first injection of BrdU was done at 6 am and the animals were sacrificed at noon.

β-cell Apoptosis Assay

Terminal deoxynucleotidyl transferase-mediated dUTP nick-end-labeling (TUNEL) was performed on paraffin sections of adult pancreata using the *In Situ* Cell Death Detection kit (Roche) and followed by immuno-fluorescent staining of insulin. Cleaved caspase-3 was detected using an antibody raised in rabbit (Cell Signaling Technology) at a dilution of 1:1,500. Three to four animals per goup were analyzed. Immunoreactivity was detected using the Vectastain ABC and DAB kits (Vector Labs).

Islet Size, β-cell Size Analysis

Pancreas sections were co-labeled for Glut2 (rabbit anti-Glut2, 1:500; Alpha Diagnostic) and insulin as described above. Every islet from one section on each slide was photographed. Using Metamorph, the area of more than 1,000 individual β-cells was determined per mouse.

In vitro Islet Culture

For pregnancy studies, islets from 8- to 12-week-old *Foxm1*$^{fl/fl}$ virgin females were cultured for 4 days in 1XRPMI 1640 supplemented with 10% horse serum, 11 mmol/l glucose, 100 units/ml penicillin, and 100 µg/ml streptomycin with or without 500 ng/ml human PL (National Hormone and Peptide Program). An average of 50–60 islets was plated in one 60X15-mm non–tissue culture–treated dish at 37°C in 5% CO_2. Cultures were replenished with fresh medium with or without PL after 48 h incubation. After 4 days, RNA was extracted and subjected to qRT-PCR analysis. At least three biological repeats were analyzed.

For aurintricarboxylic acid (ATA) studies, freshly isolated islets from 8-12 week-old *Foxm1*$^{fl/fl}$ or WT mice were cultured in 1XPPMI 1640 supplemented with 10% horse serum, 11 mmol/l glucose, 100 units/ml penicillin,100 µg/ml streptomycin and 0.1%

dimethyl sulfoxide (DMSO) with or without different concentration of ATA (Sigma). ATA stock (1000X) was made by dissolving ATA powder in DMSO.

Dispersed Islet Cell Culture with HGF

Freshly isolated islets were cultured overnight in 1XRPMI, 5mM glucose and 10% FBS (fetal bovine serum) before trypsinization. Glass coverslips were trimmed into squares slightly smaller than one well in a 24-well plate and rinsed in 100% ethanol in the hood for 30 minutes and air-dried for later plating. After one wash with PBS, islets were trypsinized (400IE in 400ul 0.05% trypsin-EDTA) in 1.5 ml tube in 37°C incubator for 7 or 8 minutes. Islets were pipetted up and down two to three times during the incubation and then 1 ml of culture medium was added to stop trypsinization. Cells were pelleted by centrifugation at 2,000 rpm for 2 minutes and re-suspended in fresh culture medium (400IE in 400 ul medium). 50 µl of suspended cells were plated on top of microscope cover glass placed in a well of a 24-well plate and incubated at 37°C to allow the attachment to the glass. One ml culture medium was then slowly added to the well for overnight culture at 37°C. On the second day, cells were cultured in 1XRPMI with 2 mM glucose (no FBS) for 24 hours followed by another 48 hours of incubation with addition of 10 µM BrdU in 10 µl volume with or without HGF (25 ng/ml in 2.5 µl or 100 ng/ml in 10 µl) to label proliferating cells. Then cells were washed in PBS and fixed with 2% paraformaldehyde for 30 minutes at room temperature in the dark before immunohistochemical analyses. Figure 17 showed an example of dispersed islet cell clusters labeled with insulin and BrdU.

Western Blotting

Islets from adult mice were lysed and sonicated with a Virsonic 100 sonicator (Virtis Company, Inc.). The supernatant was quantified using the Bio-Rad DC protein

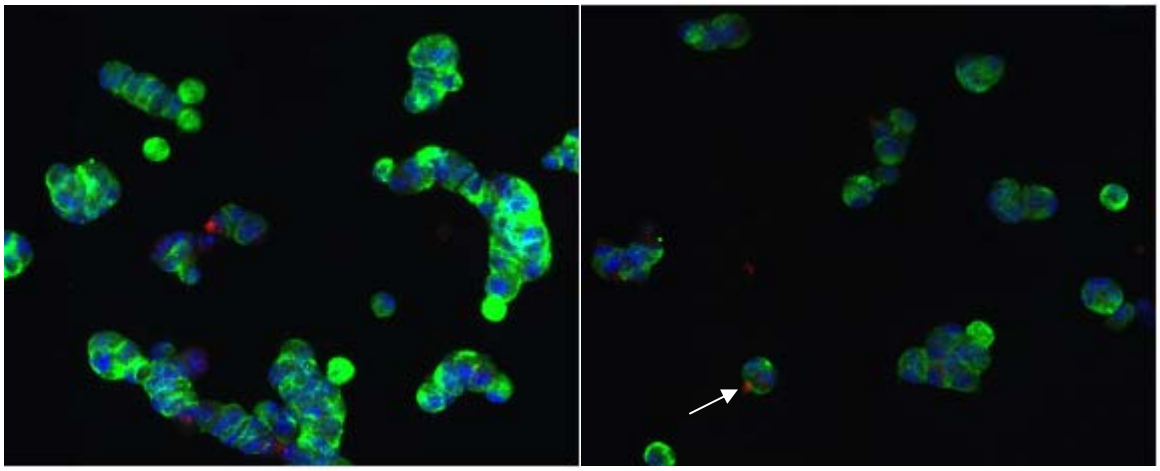


Figure 17. Dispersed islet cell clusters immunolabeled with BrdU (red) and insulin (green). DAPI (blue) labeled nuclei. Arrow denoted a cell positive for BrdU. (Magnification: 400X).

Table 8. Primers for ChIP assays

Gene ID	Species	Forward Primer	Reverse Primer	Annealing (°C)	Product
Stat5 ChIP 1	rat	CAGTCCCCAGAACTGCAAAT	GATCCTCCTGTCTCCCTTCC	59	392bp
Stat5 ChIP 2	rat	AGCTCTCAAAAGCCAAACCA	ACACATGCCTGTGAGGACAC	59	352bp
Stat5 ChIP 3	rat	CCCGCTGAACTCATGGTATT	CTCAGCCTCCAGTGACCTTC	59	378bp
Stat5 ChIP 4	rat	CTGGGACCTATGCAAGCAAT	CCACAGTGTGAAGAGGCTGA	59	306bp
Bcl6 ChIP 1	rat	CCAACAGCTGTGATCAAGGA	ATGCGCTCACCTTTCAGTTT	59	399bp
Bcl6 ChIP 2	rat	GGCTGATCCCTTCAACACAT	GCCACCTATAACCCCAACCT	59	414bp
Bcl6 ChIP 3	rat	TCCCTCACTCGCCTCTCTAA	TACGATGTGGATTGGGGTTC	59	380bp
PEPCK ChIP1	rat	GAGTGACACCTCACAGCTGTGG	GATCATAGCCATGGTCAGCA	59	328bp
Bcl6 ChIP 1	mouse	CTGTAGACCCTGGTGATGTT	AGATGATCTTCCAACACTGG	59	503bp
Bcl6 ChIP 2	mouse	GCACTAGTGGATGAGAGAGG	AGTGGAAGCCAAGATTTGTA	59	428bp
Bcl6 ChIP 3	mouse	CCATTACCAGAACCACACTT	TTTTCTTTACTTGGCTACGC	59	401bp
PEPCK ChIP 1	mouse	GAGTGACACCTCACAGCTGTGG	GGCAGGCCTTTGGATCATAGCC	59	339bp

assay (Bio-Rad). Western blotting was done using approximately 20 µg islet protein per lane from one animal. After blocking in 5% non-fat milk at room temperature for one hour, membranes were probed with primary antibodies diluted in 3% non-fat milk in Tris-buffered saline (TBS) overnight at 4°C. Peroxidase-conjugated species specific secondary antibodies (Jackson ImmunoResearch Laboratories) were diluted to 1:5,000 in 3% non-fat milk in TBS and incubated for 1 hour at room temperature. Protein levels in individual lanes were quantified using Image J 1.42 and normalized to the level of β-actin signal in corresponding lanes.

Chromatin Immunoprecipitation (ChIP) Assays

Three 10 cm dishes of INS-1 or MIN6 cells (4 X 10^6 cells per dish) were required for one ChIP experiment. After two washes with PBS, cells were fixed in 5 ml 1% formaldehyde in DMEM with no serum for 5 minutes at room temperature. Crosslinking time was optimized for different cell lines. To stop the crosslinking reaction, 250 µl 2.5 M glycine was added to each dish and incubated for 2 minutes at room temperature. After two washes with cold PBS (high temperature may reverse the crosslinking), cells were collected into 1.5 ml eppendorf tubes in 1.4 ml cold PBS and immediately placed on ice. After centrifugation at 5,000 rpm for 5 minutes at 4°C, cell pellet was resuspended in 300 µl SDS lysis buffer (1% SDS, 10 mM EDTA and 50 mM Tris pH 8) supplemented with fresh added Phenylmethanesulphonylfluoride (PMSF, 1 mM) and proteinase inhibitor cocktail (1:100, Sigma) and then incubated on ice for 10 minutes. Cell lysate was sonicated using at power setting “High” with a Virsonic 100 sonicator (Virtis Company, Inc.) for 30 second pulses with 30 second intervals for 15 minutes total at 4°C. The reactions were centrifuged for 10 minutes at 4°C to remove debris and then aliquoted at 10 µl for storage at -80°C.

One aliquot of sonicated chromatin was taken to examine the extent of fragmentation and DNA concentration. 400 µl H_2O and 20 µl 5 M NaCl was added to the chromatin aliquot and incubated at 65°C overnight to reverse crosslinking the DNA and protein. DNA was extracted with 1:1 phenol:$CHCl_3$ twice after an 1-2 hour incubation with 20 µl 1.0 M Tris, pH 6.5; 10 µl 0.5M EDTA, pH 8.0; 5 µl 10 mg/ml proteinase K; and 2 µl 10 mg/ml RNase A. $CHCl_3$ extaction was done to remove the last trace of phenol followed by ethanol precipitation with 30 minutes centrifugation at room temperature in 1 ml 100% ethanol and 1 µl glycogen azure (Pellet Paint) to visualize the pellet. After washing in 70% ethanol and vacuum drying, pellet was dissolved in 100 µl nuclease-free H_2O at room temperature overnight. Fragmented DNA was analyzed by 1% [w/v] agarose gel electrophoresis. DNA concentration was measured by NanoDrop 3300 fluorospectrometer (Thermo Scientific).

Two 100 µl-aliquots of chromatin (approximately 50-60 µg per aliquot) were quickly thawed at 37°C and placed on ice (one aliquot for experimental immunoprecipitation, the other one for control immunoprecipitation using IgG). 0.9 ml cold dilution buffer (0.01% SDS; 1.1% Triton X-100; 1.2 mM EDTA; 16.7 mM Tris, pH 8; and 167 mM NaCl) supplemented with fresh Phenylmethanesulphonylfluoride (PMSF, 1mM) and proteinase inhibitor cocktail (1:100, Sigma) was added to each aliquot. Chromatin was precleared by incubation with 25 µl (50/50 slurry) protein A/G agarose beads (Millipore) for 1 hour at 4° C on a nutator. After removal of agarose beads by centrifugation at 1,000 rpm for 30 seconds at 4°C, approximately 2 µg antibody of interest and 10 µg of IgG (Santa Cruz Biotechnology) were added to the supernatants in experimental and control tubes, respectively. The reactions incubated overnight at 4°C. 80 µl (50/50 slurry) of protein A/G agarose beads were incubated with the reactions for 3 hours on a nutator at 4°C to pull down the chromatin recognized by antibodies. The beads were then pellet by centrifugation at 10,000 rpm for 30 seconds. 0.5 ml

supernatant from reaction incubated with IgG was saved as input control. After removal of the supernatant, the beads were washed in 1 ml of each following solution for 5 minutes on a nutator at 4°C: low salt buffer (0.1% SDS; 1% Triton X-100; 2 mM EDTA; 20 mM Tris, pH 8 and 150 mM NaCl), high salt buffer (0.1% SDS; 1% Triton X-100; 2 mM EDTA; 20 mM Tris, pH 8 and 0.5 M NaCl), LiCl buffer [0.25 M LiCl; 1% Np-40; 1% sodium deoxycholate (Fisher); 1 mM EDTA and 10 mM Tris, pH8)] and two washes with TE buffer (10 mM Tris, pH 8 and 1 mM EDTA). After each washing, beads were pelleted by centrifugation at 10,000 rpm for 30 seconds. 20ul 5 M NaCl was added to 0.5 ml of supernatant saved from IgG reaction and to washed beads diluted in 0.5 ml freshly made elution buffer (0.2% SDS and 0.1 M $NaHCO_3$) and reactions were incubated at 65°C overnight for decrosslinking. DNA was extracted with phenol/$CHCl_3$ as previously described. DNA was resuspended in 100 µl nuclease-free H_2O at room temperature overnight and analyzed by PCR. The PCR reaction contained 15 pmol of each primer, 2X FailSafe PCR PreMix C (Epicenter Technologies), and 0.5 µl REDTaq DNA polymerase (Sigma), and 2.5 µl DNA. The input DNA usually needed to be diluted 100 fold for PCR. Detailed information about primers and PCR cycling parameters is listed in Table 8.

For ChIP using isolated islets, approximately 500 islets were pooled for one chromatin preparation. Islets were pelleted by centrifugation at 13,000 rpm for 10 seconds and then resuspended in 500 µl PBS. 500 µl 2.22% formaldehyde in PBS was added to the system to fix islets for 10 minutes at room temperature on a nutator. Cross-linking was stopped by adding 59 ul 2.5 M glycine to a final concentration of 0.14 M and incubating for 5 minutes at room temperature. After washing with 1 ml PBS, islets were diluted in 200 µl cold ChIP whole cell lysis buffer [1 M Tris, pH 8.1; 5 M NaCl; 1 M $MgCl_2$; 10% NP-40; 10% SDS; 10% sodium deoxycholate; proteinase inhibitor cocktail (1:100, Sigma); and phosphatase inhibitor cocktail 2 (1:100, Sigma)] and homogenized with

small plastic pellet pestle (Kimble Chase Life Science) by hand and incubated on ice for 10 minutes before sonication. Sonication, immunoprecipitation and PCR were performed as stated above.

Statistical Analysis

Data were analyzed by unpaired t-test or two-way ANOVA with Bonferroni's post-tests, using GraphPad Prism v5.01. A value of $P \leq 0.05$ was considered significant.

CHAPTER III

FOXM1 STIMULATES β-CELL REPLICATION DOWNSTREAM OF PL SIGNALING IN ADULTS

Introduction

Maintenance of normal β-cell mass in adults is crucial for body glucose homeostasis. A thorough understanding of the factors and signaling pathways that regulate adult β-cell mass will foster new methods and techniques for both *in vitro* generation of new β-cells and *in vivo* manipulation of the β-cell population to combat diabetes. In adult rodents and humans, the β-cell population is replenished primarily through β-cell proliferation (Dor et al., 2004; Meier et al., 2008b) .

Of the several growth factors known to promote islet growth and function *in vitro*, PL, a member of the lactogen family is one of the most potent in increasing β-cell proliferation *in vitro* (Ogren et al., 1989; Brelje et al., 1993). Both *in vivo* and *in vitro* evidence have implicated PL as the primary factor responsible for the increase in β-cell mass during pregnancy in rodents and humans (Parsons et al., 1992; Brelje et al., 1993; Sorenson & Brelje, 1997). As a proof of principle for direct stimulation of β-cell proliferation by PL, over-expression of PL in β-cells leads to a two-fold increase in β-cell proliferation and β-cell mass, even resulting in mild hypoglycemia (Vasavada, Garcia-Ocana et al., 2000). Gestational changes in β-cell proliferation/mass are transient and islets return to the pre-pregnancy state in response to reduced PL activity 8 days after delivery due to the activity of its primary counteracting hormone, progesterone (Sorenson et al., 1993).

PL and its closely-related family member prolactin (PRL) signal through a common receptor (PRLR). PRL receptors are present in islet cells and their expression

is upregulated during pregnancy (Galsgaard et al., 1999). β-cell proliferation stimulated through the PRLR is dependent on Jak2/Stat5 signaling pathway activation and independent of ERK1/ERK2, PI3K and PKC signaling pathways (Friedrichsen et al., 2001). Global deletion of the PRLR reduces β-cell mass (Freemark et al., 2002). Moreover, pregnant mice heterozygous for a PRLR null mutation exhibited reduced β-cell proliferation, decreased β-cell size and mass and impaired glucose tolerance (Huang et al., 2009). A gestational diabetic phenotype was also observed in a mouse model with compromised PL signaling due to the β-cell specific over-expression of Menin, a cell cycle inhibitor normally repressed by PL in pregnancy (Karnik et al., 2007). These observations demonstrated the essential role of PL signaling in the facultative increase in maternal β-cell proliferation, and thus glucose homeostasis during pregnancy.

Despite the established role of PL in β-cell replication in pregnancy, intrinsic factors in the β-cell transducing the mitogenic signal of PL have not been well identified. Seung Kim's group reported Menin as a downstream target of PL based on the fact that the *Men1* gene is transcriptionally repressed by *Bcl6*, a direct target of Stat5 during pregnancy to allow the increase in maternal β-cell proliferation (Karnik et al., 2007). However, no positive regulator of β-cell proliferation has been identified as downstream effectors of PL signaling.

Foxm1 is a crucial β-cell cell cycle regulator. It promotes cell cycle progression through transcriptional upregulation of many positive cell cycle regulators: Cdc25A (G1/S transition); Cdc25B (G2/S transition) (Wang et al., 2002b); Aurora B kinase, Survivin, PLK-1 (chromosome segregation and cytokinesis) (Krupczak-Hollis et al., 2004; Kim et al., 2005); Cenp-A and Cenp-B (kinetochore-assembly) (Wang et al., 2005; Wonsey & Follettie, 2005) (Figure 5) .

Foxm1 also indirectly represses CDKIs, $p21^{Cip1}$, $p27^{Kip1}$, $p16^{INK4A}$ and $p19^{ARF}$ (Wang et al., 2005). Foxm1 prevents nuclear accumulation of p21 and p27 by directly activating transcription of the SCF ubiquitin ligase complex that targets p27/p21 for degradation (Wang, Krupczak-Hollis et al., 2002; Krupczak-Hollis et al., 2003; Wang et al., 2005). In addition, Foxm1, by stimulating growth factor induced expression of its direct target KIS, enhances the phosphorylation of p27 leading to increased proteolysis of p27 (Petrovic et al., 2008). The mechanism by which Foxm1 inhibits *p16* and *p19* is less clear. Li et al. recently reported that, Foxm1 activates the polycomb group protein coding gene *Bmi-1* via its direct target c-Myc, to suppresse *p16/p19* (Li et al., 2008).

Accumulating evidence has demonstrated that perturbing the expression or function of major players in the cell cycle, such as cyclins and CDKs, in β-cells causes impaired β-cell growth and in many cases glucose intolerance or diabetes. In contrast, over-expression of cyclins, CDKs or deletion of the CDKIs leads to increased β-cell proliferation/mass and improved islet function (Heit et al., 2006). However, very little is known about the factors activating β-cell cell cycle machinery in response to external stimuli. Our laboratory first reported the role of FoxM1 in the pancreas as an important driver of β-cell cell cycle. Foxm1 is essential for β-cell proliferation and maintenance of β-cell mass in adults. Male mice with pancreas-wide deletion of Foxm1 developed diabetes by 9 weeks of age due to impaired β-cell proliferation and dramatic loss in β-cell mass (Zhang et al., 2006b). More recently, we found that Foxm1 is also partially required for β-cell proliferation following 60% partial pancreatectomy, an injury model that is known to stimulate an increase in β-cell proliferation/mass and neogenesis. (Ackermann Misfeldt et al., 2008). In addition, unpublished results from our laboratory suggested that Foxm1 is important for high fat diet induced β-cell expansion. Therefore, we propose Foxm1 as a common target of multiple β-cell mitogens to provoke β-cell cycle progression.

The aim of this project is to understand whether and how Foxm1 mediates pregnancy-induced β-cell proliferation. The euglycemic phenotype in virgin *Foxm1*$^{\Delta panc}$ females, despite their defects in β-cell proliferation, allows us to study the role of Foxm1 in this mutant mouse model without the confounding effects of hyperglycemia. Once challenged by pregnancy, *Foxm1*$^{\Delta panc}$ females became glucose intolerant by GD12.5 and gestationally diabetic by GD15.5 (Figure 18A and B). Our data demonstrated that Foxm1 is required for the increase in β-cell proliferation and mass during pregnancy. We further provided both *in vitro* and *in vivo* evidence suggesting that Foxm1 functions downstream of PL, as a target of Stat5 to repress Menin and p27.

Results

Impaired β-cell Proliferation in Pregnant *Foxm1*$^{\Delta panc}$ Mice

Our laboratory has shown previously that Foxm1 is vital for postnatal β-cell proliferation. Since *Foxm1*$^{\Delta panc}$ females are gestationally diabetic, we sought to examine whether the malfunction of islets was due to defects in maternal β-cell proliferation in *Foxm1*$^{\Delta panc}$ females during pregnancy. In control mice, maternal β-cell proliferation gradually increased as pregnancy proceeded. Compared to virgins, β-cell proliferation doubled by GD12.5, and increased more than three fold by GD15.5 (peak of proliferation in pregnancy) (Figure 19). After pregnancy, at postpartum day 8 (P8), β-cell proliferation declined to pre-pregnancy level. However, in *Foxm1*$^{\Delta panc}$ females, β-cell proliferation at virgin stage is only approximately a third of that in virgin controls. No increase in β-cell proliferation was observed in these mutants throughout pregnancy. After parturition, at P8, β-cell proliferation in mutants remained significantly lower than controls (Figure 18).

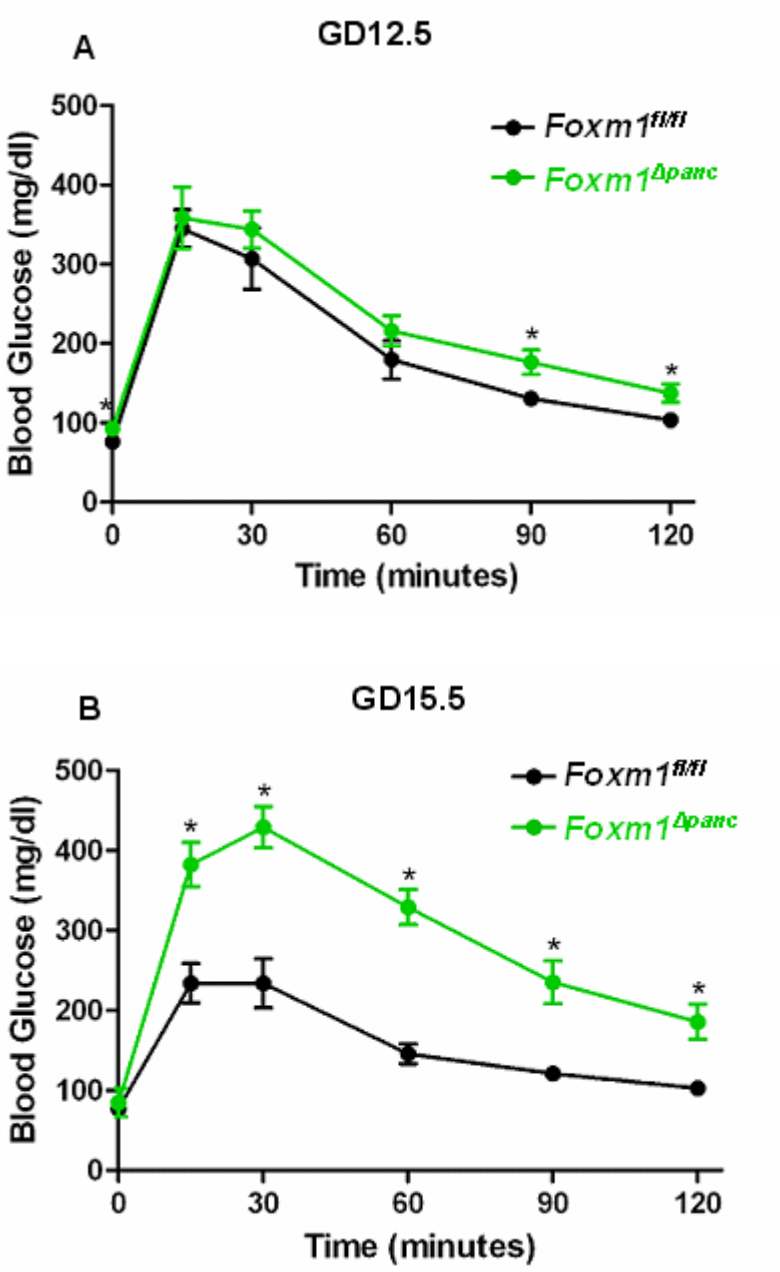


Figure 18. The absence of Foxm1 caused glucose intolerance at GD12.5 and GDM at GD15.5. IPGTTs were performed on *Foxm1*$^{fl/fl}$ (black) and *Foxm1*$^{\Delta panc}$ (green) females at GD12.5 **(A)** and GD15.5 **(B)** after a 16 h fast. At GD12.5, the mutant females displayed impaired glucose tolerance at 90 and 120 minutes **(A)**. Mutant females progressed to gestational diabetes by GD15.5 **(B)**. Two-way ANOVA was used to measure significance of difference. n = 5-10 per group. * P<0.05. Error bars represent SD. (Zhang et al., 2010, used with permission)

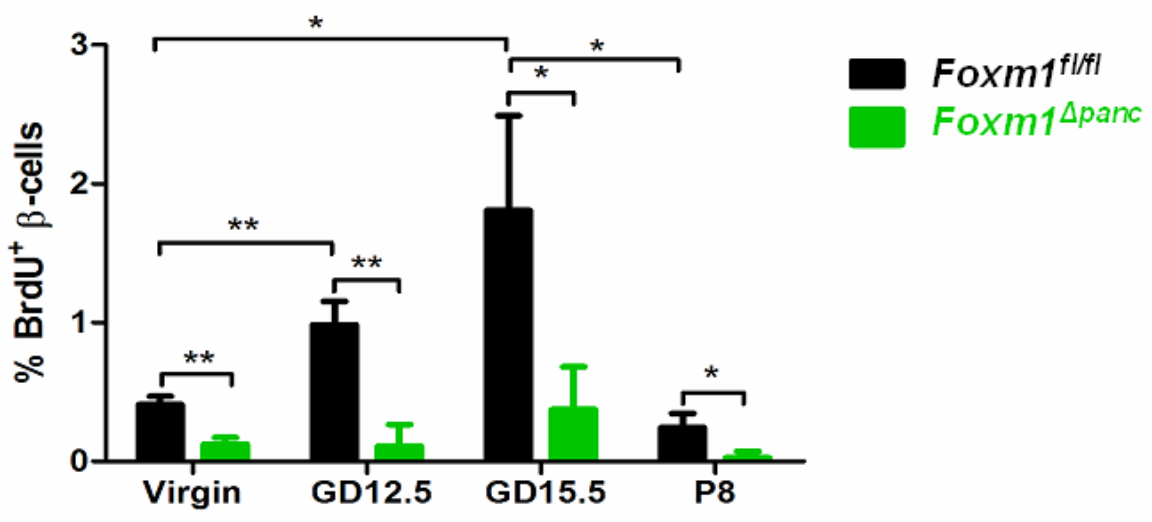


Figure 19. *Foxm1*$^{\Delta panc}$ females exhibited impaired β-cell proliferation. *Foxm1*$^{fl/fl}$ mice (black bar) showed a significant increase in β-cell proliferation at mid-gestation compared with prepregnancy. *Foxm1*$^{\Delta panc}$ females (green bar) showed much lower β-cell proliferation than control mice at every time point, and there was no increase in β-cell proliferation during pregnancy in mutant mice (evaluated by student t-test). Two-way ANOVA with Bonferroni's post-tests and two tailed student's t test were used to measure significance. n = 3-4 per group. * P<0.05, ** P<0.01. Error bars represent SD. (Zhang et al., 2010, used with permission)

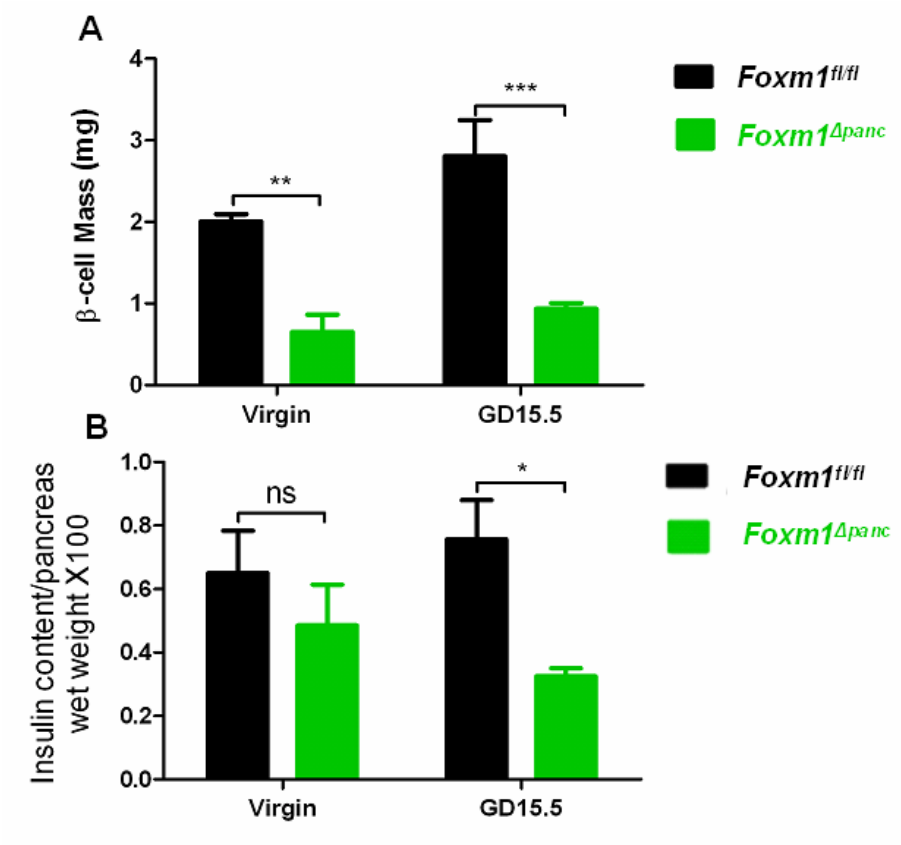
Defective β-cell Mass Expansion in Pregnant *Foxm1*$^{\Delta panc}$ Mice

We predicted that impaired β-cell proliferation would cause a defect in facultative expansion of β-cell mass during pregnancy. Indeed, at GD15.5, there was an approximately 70% reduction in β-cell mass in mutant females compared to control females, which is even larger than observed at virgin stage (Figure 20A). Consistent with defective β-cell mass, we also observed a more than 50% decrease in total pancreatic insulin content in the pregnant mutants compared to pregnant controls. In virgins, however, we did not detect such a difference despite the significantly reduced β-cell mass in mutants compared to controls (Figure 20B). This could be explained by a potential compensatory increase in insulin production by individual β-cells in mutants.

To examine whether the absence of Foxm1 would affect islet morphology, we performed insulin immunofluorescent staining. In contrast to the uniform insulin signal in the pregnant control islets, islets from pregnant mutants displayed uneven insulin reactivity (Figure 20C-E). Some cells, denoted by arrows in Figure 20E, are labeled much stronger than others, indicating β-cells with compensatory high insulin production.

***Foxm1* islet mRNA Increased During Pregnancy**

To investigate whether *Foxm1* expression changes in response to pregnancy, we performed qRT-PCR to examine *Foxm1* expression level throughout pregnancy using whole islet RNA from wild type female mice at virgin, GD14.5 (peak of β-cell proliferation) and P8. *Foxm1* mRNA increased about 1.7-fold at GD14.5 compared to virgin and declined to pre-pregnancy level at P8 (Figure 21). The dynamic change in *Foxm1* expression correlates with the dynamics of β-cell proliferation during pregnancy (Figure 4 and 19).



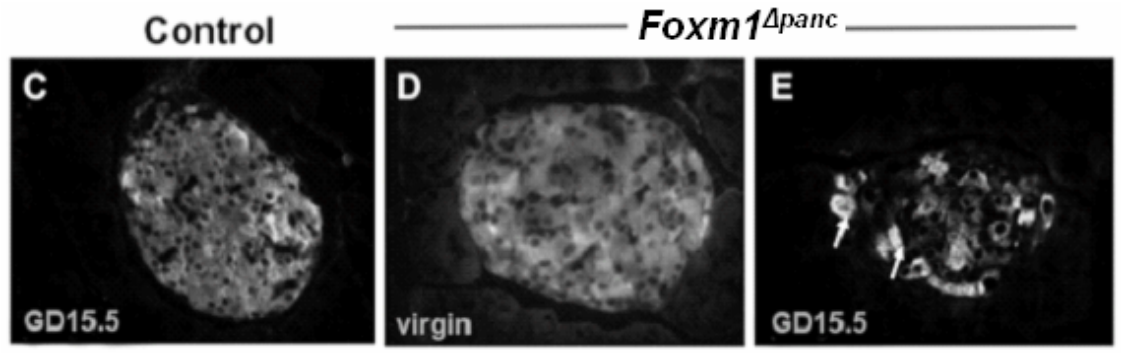


Figure 20. Decreased β-cell mass in virgin and pregnant *Foxm1*$^{\Delta panc}$ mice. (A) Compared to controls (black bar), β-cell mass was significantly decreased in virgin and GD15.5 mutant females (green bar; n = 3-4 per group). **(B)** Virgin mutant females have similar total pancreatic insulin content to virgin controls. Insulin content in mutants decreased slightly during pregnancy. Two-way ANOVA with Bonferroni's post-tests was used to measure significance. n = 4-7 per group. **(C)** Islets from control female mice at GD15.5 showed uniform insulin immunoreactivity as did islets from virgin mutants **(D)**. **(E)** Islets from female mutant mice showed uneven insulin reactivity at GD15.5 with some insulin-producing cells labeling more strongly than others (arrows). * P<0.05, ** P<0.01. *** P<0.001. ns: not significant. Error bars represent SD. (Zhang et al., 2010, used with permission)

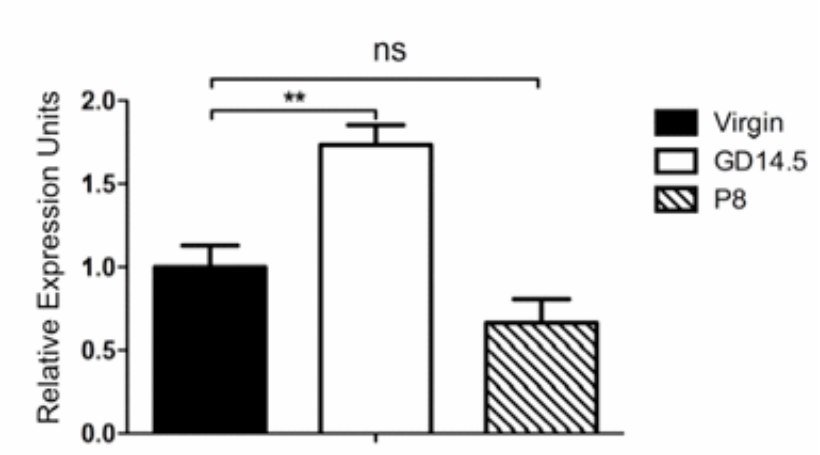


Figure 21. Elevated *Foxm1* expression in pregnancy. qRT-PCR was performed on isolated islets from 8-12 week-old virgin (black bar), GD14.5 (white bar) and P8 *Foxm1*$^{fl/fl}$ females (hatched bar). The relative expression units at GD14.5 and P8 were normalized to that of virgin mice. A 1.7-fold increase in *Foxm1* mRNA was observed in islets from GD14.5 females, compared with islets from virgin females. Unpaired t-tests were used to measure significance. n = 3-6 per group. Error bars represent SD. ** P<0.01. (Zhang et al., 2010, used with permission)

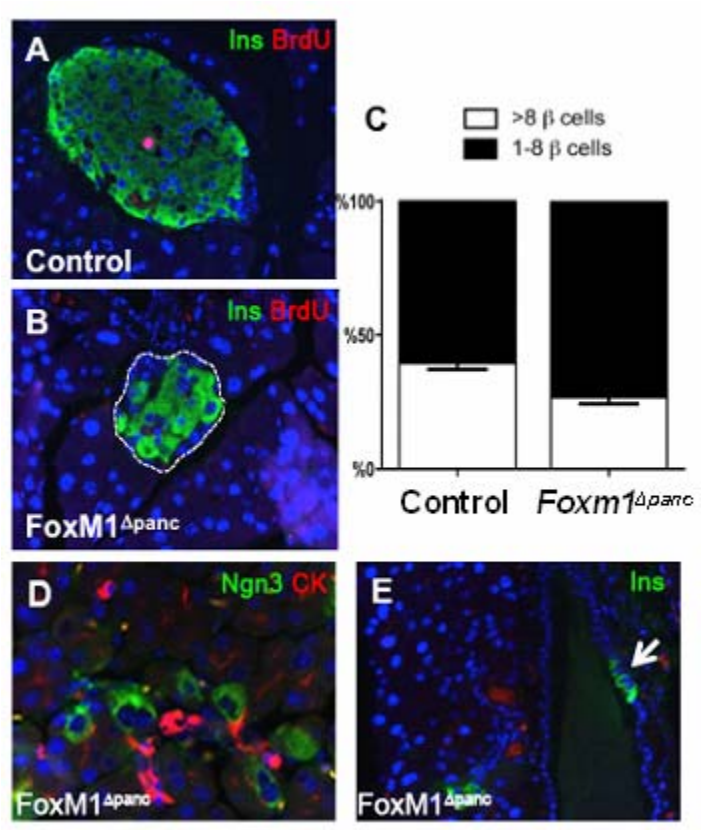


Figure 22. Lasting post-partum changes in *Foxm1*$^{\Delta panc}$ female pancreata. BrDU incorporation (red) was used to assess active β-cell proliferation (insulin: green) in maternal islets at P8. Control islets **(A)** were larger and showed increased BrDU incorporation compared with mutant islets **(B)**. **(C)** The proportion of small islet clusters (1-8 cells) was significantly increased in mutant pancreata at P8 compared with controls (n = 3; P<0.05). Rare Neurog 3^{+} cells (green) were observed adjacent to ducts cytokeratin: red) at P2 (not shown) and P4 **(D)**. Juxtaductal insulin-positive cells were observed in *Foxm1*$^{\Delta panc}$ pancreata at P8 (arrow in **E**). Magnification: 400X. (Zhang et al., 2010, used with permission)

Post-partum Islet Changes in *Foxm1*$^{\Delta panc}$ Females

GDM predisposes human patients to T2DM later in life. Therefore, we examined whether there were any lasting consequences of the absence of Foxm1 after pregnancy other than sustained defects in β-cell proliferation at P8 (Figure 18, 21A and B), islets from *Foxm1*$^{\Delta panc}$ females exhibited a significantly higher percentage of small endocrine cell clusters (1-8 β-cells) compared to controls (Figure 22C). In addition, the average islet size in P8 mutant females was less than 50% of that in the controls (Figure 22A). Despite the severely impaired β-cell mass through late pregnancy and decreased postpartum islet size, β-cell mass was restored to a normal level in the mutants at P8 as revealed by two independent methods (Figure 23B and C). Since dynamic turnover of adult β-cell mass can be regulated through processes including proliferation, hypertrophy, apoptosis and neogenesis, we sought to evaluate these events in both mutants and controls at P8. Glut2 and insulin double immunofluorescence was performed to examine β-cell size. The mutant pancreata showed about a one-third increase in β-cell size in comparison to control pancreata (Figure 23E), suggesting the contribution of hypertrophy to normalized β-cell mass in these mutants at P8. We also examined β-cell apoptosis by both cleaved caspase 3 staining and TUNEL labeling. In both experiments, the extent of apoptosis was indistinguishable between mutants and controls at P4 and P8 (data not shown). Neurog 3 and cytokeratin double labeling revealed Neurog 3^{+} cells adjacent to ducts in mutant pancreata only at P2 and P4 at a rare frequency (approximately 2 to 3 cells per pancreatic section) but not any other stage examined (virgin, GD15.5 and P8) and never in control pancreata (Figure 22D). At later stage (P8), we observed juxtaductal insulin^{+} cells in mutant pancreata, suggesting newborn β-cells (Figure 22E). This evidence further suggests that β-cell neogenesis is induced in the mutant pancreata after pregnancy as a compensatory mechanism to restore β-cell mass. Thus, the increase in small islet endocrine clusters observed in

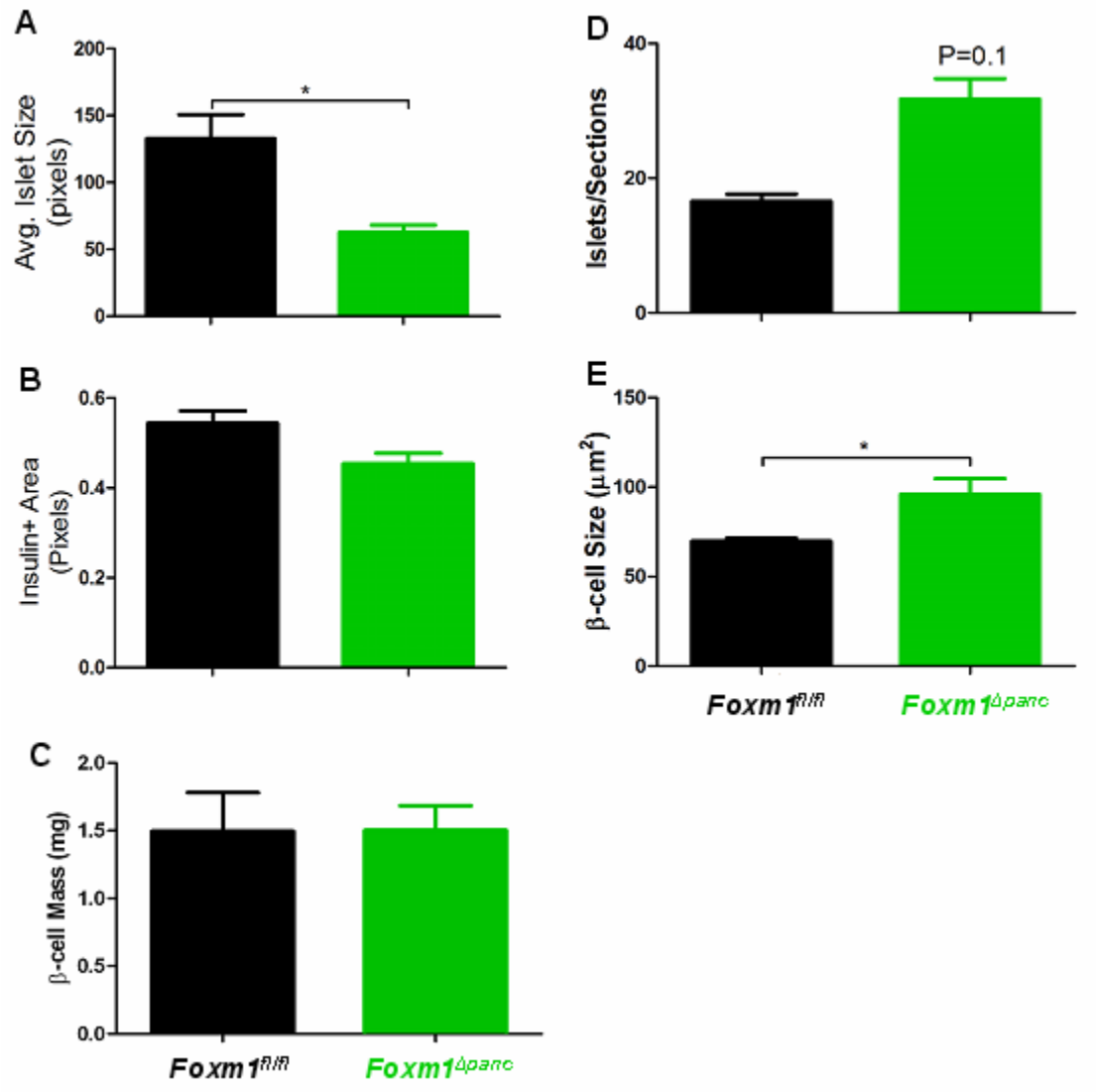


Figure 23. Post-partum β-cell mass is restored in *Foxm1*$^{\Delta panc}$ female mice due to increased islet neogenesis and β-cell hypertrophy. (A) Average islet size was decreased in mutant pancreata, although β-cell mass was normal as assessed by two different calculations **(B,C)**. This was likely due to an increase in the number of insulin^{+} clusters **(D)** and increased average β-cell size **(E)** in mutant pancreata compared with controls. n = 3-4 per group. * P<0.05. (Zhang et al., 2010, used with permission)

mutant pancreata may represent newborn islets. In addition, there were more islets per section in mutants compared to controls (Figure 23D). In conclusion, these results suggest existence of β-cell/islet neogenesis in *Foxm1*$^{\Delta panc}$ female pancreata after pregnancy, which compensated for the impaired capacity to expand β-cell mass due to severely defective β-cell proliferation.

Since an important diagnostic criterion for human GDM is that hyperglycemia resolves after pregnancy, we also evaluated islet function in mutant females at P8 byIPGTT. Consistent with its normalized β-cell mass, the mutants displayed normal blood glucose curve at P8 (Figure 24). *Foxm1*$^{\Delta panc}$ females mimic the human GDM symptoms [(hyperglycemia occurring only during but not before or after pregnancy as judged by GTT (Table 1)]. Therefore, we propose that *Foxm1*$^{\Delta panc}$ mice represent a valuable genetic model for human GDM.

Increased Expression of Cell Cycle Inhibitors in Pregnant *Foxm1*$^{\Delta panc}$ Islets

Since *Foxm1* negatively regulates p27 to promote cell cycle progression and the level of p27 was elevated in postnatal day 1 *Foxm1*$^{\Delta panc}$ islets (Zhang et al., 2006b) we predicted that absence of Foxm1 would cause nuclear accumulation of p27 during pregnancy, thus impairing β-cell proliferation. In normal pregnancy, consistent with the augmentation in β-cell proliferation, lower nuclear p27 level was detected in the GD15.5 control islets compared to virgin controls (Figure 25A and B). *Foxm1*$^{\Delta panc}$ islets at GD15.5 showed a substantial number of endocrine cells with a strong nuclear signal for p27 in contrast with the weak signal in only a few endocrine cells in control islets at this stage (Figure 25B and D). There was no change in total p27 protein between control and mutants at both virgin and GD15.5 as revealed by western blotting using whole islet protein (Figure 25E). The abnormal nuclear accumulation of p27 in islets from pregnant mutants could partially explain the defective β-cell proliferation.

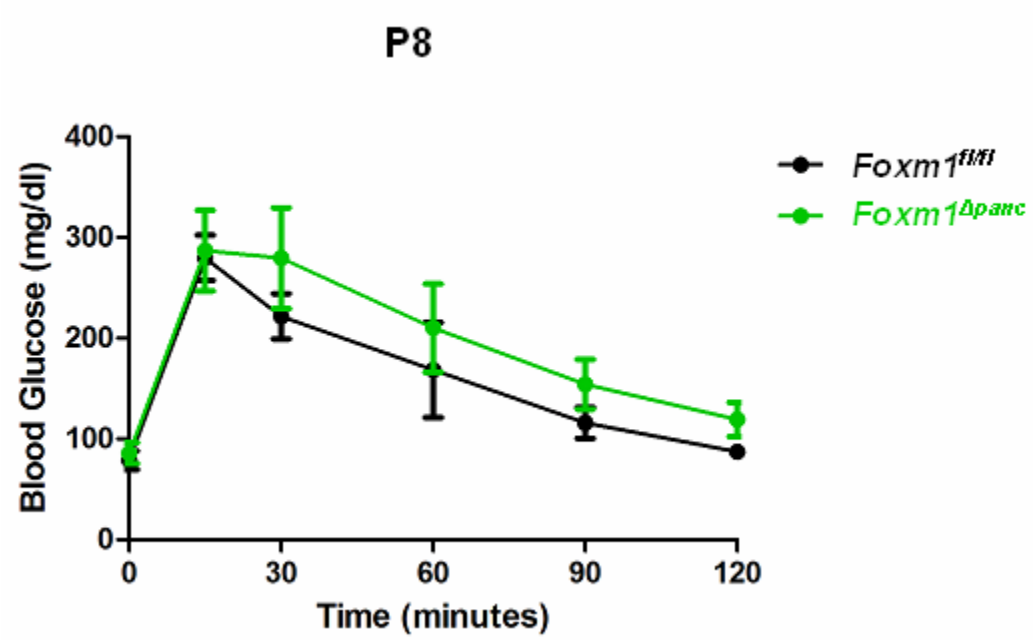


Figure 24. *Foxm1*$^{\Delta panc}$ female mice were euglycemic after pregnancy. IPGTT was performed on P8 *Foxm1*$^{fl/fl}$ (black) and *Foxm1*$^{\Delta panc}$ (green) female mice. The mutants exhibited slightly elevated blood glucose curve but not significantly different from controls. Two-way ANOVA was used to measure significance of difference. n = 5-10 per group. Error bars represent SD. (Zhang et al., 2010, used with permission)

Menin is a downstream effector of PL signaling and a crucial negative regulator of β-cell proliferation during pregnancy (Karnik et al., 2007). Since β-cell specific overexpression of Menin also caused impaired β-cell mass expansion primarily attributed to defective β-cell proliferation, we sought to determine whether Foxm1 negatively regulates Menin to allow the gestational increase in β-cell proliferation. Islets from control mice exhibited significantly lower Menin protein level at GD15.5 compared to virgin stage. In contrast, Menin protein level in *Foxm1*$^{\Delta panc}$ islets did not decrease upon pregnancy. Moreover, compared to pregnant controls, Menin expression was higher in mutant islets than WT by approximate 50% at GD15.5 (Figure 25F). Immunofluorescent labeling of Menin was also performed to understand sub-cellular changes in Menin protein. However, we were not able to repeat the results from the report of Karnik *et al.* (Karnik et al., 2005), which showed no islet signal of Menin at GD15.5 compared to robust Menin nuclear staining at virgin stage. We consistently observed clear and strong nuclear Menin signal even in islets from GD15.5 C57BL/6J females, the same mouse strain used in the study from Karnik et al.

To summarize, these results demonstrated that during pregnancy, Foxm1 promotes maternal β-cell proliferation possibly through its direct or indirect repression on p27 and Menin, two crucial inhibitors of the β-cell cell cycle.

Foxm1 Functions Downstream of PL

PL is the primary stimulatory hormone accounting for the increase in β-cell proliferation/mass during pregnancy. Since *Foxm1* is essential for augmentation of β-cell proliferation in gestation, we hypothesized that *Foxm1* might operate downstream of PL to mediate its proliferative effects on β-cells. *In vitro*, we cultured freshly isolated mouse islets with or without PL treatment for 4-days and then examined *Foxm1* expression in these islets using qRT-PCR. The results showed a two-fold increase in

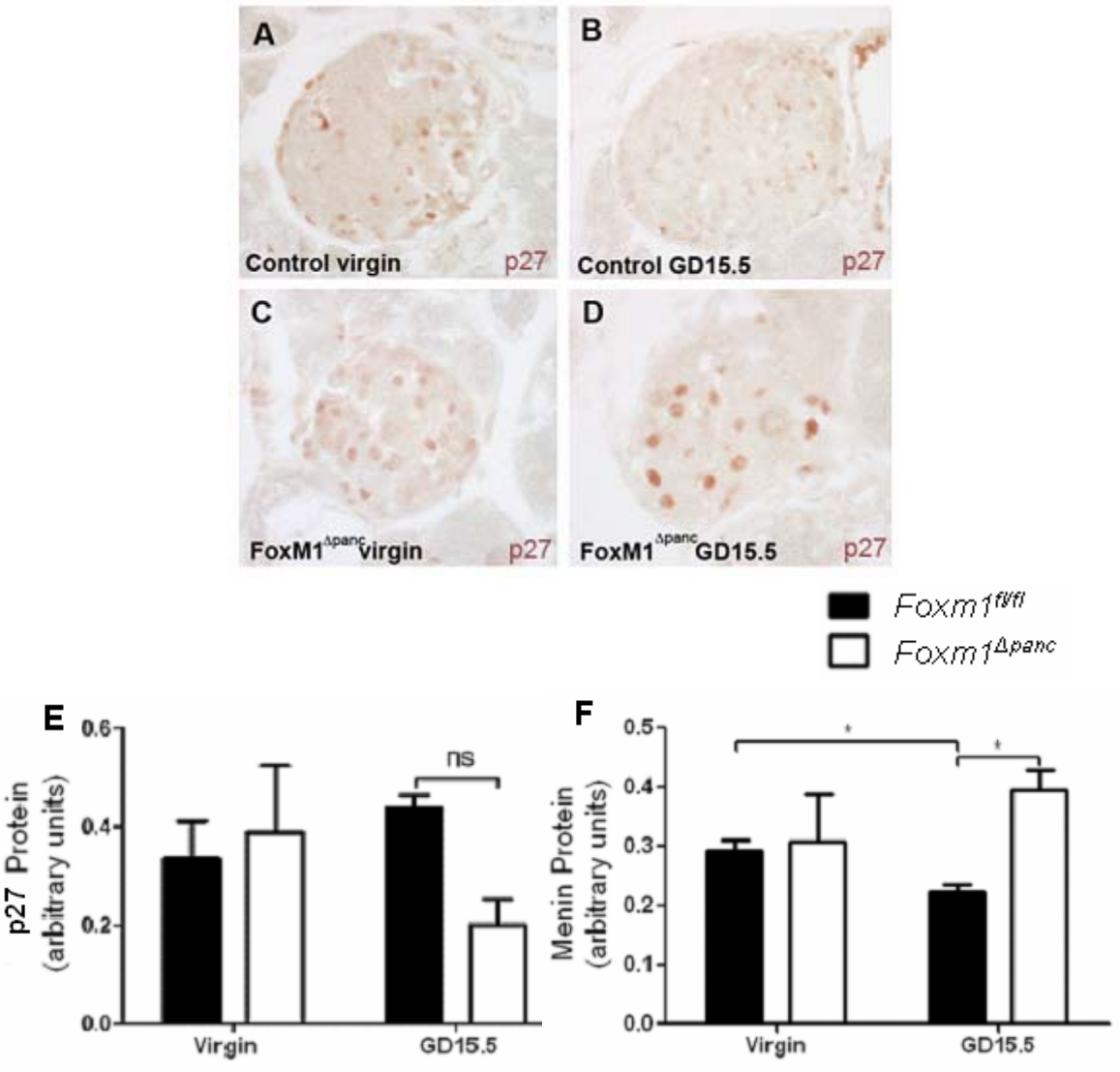


Figure 25. Increased p27 and Menin in *Foxm1*$^{\Delta panc}$ female during pregnancy. p27 (brown) was detected in some islet nuclei in virgin control and mutant pancreata at 8-12 weeks of age **(A,C)**. The number of nuclei strongly positive for p27 decreased in control pregnant females at GD15.5 **(B)** compared with virgin controls **(A)**. In both virgin and GD15.5 mutant islets **(C,D)**, nuclear localization of p27 was increased compared with control islets at GD15.5 **(B)**. The larger nuclei in mutant pancreata are due to the endoreduplication known to occur in the absence of *Foxm1*. Representative images are shown (N = 3 animals per group). Magnification: 400X. **(E)** Quantification of western blotted p27 protein from isolated islets. p27 protein level did not change in mutant islets compared to control at both virgin and GD15.5. **(F)** Quantification of western blotted Menin protein from isolated islets. Menin protein decreased in control islets during pregnancy, but remained elevated in mutant islets. N = 3-4 animals per group. * P<0.05. ns= not significant. Error bars represent SD. (Zhang et al., 2010, used with permission)

Foxm1 mRNA levels in PL- treated islets compared to non-treated islets (Figure 26A), demonstrating that PL induces *Foxm1* expression in this system. We further tested our hypothesis by determining whether β-cell proliferation induced by PL requires Foxm1. To do that, we interbred *Foxm1*$^{\Delta panc}$ mice with *RIP-PL* mice to generate *Foxm1*$^{\Delta panc}$; *RIP-PL* mice, and examined the β-cell proliferation in these mice in comparison to their littermates. Consistent with its established role as a potent stimulus for β-cell proliferation as well as previous published report (Vasavada et al., 2000), overexpression of PL in the β-cells (*RIP-PL*) caused a dramatic increase (approximately 6 fold) in β-cell proliferation in 6wks old females compared to control female littermates (*Foxm1*$^{fl/fl}$) (Figure 26B). The increase in β-cell proliferation was translated into a more than 50% increase in β-cell mass in *RIP-PL* transgenics (Figure 26C). However, despite overexpression of PL in *Foxm1*$^{\Delta panc}$; *RIP-PL* mice, β-cell proliferation was significantly lower than that in *RIP-PL* littermates, suggesting that Foxm1 is required for PL-stimulated β-cell proliferation (Figure 26B). To our surprise, β-cell mass in *Foxm1*$^{\Delta panc}$; *RIP-PL* mice was not significantly different from that in *RIP-PL* (n=2) (Figure 26C). This could be explained by two possibilities: first, since Foxm1 is not required for β-cell proliferation prior to weaning, while *RIP-PL* turned on as early as 12.5 dpc, the embryonic and neonatal expansion of β-cell mass under the stimulation of PL could be dramatic enough to mask the later defects in β-cell mass expansion due to the absence of Foxm1. Second, changes in β-cell mass usually lag behind alterations in β-cell proliferation. It is possible that we would detect a change in β-cell mass consistent with what we saw in β-cell proliferation if we were to assay β-cell mass at a later time point. In conclusion, the above results support our hypothesis that Foxm1 functions downstream of PL to provoke β-cell proliferation.

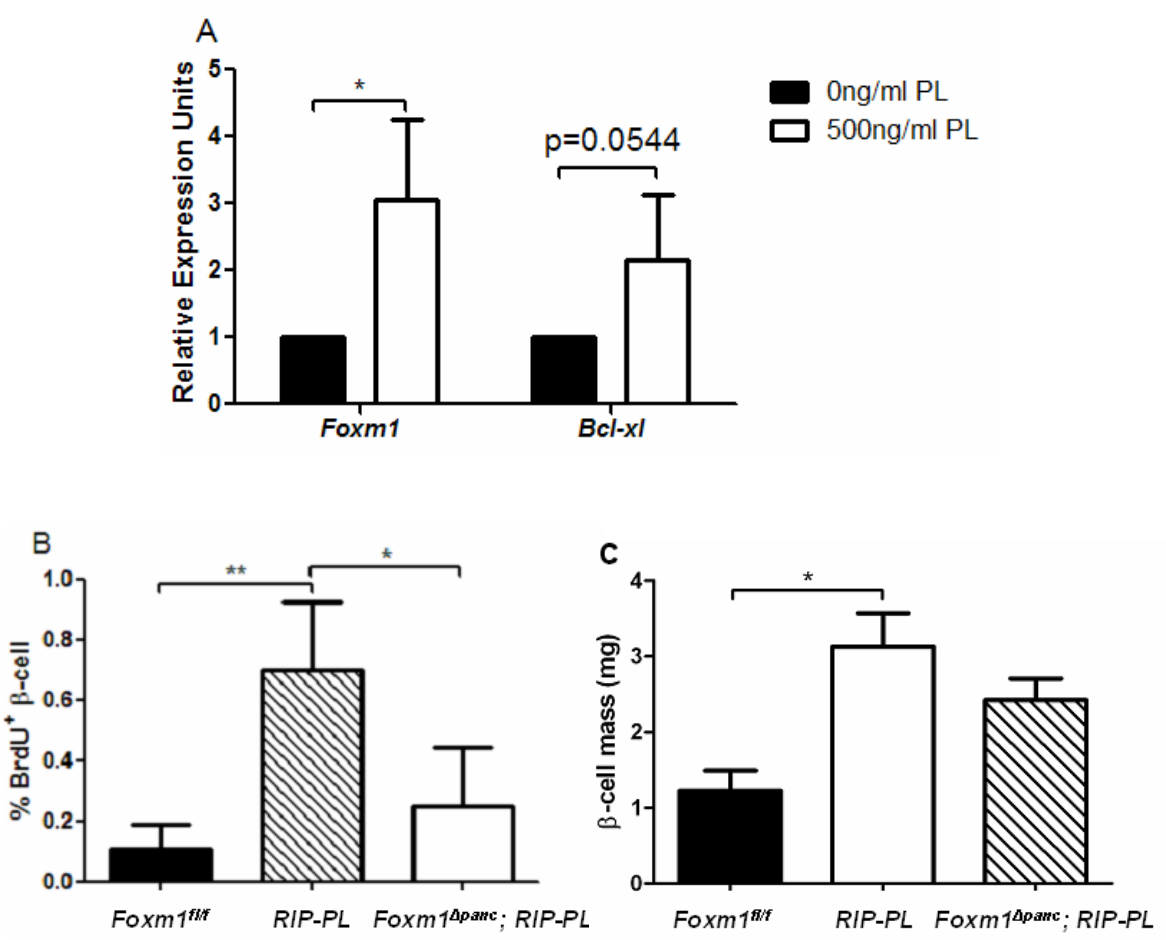


Figure 26. Foxm1 acts downstream of PL to mediate increases in β-cell proliferation and β-cell mass. (A) Expression of *Foxm1* and *Bcl-xl* was elevated in isolated islets in response to four days of PL treatment. n = 5 per group. **(B)** *Foxm1* inactivation completely inhibited PL-mediated induction of β-cell proliferation in *RIP-PL* transgenic mice at 6 weeks of age. n = 3-4 per group. **(C)** Despite lower β-cell proliferation, β-cell mass in *Foxm1*$^{\Delta panc}$; *RIP-PL* mice (n=2) was similar to that of *RIP-PL* (n=4) mice and higher than controls (*Foxm1*$^{fl/fl}$, n=4). Paired, two tailed student t test was used to measure significance. * P<0.05, ** P<0.01. Error bars represent SD. Figure A and B are from (Zhang et al., 2010, used with permission)

Next, we asked whether PL regulates Foxm1 at transcriptional and/or post-translational level. PL signals through the Jak2/Stat5 signaling pathway. Using the TRANSFAC online database, we identified four potential Stat5 binding sites within the 5kb 5' promoter region of the mouse *Foxm1* locus (Figure 27). Therefore, we predicated that *Foxm1* might be directly regulated by Stat5 upon PL stimulation. To test this hypothesis, we performed ChIP assay using both β-cell lines and isolated islets. Our preliminary results demonstrated that STAT5 binds the *Foxm1* promoter region in INS-1 cells treated with human PL for 48 h. We also observed a weaker binding of STAT5 to the promoter of *Bcl-6*, a known target of PL, indicating intact PL signaling in this experimental system (Figure 28).

In conclusion, we provide evidence supporting a model of Foxm1 operating downstream of PL to regulate maternal β-cell proliferation during pregnancy (Figure 29).

Overexpression of PL did not Induce *Foxm1* or its Target Gene Expression

To determine whether PL can regulate *Foxm1*, we examined expression of *Foxm1* and its target genes in *RIP-PL* mice in comparison to wild type littermates. To our surprise, despite the induction of Foxm1 in response to PL treatment in *in vitro* islet culture, we did not observe a change in expression of *Foxm1* or its target genes (*Survivin, Cenp-a* and *Plk-1*) at both postnatal day 2 and 9 weeks of age in this transgenic model (Figure 29).

Discussion

Pregnancy, which naturally induces metabolic demand, represents a valuable model for study of facultative β-cell mass expansion in adults. Previous studies from our laboratory highlighted the importance of *Foxm1*, a pro-proliferation transcription factor, in β-cell replication and mass expansion not only under both normal condition but also in

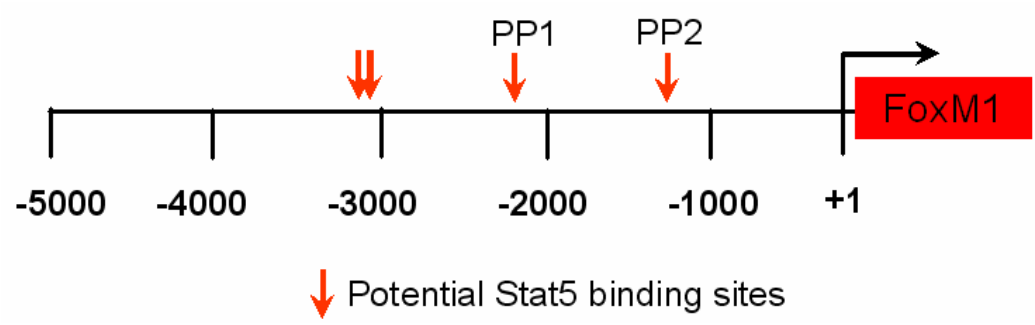


Figure 27. Potential Stat5 binding sites in the mouse *Foxm1* 5' promoter region. TRANSFAC online database was used to search for potential Stat5 binding sites within 5kb 5' promoter region of *Foxm1*. Four potential binding sites were found as the following positions: -3170 to -3150 (ATATTCAAGG AAACT), -3110 to -3009 (AAATGCCAGGAA TTA), -2230 to -2115 (GAATTCAGAGAATTA) and -1270 to -1265 (AGGAAAT).

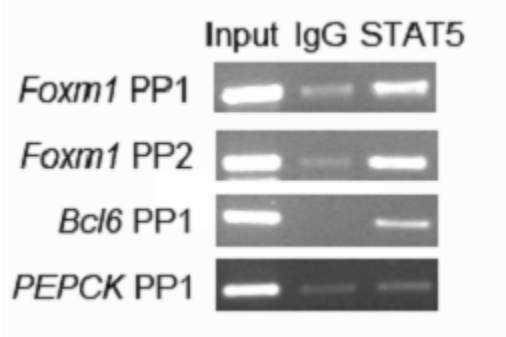


Figure 28. STAT5 binds to the *Foxm1* promoter in PL treated INS-1 cells. ChIP assay was performed using INS-1 cells treated with 500 ng/ml human PL for 16 hours. *Foxm1* PP1 primers amplify a 378 bp fragment spanning the putative binding site of STAT5 located at -2230 to -2115bp of *Foxm1* locus. *Foxm1* PP2 primers amplify a 306 bp fragment spanning the putative binding site located at -1270 to -1265bp of *Foxm1* locus. *Bcl6* PP1 primer set produces a 380 bp fragment spanning a STAT5 binding site within its 5' promoter region. As a negative control, PEPCK PP1 primer set was used to amplify the STAT5 binding site within the *Phosphoenolpyruvate carboxykinase (PEPCK)* promoter.

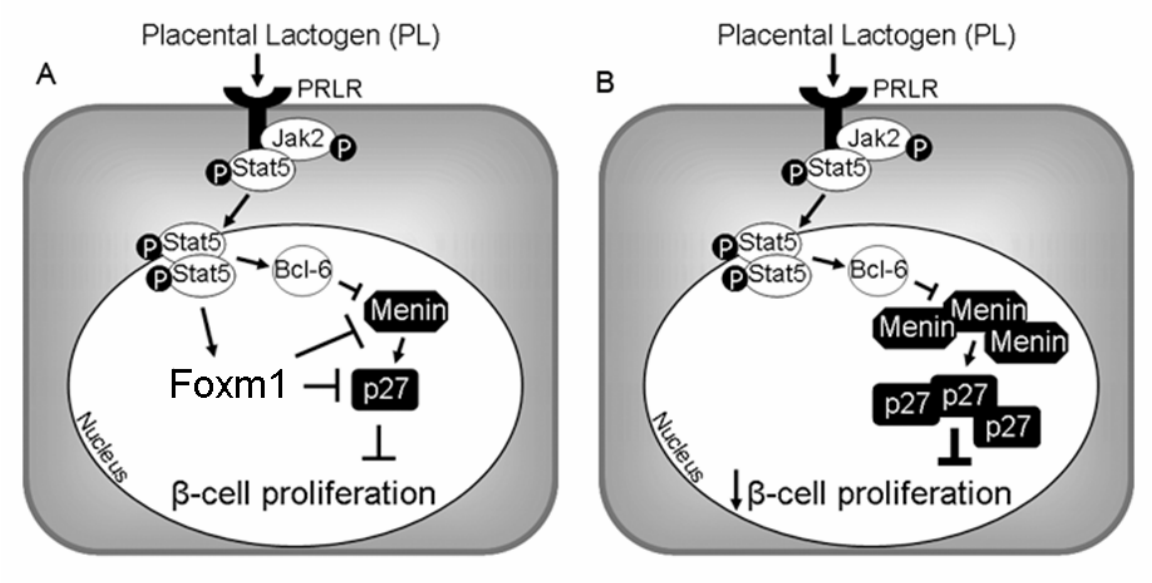


Figure 29. Model of PL and Foxm1 regulation of β-cell proliferation during pregnancy. (A) In β-cells of normal pregnant mice, PL activates Jak2/Stat5 signaling pathway through the binding to its receptor (PRLR). Nuclear accumulation of phospho-Stat5 transcriptionally inhibits Menin via its direct target Bcl-6. Attenuated Menin levels results in reduced p27 expression, thereby promoting the increase of maternal β-cell proliferation. In response to PL, Foxm1 functions as a direct target of phospho-Stat5 to stimulate β-cell replication via its inhibitory effects on Menin and p27. **(B)** In the absence of Foxm1, increased nuclear Menin and p27 prevent the augmentation of β-cell proliferation in pregnancy, thereby promoting GDM.

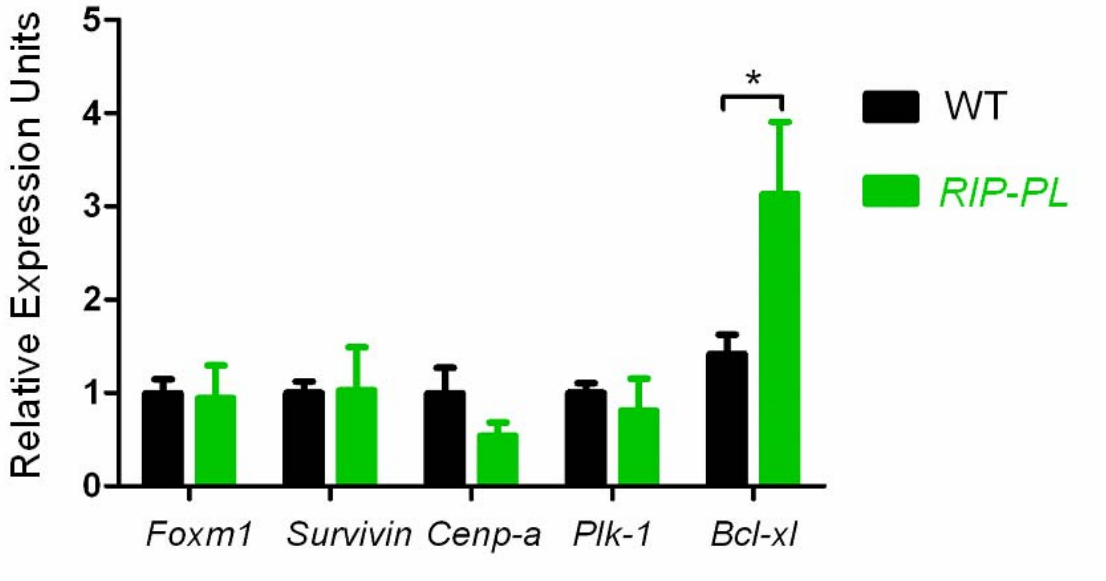


Figure 30. PL overexpression did not increase expression of *Foxm1* or its target genes. Quantitative RT-PCR revealed that *Foxm1* message levels were not significantly different between *RIP-PL* transgenic (green bar) and non-transgenic (black bar) littermates at 9 weeks of age. Likewise, Foxm1 target gene expression was unchanged in *RIP-PL* islets, although *Bcl-xl* expression was significantly increased, indicating intact PL signaling. Relative expression units of *RIP-PL* samples were normalized to those of wild type samples. Unpaired student's t-tests were used to measure significance. n= 4 per group. Error bars represent SD. *P<0.05.

response to stimuli such as PPx and high fat diet. This study presented here demonstrated that during pregnancy, *Foxm1* is essential for maternal β-cell replication and mass expansion, at least partly through repressing Menin and p27, two crucial β-cell cell cycle inhibitors. *Foxm1*$^{\Delta panc}$ females developed overt GDM. Moreover, we identified *Foxm1* as a novel distal effector of PL signaling in the β-cell based on the following observations: (a): Islet *Foxm1* is induced during pregnancy or by *in vitro* PL treatment. (b): *Foxm1* is absolutely required for PL stimulated β-cell proliferation in a transgenic mouse model overexpressing PL specifically in theβ-cell. (c): *Foxm1* is a direct target of phospho-Stat5.

In islets from pregnant *Foxm1*$^{\Delta panc}$ females, western blotting revealed significantly increased Menin levels compared to pregnant controls. However, comparing islets from control virgins to those from pregnant control mice (GD15.5), we only observed a significant yet mild decrease in Menin protein levels. This is different from a previous report from Seung Kim's group which showed more than 60% reduction in Menin mRNA and an even more dramatic decrease in Menin protein levels at GD16 compared to virgin stage. This discrepancy can be explained by the possibility that *Foxm1* regulates Menin primarily at the post-translational level, which promotes the nuclear export of Menin instead of affecting its total protein levels. Future experiments compartmentalizing the nuclear versus the cytoplasmic protein extracts would help to clarify this issue.

Similar to the current view on the pathogenesis of human GDM, GDM in *Foxm1*$^{\Delta panc}$ females is likely due to a combination of blunted β-cell mass expansion, impaired insulin production and presumably islet function. Uneven insulin reactivity in GD15.5 mutants as revealed in immunolabeling suggested defects in insulin biosynthesis (Figure 20E, note the weakly labeled cells). This notion is also supported by the dramatic reduction in total pancreatic insulin contents in these mutants (Figure 20B). In addition, preliminary data from our laboratory showed impaired insulin secretion in

Foxm1$^{\Delta panc}$ females fed a high fat diet (Dr. A. Ackermann Misfeldt, U.G. Kopsombut, and Dr. M.Gannon., unpublished observations). It would be interesting to assess islet function of *Foxm1*$^{\Delta panc}$ females under the challenge of pregnancy using an islet perifusion system and examine β-cell morphology using electron microscopy. It is unclear whether compromised β-cell function in the mutants can be directly or indirectly attributed to loss of Foxm1 activity. Future microarray and ChIP-seq studies would provide insights into new Foxm1 targets in the β-cell indicative of Foxm1 function other than cell proliferation.

The patchy immunolabeling of insulin in the GD15.5 mutant islets may also represent unhealthy or dedifferentiated β-cells. To determine whether the β-cells in *Foxm1*$^{\Delta panc}$ females are dying, we performed cleaved caspase 3 staining. The results did not show a significant difference in pregnant mutant compared to pregnant control mice, consistent with the notion that Foxm1 is not involved in cell apoptosis in other systems (Wierstra & Alves, 2007) and the loss of Foxm1 did not affect β-cell apoptosis in *Foxm1*$^{\Delta panc}$ male mice reported by our laboratory (Zhang et al., 2006b). To examine whether deletion of Foxm1 causes fate transformation of β-cells, we assessed Pdx1 expression by both immunofluorescence and western blotting. Neither revealed a change of Pdx1 expression in *Foxm1*$^{\Delta panc}$ female islets compared to controls at both virgin and GD15.5. This result indicated that despite the stress due to the loss of FoxM1, β-cells are still able to maintain their identity, at least at the time points examined. To strengthen this conclusion, other mature β-cell markers should be assessed including MafA, Pax4 and Nkx6.2. In *Foxm1*$^{\Delta panc}$ males, we oberseved a change in ratio of glucagon versus insulin positive cells due to the loss of insulin+ cells driven by senescense (Zhang et al., 2006b) rather than a absolute decrease in glucagon^{+} cell number. Therefore, fate conversion among endocrine cell types is not likely to occur in our *Foxm1*$^{\Delta panc}$ female with diabetic pregnancy. However, it is possible that gender

difference leads to new phenotypes we did not anticipate. Immunolabeling of different endocrine cell types will help to clarify this concern.

In the mutant pancreas after pregnancy, we observed several lines of evidence of putative β-cell/islet neogenesis as a compensation for deficient islet growth: (a): Neurog 3^+ cells adjacent to the duct cells in P4 mutant pancreata. (b): Insulin$^+$ cells located in the ductal epithelium in P8 mutant pancreata. (c): A proportional increase in small islet clusters (1-8 β-cells) in mutants compared to controls at P8. (d): Increased islet number per section in mutants compared to controls at P8. These results are consistent with the previous finding from our laboratory that *Foxm1* is not required for β-cell neogenesis (Ackermann Misfeldt et al., 2008). Neurog 3, a marker of endocrine cell progenitors, is robustly expressed in developing pancreas and remains active in adult pancreas at a very low level (Wang et al., 2009b). Neurog 3 expression is reactivated in pancreatic ductal epithelium in some scenarios of β-cell neogenesis in adults, such as 60% partial pancreatectomy (Ackermann Misfeldt et al., 2008) and partial duct ligation (Xu et al., 2008), as well as in α-cell neogenesis models, such as mice with *Pax4* overexpression (Collombat et al., 2009) and with *Glucagon receptor (Gcgr)* deficiency (Furuta et al., 1997), marking endocrine progenitors in adults. However, immunolabeling of Neurog 3 is traditionally challenging (Collombat et al., 2009). We never observed Neurog 3^+ signal in control pancreata. In the mutant pancreata, Neurog 3^+ cells only appeared at P2 and P4 but not other stages examined in cells adjacent to CK19$^+$ ductal cells (virgin, GD15.5 and P8), preceding the expression of insulin in the ductal epithelium at P8. This population of Neurog 3^+ detected in our experiments may represent a snapshot of newborn progenitors just delaminating from the ductal epithilum. These cells go on to differentiate into insulin$^+$ cells. We did detect insulin$^+$ cells within the ductal epithelium at a later stage, P8. The cytoplasmic signal of Neurog 3^+ we detected could be due to the difference in tissue embedding and fixation conditions. In addition, due to

poor quality of adult pancreas RNA, we were unable to quantify the induction of *Neurog 3* in postpartum *Foxm1*$^{\Delta panc}$ females. β-cell neogenesis has not been reported during normal pregnancy in mice but recently observed in human by the Butler group (Minh, 2008). Therefore, it is meaningful to further characterize the neogenesis occurring in post-patrum Foxm1 mutants. Future experiments using a *Neurog 3-GFP* transgene to label endocrine progenitors in pregnancy would not only determine the existence of β-cell neogenesis during pregnancy, but also confirm whether the inactivation of *Foxm1* in gestation activates the β-cell neogenesis program.

In our study, Foxm1 is efficiently inactivated as early as 15.5 dpc (Zhang et al., 2006b). The loss of Foxm1 may have certain cumulative effects starting from embryogenesis that would complicate the phenotype we observed in pregnancy. To address this concern, we sought to inactivate *Foxm1* after weaning using the CreER system and then examine glucose levels during pregnancy. Total 4mg Tamoxifen (TM) was injected into 4 week-old *Foxm1*$^{fl/fl}$; *Pdx1*^{PB}*-CreER* mice over the course of five days. Then these mice were mated at 8 week of age, sacrified and analyzed at GD 15.5. Previous unpublished results from our laboratory have confirmed that this regimen results in recombination of the R26R reporter in approximately 90% of the β-cells. Unfortunately, due to the harmful effect of TM during pregnancy, we found that 3 out of 7 mice (both controls and mutants) that received TM showed no embryos in the uterus at all despite a vaginal plug, the remaining four mice had very small litter sizes (approximately 4 embryos per litter). Moreover, 50% of the embryos were in the process of being resorbed and likely died at a very early stage in embryogenesis. Due to these obstacles, we were unable to complete this experiment.

The crucial pro-proliferation role of Foxm1 in the β-cell makes it a promising candidate to enhance adult β-cell mass. In the liver, overexpression of *FOXM1C* (*TTR-FOXM1C*) accelerates hepatocyte proliferation following PHx (Ye et al., 1999) with no

association with hepatocellular carcinoma (HCC) progression (Kalinina et al., 2003). However, in the pancreas, overexpression of *FOXM1C* in *ROSA26-FOXM1*C transgenic mice following PPx does not cause any detectable difference in β-cell proliferation, transcription levels of *Foxm1* targets and glucose tolerance compared to WT littermates, despite the over a thousand-fold increase in islet *Foxm1* expression levels in the transgenic animals (Ackermann Misfeldt et al., 2008). These results demonstrated that simply increasing the amount of *Foxm1* protein, even in conjunction with PPx as an external stimulus, is not sufficient to enhance Foxm1 function in the pancreas. Other stimuli (kinases) are needed to post-translationaly modify thus activate Foxm1 (presumably through phosphorylation). On the other hand, some unknown negative feedback machinery may exist in the β-cell to restrain the expression/activity of Foxm1. Release of these "brakes" on top of Foxm1 overexpression may be necessary to achieve the full potential of Foxm1 in promoting β-cell cell cycle. Alternatively, manipulation of Foxm1 itself, such as overexpression of a constitutively active version of Foxm1 missing the N-terminal inhibitory domain thus bypassing the need for kinases (Park et al., 2008b), could be a promosing avenue to augment the activity of Foxm1.

β-cell proliferation normally decreases as animals age. This is partly due to progressively increased p16 and decreased expression of Bmi-1 (a component of a histone H2A ubiquitin complex) which represses p16 expression (Dhawan et al., 2009). In the liver, overexpression of *Foxm1* can reverse the age-associated reduction in hepatocyte proliferation (Wang, Kiyokawa et al., 2002; Wang et al., 2002b). In the pancreas, Foxm1 expression decreases with age (Zhang et al., 2006b). Therefore, elevated Foxm1 function may be able to make older β-cells more responsive to proliferative cues. The connection of Foxm1 and Bmi-1 was first studied in mouse embryonic fibroblast cells. One of the isoforms of FOXM1, FOXM1c counteracts the oxidative stress induced cell senescence through upregulating Bmi-1, thus repressing

p16/p19 (Li et al., 2008). It is reasonable to speculate that Foxm1 also positively regulates Bmi-1 in the β-cells. qRT-PCR or immunolabeling to examine the Bmi-1 expression in *Foxm1*$^{\Delta panc}$ islets compared to control islet would be informative experiments. Future studies such as temporary overexpression of the active form of Foxm1 in older islets either *in vitro* or *in vivo*, and transplantation of human islets overexpressing Foxm1 into mice would further assess whether Foxm1 could be the "fountain of youth" for the β-cell without severe adverse side-effects such as tumorgenesis.

Overexpression of PL *in vivo* (*RIP-PL*) induced *Bcl-x*$_L$, but not *Foxm1* and its target genes at both postnatal day 2 (the lasted time point we are able to get good quality pancreatic RNA) and 9 weeks of age (representing adult stage). However, in *in vitro* cultured islets, PL treatment caused significant increase in *Foxm1* expression. This discrepancy could be explained by two possibilities: first, long term *in vivo* exposure to PL may elicit certain negative feedback regulation that downregulates *Foxm1* expression. Second, steroid hormones, such as progesterone and glucocorticoids (GC) counteract the mitogenic effects of PL during late pregnancy (Weinhaus et al., 2000). Therefore, *in vivo*, the effect of long term overexpression of PL on *Foxm1* expression may be attenuated due to the activity of progesterone. This possibility is evident by one study investigating the role of pregnancy hormones in regulation of *Pdx1* expression. Similar to our results, this study also reported an induction of *Pdx1* by prolactin *in vitro* but not *in vivo* (in islets from pregnant mice) (Nasir et al., 2005). Dexamethasone (DEX), the synthetic glucocorticoid class of steroids, opposes the prolactin-induced increase of *Pdx1* expression at the concentration similar to that present in pregnancy. This inhibitory effect of DEX on PL-induced gene expression was reiterated by the finding that simultaneous exposure of isolated mouse islets to prolactin and DEX attenuated changes in *Men1, p18 and p27* expressions provoked by prolactin alone (Karnik et al.,

2007). In addition, two members of the suppressor of cytokine signaling family of proteins, *Cytokine-inducible SH2-containing protein (Cish)* and *Suppressor of cytokine signaling 2 (Socs2)* are robustly induced at the transcriptional level in pancreatic islets during pregnancy as revealed by a microarray study (Rieck et al., 2009b). Cish and Socs2 interact with PRLR to decrease the activation of STAT5 (Greenhalgh et al., 2002; Endo et al., 2003), representing a new regulatory mechanism by which the system prevents excess PL activity. It is possible that *Cish* and *Socs2* are induced in the *RIP-PL* mice in response to prolonged PL overexpression to a significant level that attenuates the effect of PL on *Foxm1* transcription and/or activity.

Our *in vivo* evidence (Figure 26B) demonstrated Foxm1 mediates the mitogenic effects of PL in the β-cell. However, loss of *Foxm1* in *Foxm1$^{\Delta panc}$; RIP-PL* animals did not completely abolish β-cell proliferation, suggesting PL may stimulate β-cell proliferation via Foxm1-independ pathways. Moreover, despite significantly lower β-cell proliferation in *Foxm1$^{\Delta panc}$; RIP-PL* compared to that in *RIP-PL* mice, β-cell mass in these two types of animals are surpisingly similar (Figure 26C). This could be explained by: first, changes in β-cell mass usually lag behind alterations in cell proliferation. Second, in *RIP-PL* mice, overexpressed of PL thus its mitogenic effects initiate as early as 12.5 dpc while the requirement of Foxm1 for proliferation does not start until approximately 3 weeks of age. In our study, β-cell proliferation and mass were assessed at the same age, 6 weeks of age. Therefore, it is likely that the adverse consequence of losing Foxm1 takes longer to be recognized. Alternatively, the anti-apoptic role of PL also contributes to the augmentation of β-cell mass in *RIP-PL* mice. This effect of PL could also attenuate the difference in β-cell mass in *Foxm1$^{\Delta panc}$; RIP-PL* and *RIP-PL* mice if Foxm1 is not involved in mediating this effect.

Several signaling pathways other than Jak2/Stat5 are implicated in mediating the mitogenic effects of lactogen hormones on the β-cell, including MAPK and PI3K/Akt *in*

vitro. It is possible that these pathways also signal through Foxm1, either by increasing its expression or by enhancing its activity, to provoke β-cell proliferation. In addition, GH, whose expression also elevated during pregnancy, induces Foxm1 in liver. Therefore, the increase in *Foxm1* levels in response to pregnancy could be due to a combinatory effect of multiple hormones.

CHAPTER IV

ANALYSIS OF FOXM1 FUNCTION IN HGF AND IGF-1 STIMULATED β-CELL PROLIFERATION IN ADULTS

Introduction

Growth factors, including HGF, IGF's, lactogens, glucagon-like peptide-1(GLP-1), and parathyroid hormone-related protein (PTHrP), are known potent β-cell mitogens. Understanding how these factors function in β-cells would not only facilitate their application as therapeutic tools for diabetes, but also broaden our knowledge of novel signaling pathways and factors governing β-cell replication in general.

HGF increases β-cell proliferation in both fetal and adult human islets (Otonkoski et al., 1994; Hayek et al., 1995; Beattie et al., 1996; Beattie et al., 2002). Both HGF and its receptor c-Met are expressed in rodent and human islet cells (Otonkoski et al., 1996) and their upregulation has been observed in multiple β-cell regeneration models such as mice treated with STZ and non-obese diabetic (NOD) mice (Vasavada et al., 2006). To study the function of HGF in the islets *in vivo*, the Garcia-Ocaña group developed and characterized *RIP-HGF* transgenic mice. These mice exhibited two to three times higher β-cell replication, resulting in a two to three-fold increase in β-cell mass and a two-fold increase in the insulin content (Figure 30) (Vasavada et al., 2006), mild hypoglycemia and inappropriate hyperinsulinemia (Gahr et al., 2002). However, β-cell specific ablation of c-Met does not affect β-cell growth. Instead, it leads to impaired islet function as manifested by decreased insulin secretion, diminished GLUT2 expression, with resultant impaired glucose tolerance (Roccisana et al., 2005). These results suggested that the HGF/c-MET signaling pathway is important for normal β-cell function but dispensable for β-cell proliferation under normal circumstances. *In vitro* experiments in INS-1 cells

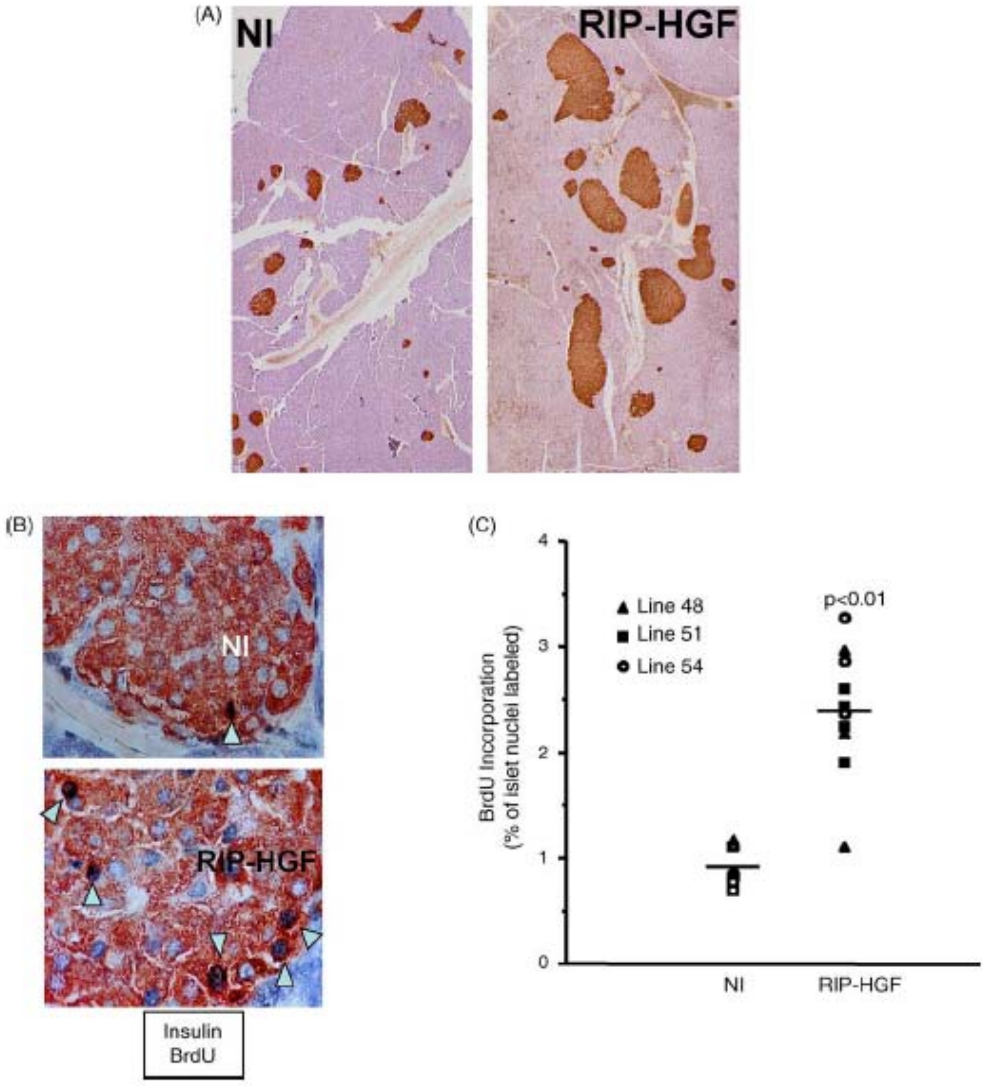


Figure 31. Overexpression of HGF in the β-cell results in increased β-cell proliferation and β-cell mass. (A) Representative pancreatic sections from normal (NI) and *RIP-HGF* mice stained for insulin. **(B)** Representative photomicrographs of pancreatic islets from normal and *RIP-HGF* mice stained for insulin and BrdU. Arrows indicate BrdU-positive β-cell nuclei. **(C)** Quantitation of β-cell proliferation rates in normal and *RIP-HGF* mice [(Vasavada et al., 2006), used with permission].

supported that HGF-induced cell proliferation requires PI3K and atypical PKCs, but not ERK1/ERK2 (Gahr et al., 2002). These pathways are downstream of multiple external stimuli for β-cell proliferation. Therefore, other signaling pathways that promote cell proliferation may compensate for loss of c-MET activity in β-cells. However, it remains possible that under metabolic demanding conditions, HGF/c-MET is required for β-cell replication.

The best studied members of the IGF family are insulin, IGF-1 and IGF-2. Both IGF-1 and 2 can induce β-cell proliferation *in vitro* as demonstrated in rat islets and insulinoma cell lines, with IGF-1 being a more potent mitogen compared to IGF-2 in neonatal rat islets (Swenne et al., 1987; Sieradzki et al., 1988; Hogg et al., 1993). Overexpression of IGF-1 in transgenic mice using the rat insulin promoter results in a three-fold increase of both β-cell proliferation and β-cell mass (George et al., 2002). ERK1/2 and PI3K and subsequent induction of mTOR/p70(S6K) appear to play an important role in mediating the mitogenic effect of IGF-1 on β-cells (Hugl et al., 1998; Dickson et al., 2001; Lingohr et al., 2002).

Most of the studies that shed light on the mechanism by which HGF and IGF-1 stimulate β-cell proliferation focused on the second messenger signaling pathways. So far, no transcription factors have been identified to link the induction of HGF and IGF-1 to β-cell proliferation. Our laboratory proposes that Foxm1 is a common target of multiple β-cell cycle stimuli. The aim of this project was to determine whether Foxm1 promotes β-cell proliferation downstream of HGF and/or IGF-1.

Results

RIP-HGF Transgene Remained Actively Expressed After Genetic Backcrossing.

We obtained *RIP-HGF* (CD-1 background) from Dr. Adolfo Garcia-Ocaña at the University of Pittsburgh (Vasavada et al., 2000). To avoid the potential confounding

effects due to mixed genetic background, we backcrossed *RIP-HGF* for 6 generations to the C57BL/6J strain. Transgenes are sometimes inatctivated after a certain numbers of generations of mating. Therefore, we sought to examine the expression of HGF after backcrossing. qRT-PCR was performed to examine HGF expression in isolated islets from 9 weeks of age *RIP-HGF* and wild type littermates. The results showed robust HGF expression in transgenics compared to wild type (Figure 32A). In addition, BrdU incorporation assay revealed a more than 50% increase in β-cell proliferation in backcrossed *RIP-HGF* compared to control littermates as early as 6 weeks of age (Figure 32B), suggesting an active mitogenic effect of HGF in *RIP-HGF* islets.

Overexpression of HGF in the β-cell did not Induce the Expression of *Foxm1* or its Target Genes.

If Foxm1 functions downstream of HGF, we predict that overexpression of HGF induces *Foxm1* expression or activity. Thus, we sought to examine the expression of *Foxm1* and its targets (as a measure of the activity of FoxM1) in isolated islets from *RIP-HGF* and wild type mice. To our surprise, we did not observe changes in RNA levels of *Foxm1* or its target genes including *Cenp-a*, *Ccnd2* and *Plk-1* (Figure 33) at 9 weeks of age, suggesting that at least at this stage, ongoing HGF expression is unable to induce *Foxm1 in vivo*. The potential upregulation of *Foxm1* by HGF could be transient, thus earlier time points should be examined in the future.

***RIP-IGF-1* Transgene was Actively Expressed After Backcrossing.**

RIP-IGF-1 mice (CD-1 background) were generously provided by Dr. Robert Ferry at the University of Tennessee Health Science Center at Memphis. *RIP-IGF-1* mice were backcrossed for 9 genearations to the C57BL/6J strain. Immunofluorescent

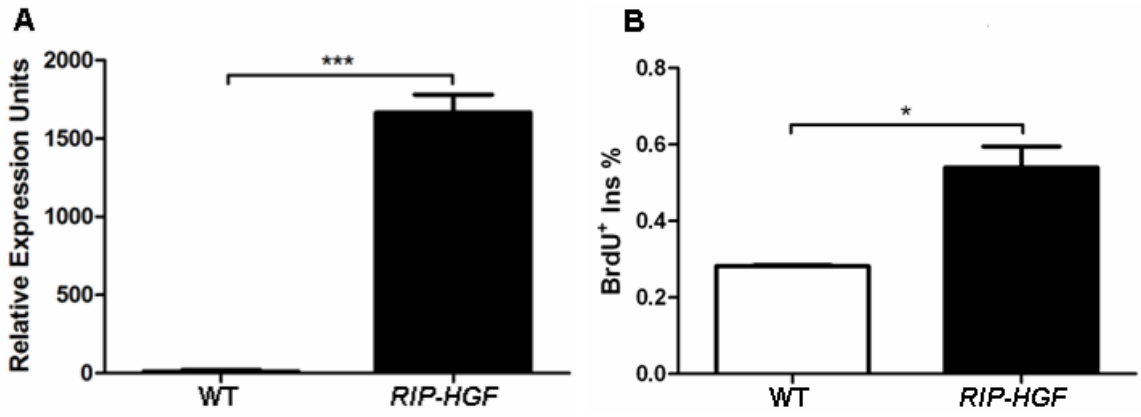


Figure 32. Active *RIP-HGF* transgene in backcrossed *RIP-HGF* mice. *RIP-HGF* mice on CD-1 background were backcrossed for 6 generation to C57BL/6J background. **(A)** qRT-PCR using islets from 8 week old male mice showed over 1000 fold higher HGF transcript level in the RIP-HGF (black bar) in comparison to wild type (WT, white bar) littermates. (n=4) **(B)** Increased β-cell proliferation in 6 week old *RIP-HGF* males (black bar) compared to WT (white bar) littermates was revealed by BrdU incorporation (n=3). Unpaired, two tailed student's t-test was used to measure significance. * P<0.05, *** P<0.001.

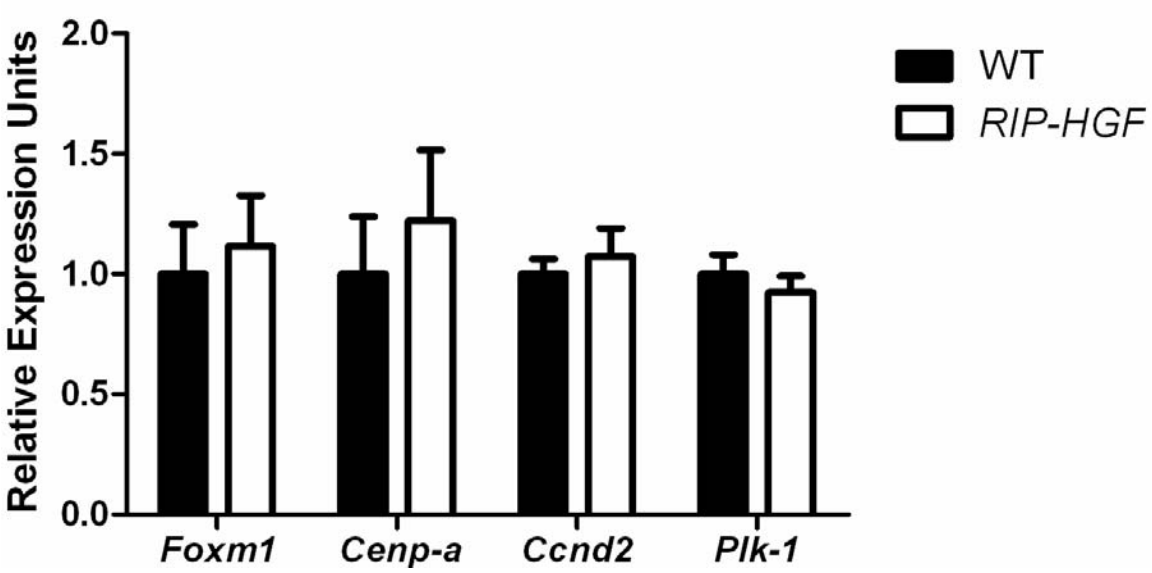


Figure 33. Overexpression of HGF was not able to induce *Foxm1* or its target genes. qRT-PCR was performed on isolated islets to evaluate the RNA levels of *Foxm1* and its target genes (*Cenp-A, Ccnd2 and PIK-1*) in *RIP-HGF* (white bar) mice in comparison to WT (black bar) littermates at 9 weeks of age. Similar expression levels of all genes examined were observed in transgenics and WT. Relative expression levels of genes in the RIP-HGF were normalized to that in the WT. n=3.

staining revealed strong IGF-1 expression in backcrossed transgenics compared to controls (Figure 34), suggesting that the transgene remained active after backcrossing.

Overexpression of IGF-1 in the β-cell did not induce the expression of *Foxm1* or its target genes.

Taking advantage of *RIP-IGF-1* mice, we sought to determine whether overexpression of IGF-1 is capable of inducing *Foxm1 in vivo*. Islet expression of *Foxm1* and its targets at 9 weeks of age were similar between *RIP-IGF-1* and WT littermates demonstrated by qRT-PCR (Figure 35).

Discussion

Both HGF and IGF-1 are promising candidates for expanding adult β-cell mass. However, their downstream effectors have been poorly understood, which hinders the clinical applications of these growth factors. Here, we initiated our effort to determine whether Foxm1, a crucial β-cell proliferative transcription factor, operates downstream of HGF and/or IGF-1. We first confirmed the mitogenic effects of these two hormones on the β-cell in C57BL/6J genetic background by examining islet expression of HGF and IGF-1 and β-cell proliferation. High level of HGF (examined by qRT-PCR) in the islets induced a significant increase in β-cell proliferation and mass. We also observed robust IGF-1 expression in transgenic islets compared to controls. The proliferation assay on *RIP-IGF-1* is in progress. We are currently testing whether *Foxm1* is essential for HGF and/ or IGF-1-induced β-cell proliferation *in vivo* by analyzing *Foxm1*$^{\Delta panc}$; *RIP-HGF* and *Foxm1*$^{\Delta panc}$; *RIP-IGF-1* mice in comparison with control animals.

Neither the *in vivo* expression of HGF or IGF-1 induced *Foxm1* or its targets, implying that HGF and IGF-1 may not signal through *Foxm1*. However, similar to our results on PL, this result could be due to the counteracting effects of other factors in the animal. To avoid this complication, we attempted to examine the induction *Foxm1*

expression by HGF or IGF-1 in dispersed islet cell clusters *in vitro*. Unfortunately, we have not been able to maintain healthy cell clusters long enough to allow the detection of *Foxm1*.

Other second messenger pathways, such as PKCζ, PI3K/Akt and MAPK/ERK stimulate β-cell proliferation downstreamd of HGF or IGF-I. If *Foxm1* is a downstream target of HGF and IGF-1 signaling, in the future, we will continue to investigate whether these pathways are responsible for *Foxm1* induction. The information obtained from this study will not only shed light on the transcriptional and/or post-translational regulatory mechanism of *Foxm1* but also uncover new therapeutic targets to the maintenance and augmentation of adult β-cell mass and function for a cure to diabetes.

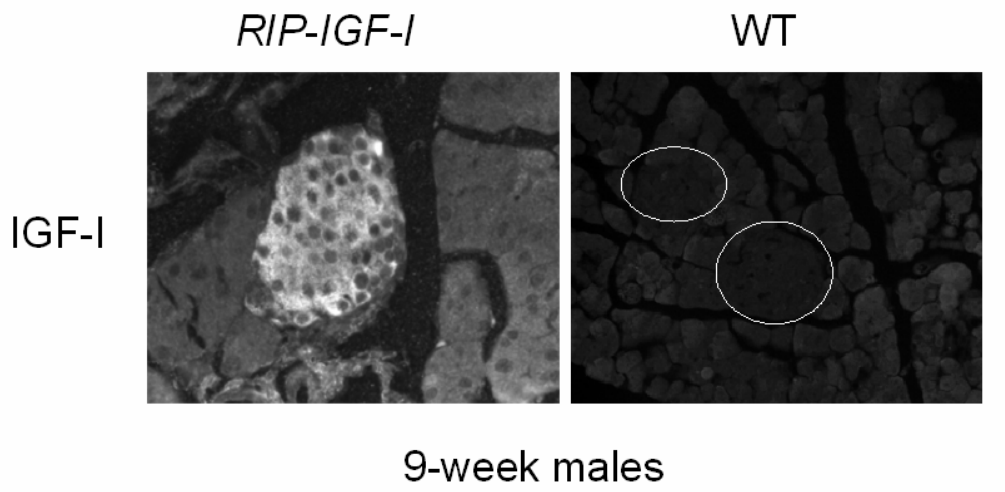


Figure 34. Robust IGF-1 expression in backcrossed *RIP-IGF-1* mice at 9 week of age. Left panel showed strong IGF-1 signal in a *RIP-IGF-I* islet as revealed by immunofluorescent staining of IGF-1. In contrast, WT (right panel) did not have any detectable signal in the islet area (White circle). Magnification: 400X.

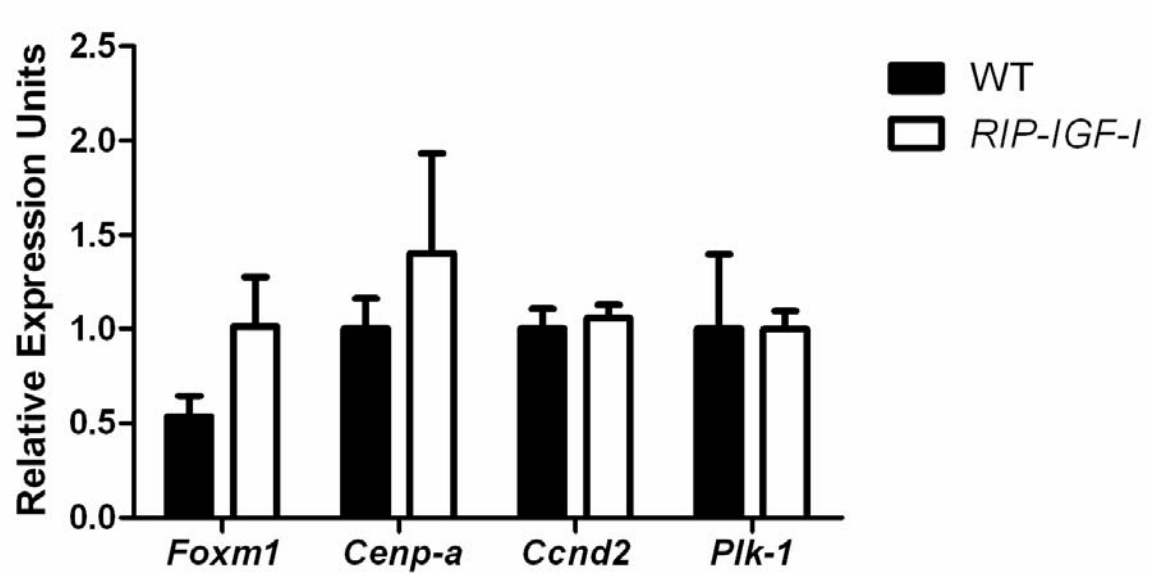


Figure 35. Overepxression of IGF-1 did not induce *Foxm1* or its target gene expression. Isolated islets from 9 week-old *RIP-IGF-1* and WT littermates were subjected to qRT-PCR. No change in *Foxm1* or its target *Cenp-A, Ccnd2* and *Plk-1* between *RIP-IGF-1* (white bar) and WT (black bar) mice were observed (n=3-4).

CHAPTER V

GENERATING A MOUSE FOXM1 ANTIBODY

Introduction

Many companies and laboratories have generated various FoxM1 antibodies. To characterize Foxm1 expression in the pancreas, our laboratory initially utilized a polyclonal antibody, a generous gift of Dr. Robert Costa at University of Illinois at Chicago. This antibody was generated by immunizing rabbits with recombinant N-terminal portion (amino acids 1 to 138) of human FOXM1B. We detected specific FoxM1 expression in both embryonic and adult mouse pancreata by immunolabeling (Figure 8), which is irreproducible when using antibodies from other sources. Unfortunately, this antibody is no longer available. Commercial antibodies from Santa Cruz Biotechnology and Abcam generated against various fragments of the Foxm1 protein failed to work on the mouse tissue. Our laboratory was unable to detect a specific signal on mouse tissue using these antibodies for immunohistochemistry despite many trials using various antigen unmasking techniques and signal amplification systems. An alternative way to detect *Foxm1* expression at a cellular level is *in situ* hybridization. Although revealing high level of *Foxm1* signal in developing endocrine cords as well as in embryonic liver, this method did not detect *Foxm1* in adult pancreatic sections, due to the enormous amount of RNase present within the pancreas after birth.

For western blotting, a different polyclonal anti-Foxm1 antibody generated against the C-terminus by the same laboratory was used to detect mouse Foxm1 (Wang *et al.* 2005). However, we found that this antibody is not specific because: first, the signal appearing at around 83 kDa (predicted size of mouse Foxm1 protein) was not

consistent between WT animals (Figure 35A); second, a weak signal at around 83 kDa also appears in lanes loaded with *Foxm1*$^{-/-}$ protein extracts (Figure 35B). To determine whether Foxm1 was actually deleted in *Foxm1*$^{-/-}$ animals, qRT-PCR was used to examine the *Foxm1* transcript level in embryonic liver in WT and *Foxm1*$^{-/-}$ mice. Compared to WT, *Foxm1* expression is nearly undetectable in the mutant liver as examined by qRT-PCR (Ackermann Misfeldt's dissertation). Therefore, this particular antibody is clearly recognizing something other than Foxm1.

No currently available antibodies were able to specifically detect the mouse Foxm1 using either immunolabeling or western blotting. This has been a real hurdle to many studies aiming to gain a better understanding of mouse Foxm1, such as cellular and sub-cellular resolution of Foxm1 expression, *in vivo* post-translational regulation of Foxm1 and the identification of novel Foxm1 targets. Therefore, our laboratory decided to generate an antibody specifically against mouse Foxm1. Although it is more time consuming, we first chose to generate a monoclonal antibody instead of a polyclonal one due to the following considerations: first, a monoclonal antibody specifically recognizes an individual epitope, whereas a polyclonal antibody is heterogeneous by nature since it recognizes multiple epitopes. Since Foxm1 belongs to a large family of proteins that shares great homology in their DNA binding domain, a polyclonal antibody is like to react with closed related members in the Fox protein family. Secondly, a monoclonal antibody can be reproduced indefinitely with consistent quality by re-expansion of the same clone of hybridoma cells. However, a polyclonal antibody, once exhausted, has to be replenished by immunizing a new animal, which creates inconsistency between batches of antibodies. Figure 37 showed a diagram of monoclonal antibody production. In collaboration with the Vanderbilt Monoclonal Antibody Core, monoclonal Foxm1 antibody was generated by first immunizing mice simultaneously with both N-terminal and C-terminal fragments of mouse FoxM1: amino acids 1-233 (fragment 1) and amino acids

1939-2445 (fragment 3) (Figure 37), both of which were fused with maltose-binding protein (MBP). These fragments were designed to exclude the DNA binding domain of Foxm1 which may raise the issue of cross-reactivity with other Fox proteins. After each bleeding, antiserum from each animal was tested by western blotting using both recombinant Foxm1 protein from HeLa cells and embryonic mouse liver extracts. After four bleedings, all anti-sera recognized recombinant Foxm1 protein, however, only two of them detected endogenous mouse Foxm1 in the WT but not in mutant tissue (Figure 39). Unfortunately, immunohistochemistry using these two antisera consistently showed signal with high background on both mutant and WT embryonic tissue sections.

In this chapter, progress on generating Foxm1 monoclonal and polyclonal antibodies will be presented and discussed.

Results

Mouse A/J-L was Chosen for Fusion in the Generation of Monoclonal Antibody.

After six bleedings, western blotting was performed to evaluate the immuno-reactivity of Foxm1 anti-sera using liver/heart/lung whole protein extracts from two *Foxm1*$^{-/-}$ and two WT e17.5 embryos. Protein extracts from HeLa cells transfected with mouse *Foxm1* full-length cDNA was also included as a positive control. Anti-serum from animal A/J-L exhibited the strongest immunoreactivity to Foxm1 in WT tissues compared with others, despite its weak bounding to Foxm1 in the mutant tissues (Figure 40). Therefore, we decided to use mouse A/J-L for the generation of hybridoma.

Screening of Hybridoma Clones

After fusion, supernatants from hybridoma clones were first screened by ELISA in the Vanderbilt Monoclonal Antibody Core and positive clones were then tested by

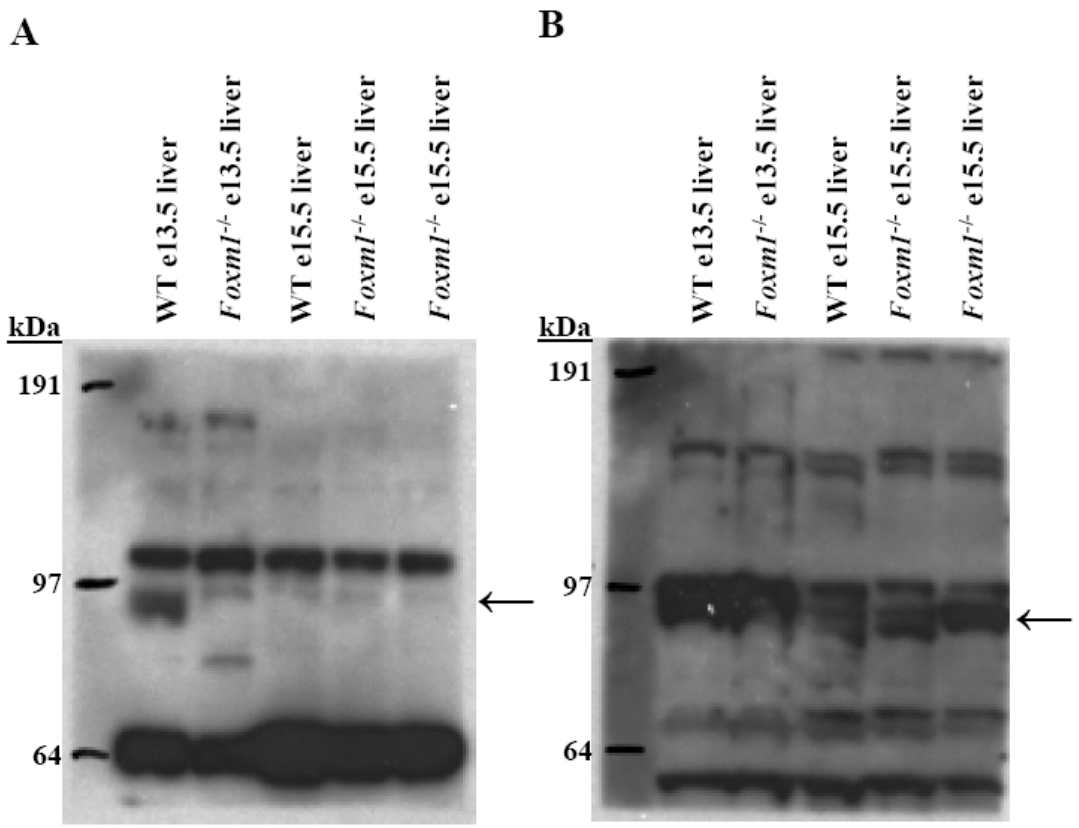


Figure 36. Available anti-Foxm1 antibodies are not specific. Western immunoblottings were performed using protein extracted from embryonic liver from WT and *Foxm1*$^{-/-}$ littermates. **(A)** A polyclonal anti-C-terminal-Foxm1 antibody provided by Dr. Robert H. Costa's laboratory bound to a protein at slightly higher than 83 kDa (arrow), but bands at this size were also observed in *Foxm1*$^{-/-}$ protein extracts, and consistent results were not observed between different WT protein samples. **(B)** A polyclonal anti-C-terminal-Foxm1 antibody from Santa Cruz Biotechnology also detect a protein at slightly higher than 83 kDa (arrow) in WT protein extract, but similar signals were also observed in the mutant protein extract. (Amanda Ackermann Misfeldt's unpublished result).

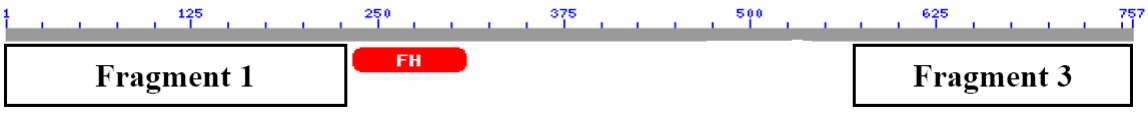
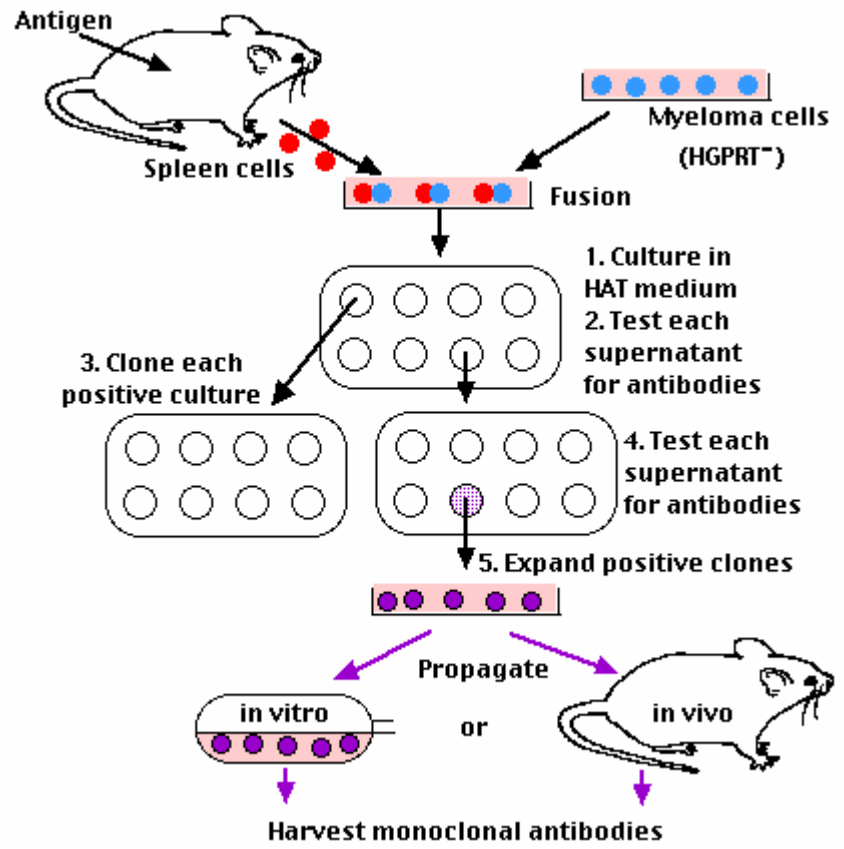


Figure 37. Fragments of mouse Foxm1 for immunization. Fragment 1 and 3, corresponding to N- and C-terminal regions of mouse Foxm1 protein were generated for immunization. The forkhead (FH) DNA binding domain was excluded from these

fragments because FoxM1 shares high sequence homology with other Fox proteins within this domain. (Adapted from Amanda Ackermann Misfeldt's dissertation)



.

Figure 38. Generation of a monoclonal antibody. Spleen cells from a mouse that has been immunized with the desired antigen are fused with myeloma cells lack the ability to synthesize purines (HGPRT-, lack hypoxanthine-guanine-phosphoribosyl transferase which catalyzes the first step in the purine pathway) to make hybridoma. After fusion, cells are cultured in HAT media containing purine precursors hypoxanthine, aminopterin and thymidine for hybridoma selection and proliferation. After tested for antibodies by enzyme-linked immunosorbent assay (ELISA), positive hybridoma clones are subcloned and subjected for another ELISA for antibodies. Positive hybridoma clones can then be expanded and propagated *in vitro* or *in vivo* to generate large amount of monoclonal antibodies (http://www.biology.iupui.edu/biocourses/Biol540/6secondwavefullCSS.html).

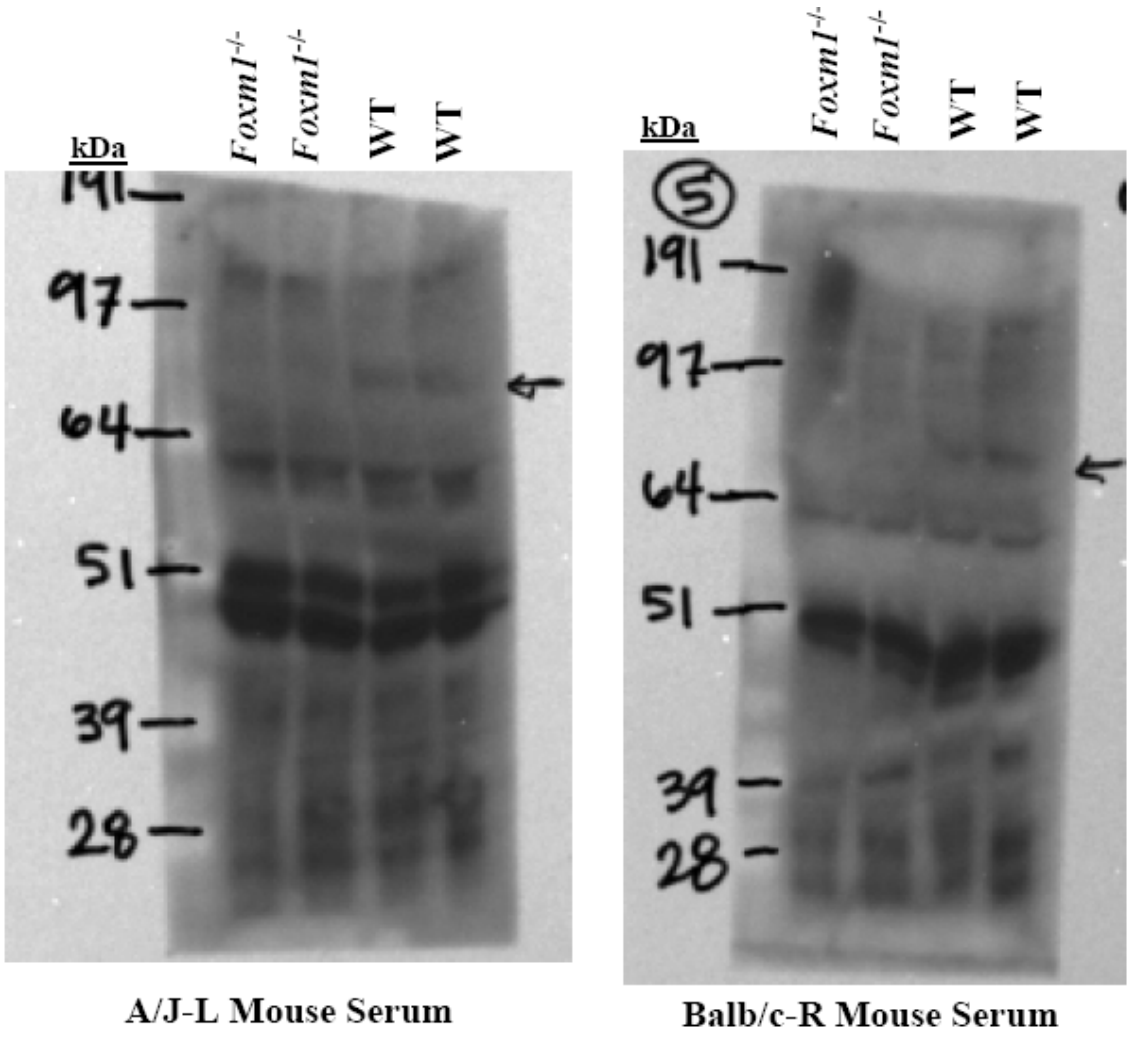


Figure 39. Endogenous mouse Foxm1 was detected by sera from two antigen-injected mice. Total protein extracts were collected from WT and *Foxm1*$^{-/-}$ embryos and used for western immunoblotting with sera from antigen-injected A/J-L and Balb/c mice. Sera from two mice produced bands at ~83 kDa (arrows) specifically in WT, but not in *Foxm1*$^{-/-}$ extracts (arrows) (Amanda Ackermann Misfeldt's unpublished result).

western blotting in our laboratory. For western blotting, we utilized *Foxm1* full length cDNA transfected HeLa cell lysate and Foxm1 antigen (fragment 1 and 3, 61 and 73 kDa in size) as positive controls and non-transfected HeLa cell lysate as negative control. Within 60 ELISA positive clones, more than 20 clones showed strong immuno-reactivity to the Foxm1 antigen. Interestingly, signal detected by these clones displayed fell into three patterns: (a), two equally intense bands, presumably binding to both Foxm1 antigen fragments judging by the size; (b), two bands with one stronger than the other, also presumably binding to both Foxm1 antigen fragments; (c), only one band (~ 60kDa), presumably binding to one Foxm1 antigen fragment. However, none of them was able to recognize endogenous Foxm1 expressed in transfected HeLa cell lysate (Figure 43). Thus we examined whether the pool of some positive clones recognizes endogenous Foxm1. With the help of the Vanderbilt Monoclonal Antibody Core, we pooled and concentrated the supernatants from clones that strongly reacted to Foxm1 antigen. Unfortunately, the pooled supernatant was still unable to detect endogenous Foxm1 in western blotting. We also assessed each ELISA positive clone by immunohistochemistry in parallel with western blotting. We failed to identify any clones that are able to specifically label Foxm1 in tissue sections.

Generating Foxm1 Polyclonal Antibodies

Since we were unable to obtain a good monoclonal antibody against murine Foxm1, we sought to generate a polyclonal antibody instead. In collaboration with the Vanderbilt Monoclonal Antibody Core, the N-terminal fragment of mouse Foxm1 (amino acids 1 to 138), the same region which Dr. Robert Costa used to generate the antibody specific for mouse Foxm1, was used to immunize mice, rats and rabbits. We tested the anti-sera from the first bleeds by western blotting and the results showed that two mice and one rabbit are promising producers of good Foxm1 antibodies (Figure 42). However,

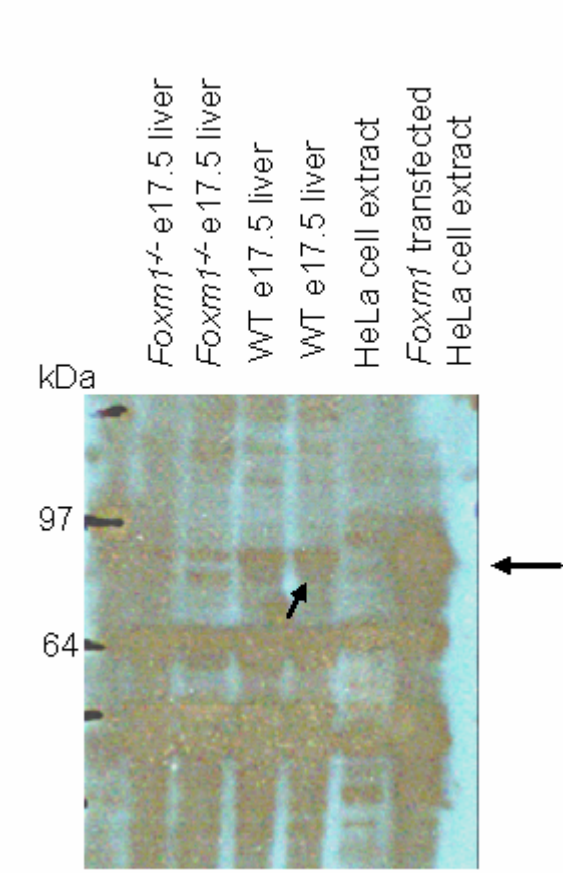


Figure 40. Endogenous Foxm1 protein was detected by the 6th bleed anti-serum from mouse A/J-L. Total liver protein extract from *Foxm1*$^{-/-}$ and control WT e17.5 embryos were used in western blotting to evaluate the immunoreactivity of the anti-serum. HeLa cell extract and *Foxm1* transfected HeLa cell extract were used as negative and positive control, respectively. Arrows denote the bands at ~83kDa (endogenous Foxm1 protein).

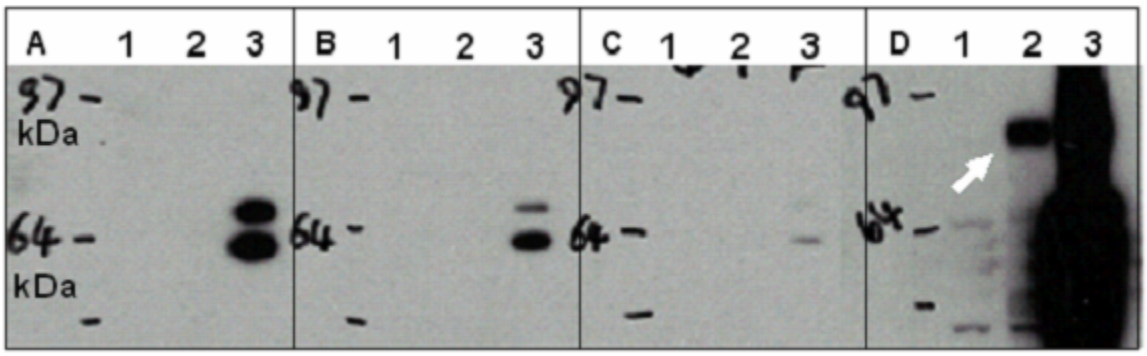


Figure 41. Representative western blotting of hybridoma producing Foxm1 antibody. Blot A, B and C were incubated with the supernatants from three different clones. Blot D was incubated with western blotting positive serum from Foxm1 antigen immunized mice. Arrow: ~ 83kDa band representing Foxm1 protein. Lane 1: non-transfected HeLa cell lysate. Lane 2: *Foxm1* full-length cDNA transfected HeLa cell lysate. Lane 3: Foxm1 protein antigen fragments (61-73kDa).

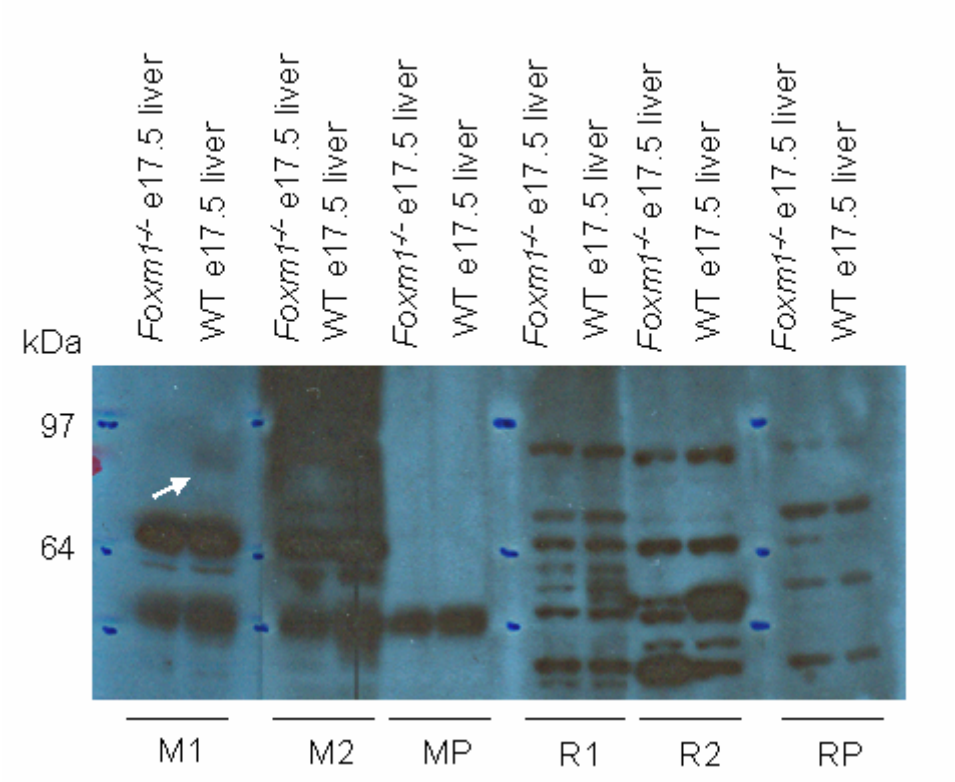


Figure 42. Westerning blotting examination of the first bleed antisera for the generation of polyclonal Foxm1 antibody in mice and rats. M1, M2 : mouse 1 and 2. R1, R2: rat 1 and 2. MP: mouse prebleed. RP; rat prebleed. Arrow denotes a ~83 band indicating endogenous mouse Foxm1.

one mouse died shortly after the first bleed for unknown reasons, we are currently testing the anti-sera from the second bleed of one mouse and one rabbit.

Simultaneously, we initiated a project of generating a rabbit polyclonal antibody against mouse Foxm1 with Bethyl Laboratories. This company immunized rabbits with two Foxm1 peptides that are predicted to be potent antigens. We are in the process of testing this affinity purified antibody by western blotting and immunohistochemistry.

Discussion

The study of mouse Foxm1 has long suffered from lacking a reliable and specific antibody. To solve this problem, we took great effort to try to generate a Foxm1 antibody. We did not succeed in generating a monoclonal Foxm1 antibody since none of the hybridoma clones reacted to endogenous Foxm1 despite strong reactivity to Foxm1 antigens. This indicates that monoclonalization of Foxm1 antibody greatly reduces the chance of Foxm1 being recognized by the antibody. It is also possible that ELISA positive clones and western blotting positive clones do not overlap, hence no western blotting positive clones identified within ELISA positive clones. In an ELISA, a positive clone produces antibodies bound to the intact form of Foxm1 antigen. In the type of western blotting we performed, a positive clone generates antibodies bound to the denatured form of Foxm1 antigen. Therefore, these two methods screen for different properties of hybridoma clones, which may lead to a selection of different populations of clones. Finally, although readings for MBP coated plates as a negative control in an ELISA screen would efficiently rule out clones that only recognize the MBP tag but not the portion of Foxm1 fragment in the Foxm1 fragment-MBP fusion protein, it can not exclude antibodies bound to the interface of MBP and Foxm1 fragment. The existence of these antibodies could explain the behavior of many hybridoma clones observed in

western blotting: the high immuno-reactivity to the recombined antigen (Foxm1 fragment-MBP fusion) but no response to endogenous Foxm1.

We are currently working on the generation of a polyclonal Foxm1 antibody with both N-terminal region (in collaboration with the Vanderbilt monoclonal antibody core) and antigenic peptides of mouse Foxm1 as antigens. Meanwhile, to avoid the issue of Foxm1 antibody, another effort in the laboratory is to generate a mouse model with a HA-tagged version of mouse Foxm1 using recombinase-mediated cassette-exchange (RMCE) technique (Feng et al., 1999; Long et al., 2004). This mouse model will open the door to versatile studies currently unfeasible due to the lack of a good antibody and greatly advance our understanding of mouse Foxm1.

CHAPTER VI

SUMMARY AND FUTURE DIRECTIONS

Foxm1 is known as a potent cell proliferative transcription factor in many tissue types, which is evident by its association with tissues/cells with high proliferating rate, its numerous targets involved in cell cycle regulation and the manipulation of this factor causing either accelerated (gain of function) or blunted (loss of function) cell replication. This characteristic of Foxm1 has drawn extensive interests to understand its role in the process of tumorigenesis and to develop strategies aiming to block its mitogenic activity thus preventing or curing human cancer (Wang et al., 2009a). On the other hand, the proliferative effect of Foxm1 could be necessary and beneficial, for instance, in establishing and/or maintaining organ mass and organ regeneration. How important Foxm1-mediated cell proliferation is for the normal function of an organ under basal and stimultative/regenerative conditions is only beginning to be understood. Prior studies in our laboratory first reported the function of Foxm1 in the pancreas. Pancreas-wide deletion of Foxm1 results in severely blunted postnatal β-cell proliferation/mass expansion, leading to progressive diabetes (Zhang et al., 2006b). Foxm1 is also important for regenerative proliferation of the β-cell as evident by the fact that the absence of Foxm1 impairs β-cell proliferation stimulated by 60% partial pancreatectomy (Ackermann Misfeldt et al., 2008). These findings prompted us to investigate the requirement of Foxm1 for β-cell proliferation/mass expansion and normal pancreas function under other stimulatory conditions.

This dissertation described our effort towards understanding the role of Foxm1 in growth factor (PL, HGF and IGF-1) -mediated β-cell proliferation. Our results in chapter

III demonstrated that Foxm1 is essential for facultative β-cell proliferation and mass expansion in response to pregnancy, a physiologically relevant condition with high metabolic demands. Foxm1-mediated β-cell proliferation is clearly crucial for maternal glucose homeostatsis during pregnancy. These findings further support the therapeutic potential of Foxm1 in generating new β-cells without lossing essential β-cell characteristics. In addition, *Foxm1*$^{\Delta panc}$ female mice are the only mouse model to date that nicely meets the diagnostic criteria for human GDM (hyperglycemia occurs only during pregnancy but not before or after, Table 1), thus representing a valuable model of GDM. Future experiments on these animals examining other maternal and fetal consequences commonly seen in humans such as marosomia, T2DM, neural or cardiac defects, would provide more thorough evalution of the clinical relavence of this mouse model. Along this line, genome wide association studies analyzing the association of genetic variants of Foxm1 with traits of GDM in human is needed.

Although multiple signaling pathways promoting cell cycle, such as Ras/MAPK, Hh, and PI3K/Akt have been implicated in *Foxm1* induction (Laoukili et al., 2007), direct evidence regarding the underlying molecular events leading to *Foxm1* transcription is largely missing. In particular, the upstream regulatory mechanism of FoxM1 in the pancreas has not been uncovered. In chapter III, we provided both *in vivo* and *in vitro* evidence demonstring that PL is an upstream signaling pathway that directs FoxM1 to provoke β-cell proliferation. The induction of *Foxm1* by PL both *in vitro* and *in vitro* is required for PL-stimulated β-cell proliferation in mice. Furthermore, ChIP assay showed that Foxm1 is a direct target of Stat5, the downstream effector of PL signaling. In chapter IV, we presented our initial steps on determining whether Foxm1 mediates the mitogenic effect of HGF and IGF-1 on β-cells using transgenic models overexpressing HGF or IGF-1. These results, combined with previous studies in the laboratory, lead us to propose **Foxm1 as a common target of multiple β-cell mitogens** (Figure 43). Further studies

on whether and how Foxm1 mediates HGF, IGF-1 and high-fat diet induced β-cell proliferation are in progress in our laboratory. It would also be interesting to explore the potential crosstalks among these signaling pathways regarding their regulation of Foxm1.

Mutiple avenues have been investigated to generate new β-cells, including differentiation from embryonic stem cells, differentiation from progenitors, transdifferentiation from other cell types such as exocrine cell, hepatocytes, intestine cells and bone borrow cells (Ackermann & Gannon, 2007; Jun, 2008). However, these methods suffer from low yield of functional β-cells. Introducing Foxm1 (ideally through a combination of increased transcription and activity of Foxm1) to these methods may greatly increase the efficiency.

Post-translational modifications, such as phosphorylation are crucial for Foxm1 activity (Figure 8) (Major et al., 2004; Ma et al., 2005). Overexpression of Foxm1 alone is not sufficient to induce β-cell proliferation in transgenic mice (Ackermann Misfeldt et al., 2008). Due to the lack of a specific and reliable antibody against murine Foxm1, we were unable to show definitively that growth factors such as PL induce Foxm1 only at the transcriptional level or also at the post-translatoinal level. To solve this problem, generation of a polyconal antibody and a mouse model expressing a HA-tagged version of Foxm1 are underway in our laboratory. In the long run, detailed mapping of phosphorylation sites of Foxm1 in the pancreas under basal and stimulatory condition utilizing mass spectrometry would be insightful to understand the behavior of Foxm1 as well as its regulatory mechnisam in the β-cell. Followup genetic manipulations of these identified phosyphorylation sites, generating either phospho-mimetic or phospho-resistant forms of Foxm1 in mice would allow a better understanding of the significance of these modifications to Foxm1activity *in vivo*. If a certain manipulation of Foxm1 leads to enhanced activity *in vivo*, further studies could be performed to evaluate whether this

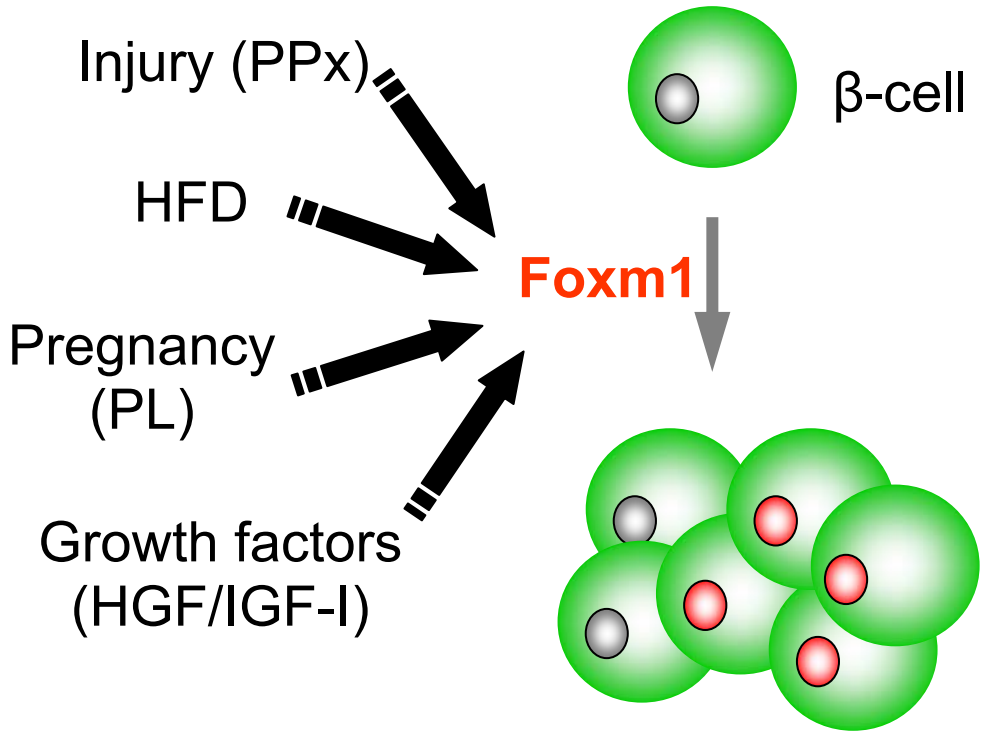


Figure 43. The central role of Foxm1 in facultative β-cell proliferation. Foxm1 may operatre downstream of multiple β-cell stimuli, such as injury (partial pancreatecomy, PPx), HFD (high fat-diet), pregnancy and growth factors including HGF and IGF-1. This dissertation investigated the role of Foxm1 in pregnancy and growth factor-stimulated β-cell replication.

form of manipulation can be used in diabetic animal models to restore functional β-cell mass thus ameliorating or curing diabetes. In addition, a specific mouse Foxm1 antibody or a tagged version of Foxm1 would also make experiments such as ChIP-seq feasible for the identification of novel targets thus novel functions of Foxm1 in β-cell survival and insulin biosythesis or secretion.

Cell cycle or size regulators other than Foxm1 could operate parallelly to promote facultative β-cell expansion. In *Foxm1*$^{\Delta panc}$; *RIP-PL* mice, without Foxm1 activity, we still observed substaintial basal proliferation, similar to that in *Foxm1*$^{fl/fl}$ mice (Figure 26B). This indicated that PL mediates Foxm1-independent β-cell proliferation. Therefore, it is possible that Foxm1 is only one downstream branch of other external stimuli.

In human, basal adult β-cell proliferate [~0.4% in lean health individual (Hanley et al., 2010)] is much lower than that in mice. However, this does not dispute the proliferative potential of human β-cell in response to stimuli. A great example came from Garcia-Ocaňa's work on HGF signaling pathway in β-cell proliferation. This group successfully enhanced human primary islet cell proliferation up to 3-fold via adenovirus-mediated delivery of constitutively active PKCζ, a key mediator of the mitogenic effects of HGF (Vasavada et al., 2007). Therefore, the insights we gain from study on murine Foxm1 could be applied to augment human β-cell replication. The role of FOXM1 in human β-cells has not been studied. We recently detected *FOXM1* transcript in islets from a 17-year-old human by qRT-PCR (Jia Zhang, unpublished observations), indicating that FOXM1 may also operate in human β-cells. It would be interesting to extend our studies in this dissertation to humans in the following aspects: first, examine whether human β-cells upregulate *Foxm1* in response to stimuli, such as pregnancy and growth factor treatments; second, determine whether human β-cell proliferation also requires *Foxm1* by siRNA knockdown or Foxm1 inhibitors and whether *Foxm1*

expression declines with age; third, evaluate whether the overexpression of *Foxm1* in human islets can promote β-cell proliferation and improve islet function; fourth, determine whether the signaling pathways the direct *Foxm1* in mice are conserved in humans.

This dissertation demonstrated that importance and the molecular mechanism of Foxm1 in promoting adaptive β-cell proliferation/ mass expansion thus glucose homeostasis in response to growth factors, in particular, placental lactogen. This study reinforced the proposal that Foxm1 is a common target of multiple β-cell mitogens. Moreover, we identified a new regulatory pathway of Foxm1 in the β-cell, PL signaling. This study on Foxm1 broadened the current knowledge about adult β-cell proliferation regulation and brought us closer to the success of increasing β-cell number *in vivo*.

REFERENCES

(2000). Gestational diabetes mellitus. *Diabetes Care*, **23 Suppl 1**: S77-9.

(2003). Report of the expert committee on the diagnosis and classification of diabetes mellitus. *Diabetes Care*, **26 Suppl 1**: S5-20.

(2007). National diabetes fact sheet.

Aberg, A., Westbom, L. and Kallen, B. (2001). Congenital malformations among infants whose mothers had gestational diabetes or preexisting diabetes. *Early Hum Dev*, **61**(2): 85-95.

Ackermann, A. M. and Gannon, M. (2007). Molecular regulation of pancreatic beta-cell mass development, maintenance, and expansion. *J Mol Endocrinol*, **38**(1-2): 193-206.

Ackermann Misfeldt, A., Costa, R. H. and Gannon, M. (2008). Beta-cell proliferation, but not neogenesis, following 60% partial pancreatectomy is impaired in the absence of FoxM1. *Diabetes*, **57**(11): 3069-77.

Altshuler, D., Hirschhorn, J. N., Klannemark, M., Lindgren, C. M., Vohl, M. C., Nemesh, J., Lane, C. R., Schaffner, S. F., Bolk, S., Brewer, C., Tuomi, T., Gaudet, D., Hudson, T. J., Daly, M., Groop, L. and Lander, E. S. (2000). The common PPARgamma Pro12Ala polymorphism is associated with decreased risk of type 2 diabetes. *Nat Genet*, **26**(1): 76-80.

Amaral, M. E., Cunha, D. A., Anhe, G. F., Ueno, M., Carneiro, E. M., Velloso, L. A., Bordin, S. and Boschero, A. C. (2004). Participation of prolactin receptors and phosphatidylinositol 3-kinase and MAP kinase pathways in the increase in pancreatic islet mass and sensitivity to glucose during pregnancy. *J Endocrinol*, **183**(3): 469-76.

Amaral, M. E., Ueno, M., Carvalheira, J. B., Carneiro, E. M., Velloso, L. A., Saad, M. J. and Boschero, A. C. (2003). Prolactin-signal transduction in neonatal rat pancreatic islets and interaction with the insulin-signaling pathway. *Horm Metab Res*, **35**(5): 282-9.

Anubhuti and Arora, S. (2008). Leptin and its metabolic interactions: an update. *Diabetes Obes Metab*, **10**(11): 973-93.

Balcazar, N., Sathyamurthy, A., Elghazi, L., Gould, A., Weiss, A., Shiojima, I., Walsh, K. and Bernal-Mizrachi, E. (2009). mTORC1 activation regulates beta-cell mass and proliferation by modulation of cyclin D2 synthesis and stability. *J Biol Chem*, **284**(12): 7832-42.

Barr, F. A., Sillje, H. H. and Nigg, E. A. (2004). Polo-like kinases and the orchestration of cell division. *Nat Rev Mol Cell Biol*, **5**(6): 429-40.

Beattie, G. M., Cirulli, V., Lopez, A. D. and Hayek, A. (1997). Ex-vivo expansion of human pancreatic endocrine cells. *J Clin Endocrinol Metab.* , **82**: 1852–6.

Beattie, G. M., Montgomery, A. M., Lopez, A. D., Hao, E., Perez, B. and Just, M. L. (2002). A novel approach to increase human islet cell mass while preserving beta-cell function. *Diabetes*, **51**: 3435–9.

Beattie, G. M., Rubin, J. S., Mally, M. I., Otonkoski, T. and Hayek, A. (1996). Regulation of proliferation and differentiation of human fetal pancreatic islet cells by extracellular matrix, hepatocyte growth factor, and cell-cell contact. *Diabetes*, **45**(9): 1223-8.

Bell, G. I., Xiang, K. S., Newman, M. V., Wu, S. H., Wright, L. G., Fajans, S. S., Spielman, R. S. and Cox, N. J. (1991). Gene for non-insulin-dependent diabetes mellitus (maturity-onset diabetes of the young subtype) is linked to DNA polymorphism on human chromosome 20q. *Proc Natl Acad Sci U S A*, **88**(4): 1484-8.

Bellamy, L., Casas, J. P., Hingorani, A. D. and Williams, D. (2009). Type 2 diabetes mellitus after gestational diabetes: a systematic review and meta-analysis. *Lancet*, **373**(9677): 1773-9.

Blanco-Bose, W. E., Murphy, M. J., Ehninger, A., Offner, S., Dubey, C., Huang, W., Moore, D. D. and Trumpp, A. (2008). C-Myc and its target FoxM1 are critical downstream effectors of constitutive androstane receptor (CAR) mediated direct liver hyperplasia. *Hepatology*, **48**(4): 1302-11.

Boura-Halfon, S. and Zick, Y. (2009). Phosphorylation of IRS proteins, insulin action, and insulin resistance. *Am J Physiol Endocrinol Metab*, **296**(4): E581-91.

Bouwens, L. and Rooman, I. (2005). Regulation of pancreatic beta-cell mass. *Physiol Rev*, **85**(4): 1255-70.

Brelje, T. C., Scharp, D. W., Lacy, P. E., Ogren, L., Talamantes, F., Robertson, M., Friesen, H. G. and Sorenson, R. L. (1993). Effect of homologous placental lactogens, prolactins, and growth hormones on islet B-cell division and insulin secretion in rat, mouse, and human islets: implication for placental lactogen regulation of islet function during pregnancy. *Endocrinology*, **132**(2): 879-87.

Brockman, J. L., Schroeder, M. D. and Schuler, L. A. (2002). PRL activates the cyclin D1 promoter via the Jak2/Stat pathway. *Mol Endocrinol*, **16**(4): 774-84.

Buchanan, T. A. and Xiang, A. H. (2005). Gestational diabetes mellitus. *J Clin Invest*, **115**(3): 485-91.

Burroughs, K. D., Dunn, S. E., Barrett, J. C. and Taylor, J. A. (1999). Insulin-like growth factor-I: a key regulator of human cancer risk? *J Natl Cancer Inst*, **91**(7): 579-81.

Buteau, J., Foisy, S., Rhodes, C. J., Carpenter, L., Biden, T. J. and Prentki, M. (2001). Protein kinase C zeta activation mediates glucagon-like peptide-1-induced pancreatic beta-cell proliferation. *Diabetes*, **50**: 2237–43.

Buteau, J., Roduit, R., Susini, S. and Prentki, M. (1999). Glucagonlike peptide-1 promotes DNA synthesis, activates phosphatidylinositol 3-kinase and increases transcription factor pancreatic and duodenal homeobox gene 1 (PDX-1) DNA binding activity in beta (INS-1)-cells. *Diabetologia*, **42**: 856–64.

Chang, T. I., Horal, M., Jain, S. K., Wang, F., Patel, R. and Loeken, M. R. (2003). Oxidant regulation of gene expression and neural tube development: Insights gained from diabetic pregnancy on molecular causes of neural tube defects. *Diabetologia*, **46**(4): 538-45.

Chaudhary, J., Mosher, R., Kim, G. and Skinner, M. K. (2000). Role of winged helix transcription factor (WIN) in the regulation of Sertoli cell differentiated functions: WIN acts as an early event gene for follicle-stimulating hormone. *Endocrinology*, **141**(8): 2758-66.

Chen, H., Gu, X., Su, I. H., Bottino, R., Contreras, J. L., Tarakhovsky, A. and Kim, S. K. (2009). Polycomb protein Ezh2 regulates pancreatic beta-cell Ink4a/Arf expression and regeneration in diabetes mellitus. *Genes Dev*, **23**(8): 975-85.

Chen, Y., Liao, W. X., Roy, A. C., Loganath, A. and Ng, S. C. (2000). Mitochondrial gene mutations in gestational diabetes mellitus. *Diabetes Res Clin Pract*, **48**(1): 29-35.

Chiu, K. C., Go, R. C., Aoki, M., Riggs, A. C., Tanizawa, Y., Acton, R. T., Bell, D. S., Goldenberg, R. L., Roseman, J. M. and Permutt, M. A. (1994). Glucokinase gene in gestational diabetes mellitus: population association study and molecular scanning. *Diabetologia*, **37**(1): 104-10.

Cho, Y. M., Kim, T. H., Lim, S., Choi, S. H., Shin, H. D., Lee, H. K., Park, K. S. and Jang, H. C. (2009). Type 2 diabetes-associated genetic variants discovered in the recent genome-wide association studies are related to gestational diabetes mellitus in the Korean population. *Diabetologia*, **52**(2): 253-61.

Cohen, P. (2006). Overview of the IGF-I system. *Horm Res.*, **65**: 3-8.

Collombat, P., Xu, X., Ravassard, P., Sosa-Pineda, B., Dussaud, S., Billestrup, N., Madsen, O. D., Serup, P., Heimberg, H. and Mansouri, A. (2009). The ectopic expression of Pax4 in the mouse pancreas converts progenitor cells into alpha and subsequently beta cells. *Cell*, **138**(3): 449-62.

Coustan, D. R. (2007). *Pharmacological management of gestational diabetes: an overview. *Diabetes Care*, **30 Suppl 2**: S206-8.

Cozar-Castellano, I., Fiaschi-Taesch, N., Bigatel, T. A., Takane, K. K., Garcia-Ocana, A., Vasavada, R. and Stewart, A. F. (2006a). Molecular control of cell cycle progression in the pancreatic beta-cell. *Endocr Rev*, **27**(4): 356-70.

Cozar-Castellano, I., Takane, K. K., Bottino, R., Balamurugan, A. N. and Stewart, A. F. (2004). Induction of beta-cell proliferation and retinoblastoma protein phosphorylation in rat and human islets using adenovirus-mediated transfer of cyclin-dependent kinase-4 and cyclin D1. *Diabetes*, **53**(1): 149-59.

Cozar-Castellano, I., Weinstock, M., Haught, M., Velazquez-Garcia, S., Sipula, D. and Stewart, A. F. (2006). Evaluation of beta-cell replication in mice transgenic for hepatocyte growth factor and placental lactogen: comprehensive characterization of the G1/S regulatory proteins reveals unique involvement of p21cip. *Diabetes*, **55**(1): 70-7.

Crawford, L. A., Guney, M. A., Oh, Y. A., Deyoung, R. A., Valenzuela, D. M., Murphy, A. J., Yancopoulos, G. D., Lyons, K. M., Brigstock, D. R., Economides, A. and Gannon, M. (2009). Connective tissue growth factor (CTGF) inactivation leads to defects in islet cell lineage allocation and beta-cell proliferation during embryogenesis. *Mol Endocrinol*, **23**(3): 324-36.

da Silva Xavier, G., Loder, M. K., McDonald, A., Tarasov, A. I., Carzaniga, R., Kronenberger, K., Barg, S. and Rutter, G. A. (2009). TCF7L2 regulates late events in insulin secretion from pancreatic islet beta-cells. *Diabetes*, **58**(4): 894-905.

Dabelea, D., Knowler, W. C. and Pettitt, D. J. (2000). Effect of diabetes in pregnancy on offspring: follow-up research in the Pima Indians (ref) *J Matern Fetal Med*, **9**(1): 83-8.

Dai, B., Kang, S. H., Gong, W., Liu, M., Aldape, K. D., Sawaya, R. and Huang, S. (2007). Aberrant FoxM1B expression increases matrix metalloproteinase-2 transcription and enhances the invasion of glioma cells. *Oncogene*, **26**(42): 6212-9.

Dai, C., Li, Y., Yang, J. and Liu, Y. (2003). Hepatocyte growth factor preserves beta cell mass and mitigates hyperglycemia in streptozotocin-induced diabetic mice *J Biol Chem.*, **278**: 27080–87.

De Leon, D. D., Deng, S., Madani, R., Ahima, R. S., Drucker, D. J. and Stoffers, D. A. (2003). Role of endogenous glucagon-like peptide-1 in islet regeneration after partial pancreatectomy. *Diabetes*, **52**(2): 365-71.

Deeb, S. S., Fajas, L., Nemoto, M., Pihlajamaki, J., Mykkanen, L., Kuusisto, J., Laakso, M., Fujimoto, W. and Auwerx, J. (1998). A Pro12Ala substitution in PPARgamma2 associated with decreased receptor activity, lower body mass index and improved insulin sensitivity. *Nat Genet*, **20**(3): 284-7.

Dhawan, S., Tschen, S. I. and Bhushan, A. (2009). Bmi-1 regulates the Ink4a/Arf locus to control pancreatic beta-cell proliferation. *Genes Dev*, **23**(8): 906-11.

Di Cianni, G., Miccoli, R., Volpe, L., Lencioni, C. and Del Prato, S. (2003). Intermediate metabolism in normal pregnancy and in gestational diabetes *Diabetes Metab Res Rev*, **19**(4): 259-70.

Dickson, L. M., Lingohr, M. K., McCuaig, J., Hugl, S. R., Snow, L. and Kahn, B. B. (2001). Differential activation of protein kinase B and p70(S6)K by glucose and insulin-like growth factor 1 in pancreatic beta-cells (INS-1). *Journal of Biological Chemistry*, **276**: 21110–20.

Dor, Y., Brown, J., Martinez, O. I. and Melton, D. A. (2004). Adult pancreatic beta-cells are formed by self-duplication rather than stem-cell differentiation. *Nature*, **429**(6987): 41-6.

Drucker, D. J. (2001). Minireview: The glucagon-like peptides. *Endocrinology*, **142**: 521–7.

Egan, J. M. (2007). Mechanisms of action of glucagons-likie peptide 1 in the pancreas. *Pharmacology & Therapeutics*, **113**: 546-93.

Egan, J. M., Clocquet, A. R. and Elahi, D. (2002). The insulinotropic effect of acute exendin-4 administered to humans: comparison of nondiabetic state to type 2 diabetes. *J Clin Endocrinol Metab.*, **87**: 1282-90.

Egan, J. M., Meneilly, G. S. and Elahi, D. (2003). Effects of 1-mo bolus subcutaneous administration of exendin-4 in type 2 diabetes. *Am J Physiol Endocrinol Metab* **284**: E1072−9.

Endo, T., Sasaki, A., Minoguchi, M., Joo, A. and Yoshimura, A. (2003). CIS1 interacts with the Y532 of the prolactin receptor and suppresses prolactin-dependent STAT5 activation. *J Biochem*, **133**(1): 109-13.

Fajas, L., Annicotte, J. S., Miard, S., Sarruf, D., Watanabe, M. and Auwerx, J. (2004). Impaired pancreatic growth, beta cell mass, and beta cell function in E2F1 (-/-)mice. *J Clin Invest*, **113**(9): 1288-95.

Fallucca, F., Dalfra, M. G., Sciullo, E., Masin, M., Buongiorno, A. M., Napoli, A., Fedele, D. and Lapolla, A. (2006). Polymorphisms of insulin receptor substrate 1 and beta3-adrenergic receptor genes in gestational diabetes and normal pregnancy. *Metabolism*, **55**(11): 1451-6.

Farilla, L., Hui, H., Bertolotto, C., Kang, E., Bulotta, A. and Di Mario, U. (2002). Glucagon-like peptide-1 promotes islet cell growth and inhibits apoptosis in Zucker diabetic rats. *Endocrinology*, **143**: 4397−408.

Feng, Y. Q., Seibler, J., Alami, R., Eisen, A., Westerman, K. A., Leboulch, P., Fiering, S. and Bouhassira, E. E. (1999). Site-specific chromosomal integration in mammalian cells: highly efficient CRE recombinase-mediated cassette exchange. *J Mol Biol*, **292**(4): 779-85.

Figeac, F., Uzan, B., Faro, M., Chelali, N., Portha, B. and Movassat, J. (2009). Neonatal growth and regeneration of {beta} cells are regulated by the Wnt/{beta}-catenin signaling in normal and diabetic rats. *Am J Physiol Endocrinol Metab*.

Finegood, D. T., Scaglia, L. and Bonner-Weir, S. (1995). Dynamics of beta-cell mass in the growing rat pancreas. Estimation with a simple mathematical model. *Diabetes*, **44**(3): 249-56.

Francis, R. E., Myatt, S. S., Krol, J., Hartman, J., Peck, B., McGovern, U. B., Wang, J., Guest, S. K., Filipovic, A., Gojis, O., Palmieri, C., Peston, D., Shousha, S., Yu, Q., Sicinski, P., Coombes, R. C. and Lam, E. W. (2009). FoxM1 is a downstream

target and marker of HER2 overexpression in breast cancer. *Int J Oncol*, **35**(1): 57-68.

Franklin, D. S., Godfrey, V. L., O'Brien, D. A., Deng, C. and Xiong, Y. (2000). Functional collaboration between different cyclin-dependent kinase inhibitors suppresses tumor growth with distinct tissue specificity. *Mol Cell Biol*, **20**(16): 6147-58.

Frayling, T. M. (2007). Genome-wide association studies provide new insights into type 2 diabetes aetiology. *Nat Rev Genet*, **8**(9): 657-62.

Freemark, M., Avril, I., Fleenor, D., Driscoll, P., Petro, A., Opara, E., Kendall, W., Oden, J., Bridges, S., Binart, N., Breant, B. and Kelly, P. A. (2002). Targeted deletion of the PRL receptor: effects on islet development, insulin production, and glucose tolerance. *Endocrinology*, **143**(4): 1378-85.

Friedman, J. E., Ishizuka, T., Shao, J., Huston, L., Highman, T. and Catalano, P. (1999). Impaired glucose transport and insulin receptor tyrosine phosphorylation in skeletal muscle from obese women with gestational diabetes. *Diabetes*, **48**(9): 1807-14.

Friedrichsen, B. N., Galsgaard, E. D., Nielsen, J. H. and Moldrup, A. (2001). Growth hormone- and prolactin-induced proliferation of insulinoma cells, INS-1, depends on activation of STAT5 (signal transducer and activator of transcription 5). *Endocrinology*, **15**: 136-48.

Froguel, P., Zouali, H., Vionnet, N., Velho, G., Vaxillaire, M., Sun, F., Lesage, S., Stoffel, M., Takeda, J., Passa, P. and et al. (1993). Familial hyperglycemia due to mutations in glucokinase. Definition of a subtype of diabetes mellitus. *N Engl J Med*, **328**(10): 697-702.

Fujinaka, Y., Takane, K., Yamashita, H. and Vasavada, R. C. (2007). Lactogens promote beta cell survival through JAK2/STAT5 activation and Bcl-XL upregulation. *J Biol Chem*, **282**(42): 30707-17.

Furuta, M., Yano, H., Zhou, A., Rouille, Y., Holst, J. J., Carroll, R., Ravazzola, M., Orci, L., Furuta, H. and Steiner, D. F. (1997). Defective prohormone processing and altered pancreatic islet morphology in mice lacking active SPC2. *Proc Natl Acad Sci U S A*, **94**(13): 6646-51.

Gahr, S., Merger, M., Bollheimer, L. C., Hammerschmied, C. G., Scholmerich, J. and Hugl, S. R. (2002). Hepatocyte growth factor stimulates proliferation of pancreatic beta-cells particularly in the presence of subphysiological glucose concentrations. *J Mol Endocrinol*, **28**(2): 99-110.

Galsgaard, E. D., Nielsen, J. H. and Moldrup, A. (1999). Regulation of prolactin receptor (PRLR) gene expression in insulin-producing cells. Prolactin and growth hormone activate one of the rat prlr gene promoters via STAT5a and STAT5b. *J Biol Chem.*, **274**(26): 18686-92.

Gannon, M. (2007). BuMP-ing up insulin secretion by pancreatic beta cells. *Cell Metab*, **5**(3): 157-9.

Gannon, M., Ables, E. T., Crawford, L., Lowe, D., Offield, M. F., Magnuson, M. A. and Wright, C. V. (2008). pdx-1 function is specifically required in embryonic beta cells to generate appropriate numbers of endocrine cell types and maintain glucose homeostasis. *Dev Biol*, **314**(2): 406-17.

Gannon, M., Gamer, L. W. and Wright, C. V. (2001). Regulatory regions driving developmental and tissue-specific expression of the essential pancreatic gene pdx1. *Dev Biol*, **238**(1): 185-201.

Gao, N., White, P., Doliba, N., Golson, M. L., Matschinsky, F. M. and Kaestner, K. H. (2007). Foxa2 controls vesicle docking and insulin secretion in mature Beta cells. *Cell Metab.*, **6**: 267-79.

Garcia-Ocana, A., Takane, K. K., Syed, M. A., Philbrick, W. M., Vasavada, R. C. and Stewart, A. F. (2000). Hepatocyte growth factor overexpression in the islet of transgenic mice increases beta cell proliferation, enhances islet mass, and induces mild hypoglycemia. *J Biol Chem*, **275**(2): 1226-32.

Garred, P., Thiel, S., Madsen, H. O., Ryder, L. P., Jensenius, J. C. and Svejgaard, A. (1992). Gene frequency and partial protein characterization of an allelic variant of mannan binding protein associated with low serum concentrations. *Clin Exp Immunol*, **90**(3): 517-21.

George, M., Ayuso, E., Casellas, A., Costa, C., Devedjian, J. C. and Bosch, F. (2002). Beta cell expression of IGF-I leads to recovery from type 1 diabetes. *J Clin Invest.* , **109**: 1153-63.

Georgia, S. and Bhushan, A. (2004). Beta cell replication is the primary mechanism for maintaining postnatal beta cell mass. *J Clin Invest*, **114**(7): 963-8.

Georgia, S. and Bhushan, A. (2006). p27 Regulates the transition of beta-cells from quiescence to proliferation. *Diabetes*, **55**(11): 2950-6.

Giuffrida, F. M. and Reis, A. F. (2005). Genetic and clinical characteristics of maturity-onset diabetes of the young. *Diabetes Obes Metab*, **7**(4): 318-26.

Gloyn, A. L., Hashim, Y., Ashcroft, S. J., Ashfield, R., Wiltshire, S. and Turner, R. C. (2001). Association studies of variants in promoter and coding regions of beta-cell ATP-sensitive K-channel genes SUR1 and Kir6.2 with Type 2 diabetes mellitus (UKPDS 53). *Diabet Med*, **18**(3): 206-12.

Gragnoli, C., Stanojevic, V., Gorini, A., Von Preussenthal, G. M., Thomas, M. K. and Habener, J. F. (2005). IPF-1/MODY4 gene missense mutation in an Italian family with type 2 and gestational diabetes. *Metabolism*, **54**(8): 983-8.

Greenhalgh, C. J., Bertolino, P., Asa, S. L., Metcalf, D., Corbin, J. E., Adams, T. E., Davey, H. W., Nicola, N. A., Hilton, D. J. and Alexander, W. S. (2002). Growth enhancement in suppressor of cytokine signaling 2 (SOCS-2)-deficient mice is dependent on signal transducer and activator of transcription 5b (STAT5b). *Mol Endocrinol*, **16**(6): 1394-406.

Gross, D. N., van den Heuvel, A. P. and Birnbaum, M. J. (2008). The role of FoxO in the regulation of metabolism. *Oncogene*, **27**(16): 2320-36.

Grunblatt, E., Salkovic-Petrisic, M., Osmanovic, J., Riederer, P. and Hoyer, S. (2007). Brain insulin system dysfunction in streptozotocin intracerebroventricularly treated rats generates hyperphosphorylated tau protein. *J Neurochem*, **101**(3): 757-70.

Gu, G., Dubauskaite, J. and Melton, D. A. (2002). Direct evidence for the pancreatic lineage: NGN3+ cells are islet progenitors and are distinct from duct progenitors. *Development*, **129**(10): 2447-57.

Gupta, R. K., Gao, N., Gorski, R. K., White, P., Hardy, O. T., Rafiq, K., Brestelli, J. E., Chen, G., Stoeckert, C. J., Jr. and Kaestner, K. H. (2007). Expansion of adult beta-cell mass in response to increased metabolic demand is dependent on HNF-4alpha. *Genes Dev*, **21**(7): 756-69.

Gusarova, G. A., Wang, I. C., Major, M. L., Kalinichenko, V. V., Ackerson, T., Petrovic, V. and Costa, R. H. (2007). A cell-penetrating ARF peptide inhibitor of FoxM1 in mouse hepatocellular carcinoma treatment. *J Clin Invest*, **117**(1): 99-111.

Hani, E. H., Boutin, P., Durand, E., Inoue, H., Permutt, M. A., Velho, G. and Froguel, P. (1998). Missense mutations in the pancreatic islet beta cell inwardly rectifying K+ channel gene (KIR6.2/BIR): a meta-analysis suggests a role in the polygenic basis of Type II diabetes mellitus in Caucasians. *Diabetologia*, **41**(12): 1511-5.

Hanley, S. C., Austin, E., Assouline-Thomas, B., Kapeluto, J., Blaichman, J., Moosavi, M., Petropavlovskaia, M. and Rosenberg, L. (2010). {beta}-Cell Mass Dynamics and Islet Cell Plasticity in Human Type 2 Diabetes. *Endocrinology*, **151**(4): 1462-72.

Hansen, L., Deacon, C. F., Orskov, C. and Holst, J. J. (1999). Glucagon-like peptide-1-(7-36)amide is transformed to glucagon-like peptide-1-(9-36) amide by dipeptidyl peptidase IV in the capillaries supplying the L cells of the porcine intestine. *Endocrinology*, **140**: 5356−63.

Harb, G., Vasavada, R. C., Cobrinik, D. and Stewart, A. F. (2009). The retinoblastoma protein and its homolog p130 regulate the G1/S transition in pancreatic beta-cells. *Diabetes*, **58**(8): 1852-62.

Harbour, J. W. and Dean, D. C. (2000). The Rb/E2F pathway: expanding roles and emerging paradigms. *Genes Dev*, **14**(19): 2393-409.

Hardt, P. D., Hauenschild, A., Nalop, J., Marzeion, A. M., Jaeger, C., Teichmann, J., Bretzel, R. G., Hollenhorst, M. and Kloer, H. U. (2003). High prevalence of exocrine pancreatic insufficiency in diabetes mellitus. A multicenter study screening fecal elastase 1 concentrations in 1,021 diabetic patients. *Pancreatology*, **3**(5): 395-402.

Hart, L. M., Dekker, J. M., van Haeften, T. W., Ruige, J. B., Stehouwer, C. D., Erkelens, D. W., Heine, R. J. and Maassen, J. A. (2000). Reduced second phase insulin

secretion in carriers of a sulphonylurea receptor gene variant associating with Type II diabetes mellitus. *Diabetologia*, **43**(4): 515-9.

Hattersley, A. T., Turner, R. C., Permutt, M. A., Patel, P., Tanizawa, Y., Chiu, K. C., O'Rahilly, S., Watkins, P. J. and Wainscoat, J. S. (1992). Linkage of type 2 diabetes to the glucokinase gene. *Lancet*, **339**(8805): 1307-10.

Havrankova, J., Brownstein, M. and Roth, J. (1981). Insulin and insulin receptors in rodent brain. *Diabetologia*, **20 Suppl**: 268-73.

Hayek, A., Beattie, G. M., Cirulli, V., Lopez, A. D., Ricordi, C. and Rubin, J. S. (1995). Growth factor/matrix-induced proliferation of human adult beta-cells. *Diabetes*, **44**(12): 1458-60.

Heit, J. J., Karnik, S. K. and Kim, S. K. (2006). Intrinsic regulators of pancreatic beta-cell proliferation. *Annu Rev Cell Dev Biol*, **22**: 311-38.

Hogg, J., Han, V. K., Clemmons, D. R. and Hill, D. J. (1993). Interactions of nutrients, insulin-like growth factors (IGFs) and IGF-binding proteins in the regulation of DNA synthesis by isolated fetal rat islets of Langerhans. *J Endocrinol*, **138**(3): 401-12.

Holmkvist, J., Almgren, P., Lyssenko, V., Lindgren, C. M., Eriksson, K. F., Isomaa, B., Tuomi, T., Nilsson, P. and Groop, L. (2008). Common variants in maturity-onset diabetes of the young genes and future risk of type 2 diabetes. *Diabetes*, **57**(6): 1738-44.

Homko, C., Sivan, E., Chen, X., Reece, E. A. and Boden, G. (2001). Insulin secretion during and after pregnancy in patients with gestational diabetes mellitus. *J Clin Endocrinol Metab*, **86**(2): 568-73.

Horikawa, Y., Iwasaki, N., Hara, M., Furuta, H., Hinokio, Y., Cockburn, B. N., Lindner, T., Yamagata, K., Ogata, M., Tomonaga, O., Kuroki, H., Kasahara, T., Iwamoto, Y. and Bell, G. I. (1997). Mutation in hepatocyte nuclear factor-1 beta gene (TCF2) associated with MODY. *Nat Genet*, **17**(4): 384-5.

Horikawa, Y., Oda, N., Cox, N. J., Li, X., Orho-Melander, M., Hara, M., Hinokio, Y., Lindner, T. H., Mashima, H., Schwarz, P. E., del Bosque-Plata, L., Oda, Y., Yoshiuchi, I., Colilla, S., Polonsky, K. S., Wei, S., Concannon, P., Iwasaki, N., Schulze, J., Baier, L. J., Bogardus, C., Groop, L., Boerwinkle, E., Hanis, C. L. and Bell, G. I. (2000). Genetic variation in the gene encoding calpain-10 is associated with type 2 diabetes mellitus. *Nat Genet*, **26**(2): 163-75.

Hotamisligil, G. S., Peraldi, P., Budavari, A., Ellis, R., White, M. F. and Spiegelman, B. M. (1996). IRS-1-mediated inhibition of insulin receptor tyrosine kinase activity in TNF-alpha- and obesity-induced insulin resistance. *Science*, **271**(5249): 665-8.

Huang, C., Snider, F. and Cross, J. C. (2009). Prolactin receptor is required for normal glucose homeostasis and modulation of beta-cell mass during pregnancy. *Endocrinology*, **150**(4): 1618-26.

Hugl, S. R., White, M. F. and Rhodes, C. J. (1998). Insulin-like growth factor I (IGF-I)-stimulated pancreatic beta-cell growth is glucosedependent. Synergistic activation of insulin receptor substratemediated signal transduction pathways by glucose and IGF-I in INS-1 cells. *Journal of Biological Chemistry*, **273**: 17771–9.

Iglesias, A., Murga, M., Laresgoiti, U., Skoudy, A., Bernales, I., Fullaondo, A., Moreno, B., Lloreta, J., Field, S. J., Real, F. X. and Zubiaga, A. M. (2004). Diabetes and exocrine pancreatic insufficiency in E2F1/E2F2 double-mutant mice. *J Clin Invest*, **113**(10): 1398-407.

Ishizuka, T., Klepcyk, P., Liu, S., Panko, L., Gibbs, E. M. and Friedman, J. E. (1999). Effects of overexpression of human GLUT4 gene on maternal diabetes and fetal growth in spontaneous gestational diabetic C57BLKS/J Lepr(db/+) mice. *Diabetes*, **48**(5): 1061-9.

Jabri, N., Schalch, D. S., Schwartz, S. L., Fischer, J. S., Kipnes, M. S., Radnik, B. J., Turman, N. J., Marcsisin, V. S. and Guler, H. P. (1994). Adverse effects of recombinant human insulin-like growth factor I in obese insulin-resistant type II diabetic patients. *Diabetes*, **43**(3): 369-74.

Jellema, A., Zeegers, M. P., Feskens, E. J., Dagnelie, P. C. and Mensink, R. P. (2003). Gly972Arg variant in the insulin receptor substrate-1 gene and association with Type 2 diabetes: a meta-analysis of 27 studies. *Diabetologia*, **46**(7): 990-5.

Joffe, G. M., Esterlitz, J. R., Levine, R. J., Clemens, J. D., Ewell, M. G., Sibai, B. M. and Catalano, P. M. (1998). The relationship between abnormal glucose tolerance and hypertensive disorders of pregnancy in healthy nulliparous women. Calcium for Preeclampsia Prevention (CPEP) Study Group. *Am J Obstet Gynecol*, **179**(4): 1032-7.

Jun, H. S. (2008). Regeneration of pancreatic beta cells. *Front Biosci*, **13**: 6170-82.

Kaestner, K. H., Knochel, W. and Martinez, D. E. (2000). Unified nomenclature for the winged helix/forkhead transcription factors. *Genes Dev*, **14**(2): 142-6.

Kalin, T. V., Wang, I. C., Ackerson, T. J., Major, M. L., Detrisac, C. J., Kalinichenko, V. V., Lyubimov, A. and Costa, R. H. (2006). Increased levels of the FoxM1 transcription factor accelerate development and progression of prostate carcinomas in both TRAMP and LADY transgenic mice. *Cancer Res*, **66**(3): 1712-20.

Kalin, T. V., Wang, I. C., Meliton, L., Zhang, Y., Wert, S. E., Ren, X., Snyder, J., Bell, S. M., Graf, L., Jr., Whitsett, J. A. and Kalinichenko, V. V. (2008). Forkhead Box m1 transcription factor is required for perinatal lung function. *Proc Natl Acad Sci U S A*, **105**(49): 19330-5.

Kalinichenko, V. V., Gusarova, G. A., Tan, Y., Wang, I. C., Major, M. L., Wang, X., Yoder, H. M. and Costa, R. H. (2003). Ubiquitous expression of the forkhead box M1B transgene accelerates proliferation of distinct pulmonary cell types following lung injury. *J Biol Chem*, **278**(39): 37888-94.

Kalinichenko, V. V., Major, M. L., Wang, X., Petrovic, V., Kuechle, J., Yoder, H. M., Dennewitz, M. B., Shin, B., Datta, A., Raychaudhuri, P. and Costa, R. H. (2004). Foxm1b transcription factor is essential for development of hepatocellular carcinomas and is negatively regulated by the p19ARF tumor suppressor. *Genes Dev*, **18**(7): 830-50.

Kalinina, O. A., Kalinin, S. A., Polack, E. W., Mikaelian, I., Panda, S., Costa, R. H. and Adami, G. R. (2003). Sustained hepatic expression of FoxM1B in transgenic mice has minimal effects on hepatocellular carcinoma development but increases cell proliferation rates in preneoplastic and early neoplastic lesions. *Oncogene*, **22**(40): 6266-76.

Karnik, S. K., Chen, H., McLean, G. W., Heit, J. J., Gu, X., Zhang, A. Y., Fontaine, M., Yen, M. H. and Kim, S. K. (2007). Menin controls growth of pancreatic beta-cells in pregnant mice and promotes gestational diabetes mellitus. *Science*, **318**(5851): 806-9.

Karnik, S. K., Hughes, C. M., Gu, X., Rozenblatt-Rosen, O., McLean, G. W., Xiong, Y., Meyerson, M. and Kim, S. K. (2005). Menin regulates pancreatic islet growth by promoting histone methylation and expression of genes encoding p27Kip1 and p18INK4c. *Proc Natl Acad Sci U S A*, **102**(41): 14659-64.

Kassem, S. A., Ariel, I., Thornton, P. S., Scheimberg, I. and Glaser, B. (2000). Beta-cell proliferation and apoptosis in the developing normal human pancreas and in hyperinsulinism of infancy. *Diabetes*, **49**(8): 1325-33.

Kaufmann, R. C., Amankwah, K. S., Colliver, J. A. and Arbuthnot, J. (1987). Diabetic pregnancy. The effect of genetic susceptibility for diabetes on fetal weight. *Am J Perinatol*, **4**(1): 72-4.

Kaufmann, R. C., Amankwah, K. S., Dunaway, G., Maroun, L., Arbuthnot, J. and Roddick, J. W., Jr. (1981). An animal model of gestational diabetes. *Am J Obstet Gynecol*, **141**(5): 479-82.

Kautzky-Willer, A., Prager, R., Waldhausl, W., Pacini, G., Thomaseth, K., Wagner, O. F., Ulm, M., Streli, C. and Ludvik, B. (1997). Pronounced insulin resistance and inadequate beta-cell secretion characterize lean gestational diabetes during and after pregnancy. *Diabetes Care*, **20**(11): 1717-23.

Kawai, M. and Kishi, K. (1999). Adaptation of pancreatic islet B-cells during the last third of pregnancy: regulation of B-cell function and proliferation by lactogenic hormones in rats. *Eur J Endocrinol*, **141**(4): 419-25.

Kido, Y., Nakae, J., Hribal, M. L., Xuan, S., Efstratiadis, A. and Accili, D. (2002). Effects of mutations in the insulin-like growth factor signaling system on embryonic pancreas development and beta-cell compensation to insulin resistance. *J Biol Chem*, **277**(39): 36740-7.

Kilpatrick, D. C. (2002). Mannan-binding lectin: clinical significance and applications. *Biochim Biophys Acta*, **1572**(2-3): 401-13.

Kim, I. M., Ackerson, T., Ramakrishna, S., Tretiakova, M., Wang, I. C., Kalin, T. V., Major, M. L., Gusarova, G. A., Yoder, H. M., Costa, R. H. and Kalinichenko, V. V. (2006). The Forkhead Box m1 transcription factor stimulates the proliferation of tumor cells during development of lung cancer. *Cancer Res*, **66**(4): 2153-61.

Kim, I. M., Ramakrishna, S., Gusarova, G. A., Yoder, H. M., Costa, R. H. and Kalinichenko, V. V. (2005). The forkhead box m1 transcription factor is essential for embryonic development of pulmonary vasculature. *J Biol Chem*, **280**(23): 22278-86.

Kim, I. M., Ramakrishna, S., Gusarova, G. A., Yoder, H. M., Costa, R. H. and Kalinichenko, V. V. (2005). The forkhead box m1 transcription factor is essential for embryonic development of pulmonary vasculature. *J. Biol. Chem.* , **280**: 22278–86.

Kioussi, C., Briata, P., Baek, S. H., Rose, D. W., Hamblet, N. S., Herman, T., Ohgi, K. A., Lin, C., Gleiberman, A., Wang, J., Brault, V., Ruiz-Lozano, P., Nguyen, H. D., Kemler, R., Glass, C. K., Wynshaw-Boris, A. and Rosenfeld, M. G. (2002). Identification of a Wnt/Dvl/beta-Catenin --> Pitx2 pathway mediating cell-type-specific proliferation during development. *Cell*, **111**(5): 673-85.

Kitamura, T., Nakae, J., Kitamura, Y., Kido, Y., Biggs, W. H., Wright , C. V., White, M. F., Arden, K. C. and Accili, D. (2002). The forkhead transcription factor Foxo1 links insulin signaling to Pdx1 regulation of pancreatic beta cell growth. *J Clin Invest.* , **110**: 1839-47.

Korver, W., Roose, J., and Clevers, H. (1997a). The winged-helix transcription factor Trident is expressed in cycling cells. *Nucleic Acids Res*, **25**(9): 1715-9.

Korver, W., Roose, J., Heinen, K., Weghuis, D. O., de Bruijn, D., van Kessel, A. G. and Clevers, H. (1997b). The human TRIDENT/HFH-11/FKHL16 gene: structure, localization, and promoter characterization. *Genomics*, **46**(3): 435-42.

Korver, W., Roose, J., Wilson, A. and Clevers, H. (1997c). The winged-helix transcription factor Trident is expressed in actively dividing lymphocytes. *Immunobiology*, **198**: 157–61.

Korver, W., Schilham, M. W., Moerer, P., van den Hoff, M. J., Dam, K., Lamers, W. H., Medema, R. H. and Clevers, H. (1998). Uncoupling of S phase and mitosis in cardiomyocytes and hepatocytes lacking the winged-helix transcription factor Trident. *Curr Biol*, **8**(24): 1327-30.

Krishnamurthy, J., Ramsey, M. R., Ligon, K. L., Torrice, C., Koh, A., Bonner-Weir, S. and Sharpless, N. E. (2006). p16INK4a induces an age-dependent decline in islet regenerative potential. *Nature*, **443**(7110): 453-7.

Krupczak-Hollis, K., Wang, X., Dennewitz, M. B. and Costa, R. H. (2003). Growth hormone stimulates proliferation of old-aged regenerating liver through forkhead box m1b. *Hepatology*, **38**: 1552–62.

Krupczak-Hollis, K., Wang, X., Dennewitz, M. B. and Costa, R. H. (2003). Growth hormone stimulates proliferation of old-aged regenerating liver through forkhead box m1b. *Hepatology*, **38**(6): 1552-62.

Krupczak-Hollis, K., Wang, X., Kalinichenko, V. V., Gusarova, G. A., Wang, I. C., Dennewitz, M. B., Yoder, H. M., Kiyokawa, H., Kaestner, K. H. and Costa, R. H. (2004). The mouse Forkhead Box m1 transcription factor is essential for hepatoblast mitosis and development of intrahepatic bile ducts and vessels during liver morphogenesis. *Dev Biol*, **276**(1): 74-88.

Kulkarni, R. N. (2005). New insights into the roles of insulin/IGF-I in the development and maintenance of beta-cell mass. *Rev Endocr Metab Disord*, **6**(3): 199-210.

Kulkarni, R. N., Bruning, J. C., Winnay, J. N., Postic, C., Magnuson, M. A. and Kahn, C. R. (1999). Tissue-specific knockout of the insulin receptor in pancreatic beta cells creates an insulin secretory defect similar to that in type 2 diabetes. *Cell*, **96**: 329-39.

Kulkarni, R. N., Holzenberger, M., Shih, D. Q., Ozcan, U., Stoffel, M., Magnuson, M. A. and Kahn, C. R. (2002). Beta-cell-specific deletion of the Igf1 receptor leads to hyperinsulinemia and glucose intolerance but does not alter beta-cell mass. *Nature Genetics*, **31**: 111-15.

Kushner, J. A., Ciemerych, M. A., Sicinska, E., Wartschow, L. M., Teta, M., Long, S. Y., Sicinski, P. and White, M. F. (2005). Cyclins D2 and D1 are essential for postnatal pancreatic beta-cell growth. *Mol Cell Biol*, **25**(9): 3752-62.

Lain, K. Y. and Catalano, P. M. (2007). *Metabolic changes in pregnancy. *Clin Obstet Gynecol*, **50**(4): 938-48.

Lantz, K. A., Vatamaniuk, M. Z., Brestelli, J. E., Friedman, J. R., Matschinsky, F. M. and Kaestner, K. H. (2004). Foxa2 regulates multiple pathways of insulin secretion. *J Clin Invest*, **114**(4): 512-20.

Laoukili, J., Kooistra, M. R., Bras, A., Kauw, J., Kerkhoven, R. M., Morrison, A., Clevers, H. and Medema, R. H. (2005). FoxM1 is required for execution of the mitotic programme and chromosome stability. *Nat Cell Biol*, **7**(2): 126-36.

Laoukili, J., Stahl, M. and Medema, R. H. (2007). FoxM1: at the crossroads of ageing and cancer. *Biochim Biophys Acta*, **1775**(1): 92-102.

Lapolla, A., Dalfra, M. G. and Fedele, D. (2005). Insulin therapy in pregnancy complicated by diabetes: are insulin analogs a new tool? *Diabetes Metab Res Rev*, **21**(3): 241-52.

Latres, E., Malumbres, M., Sotillo, R., Martin, J., Ortega, S., Martin-Caballero, J., Flores, J. M., Cordon-Cardo, C. and Barbacid, M. (2000). Limited overlapping roles of P15(INK4b) and P18(INK4c) cell cycle inhibitors in proliferation and tumorigenesis. *EMBO J*, **19**(13): 3496-506.

Lauenborg, J., Damm, P., Ek, J., Glumer, C., Jorgensen, T., Borch-Johnsen, K., Vestergaard, H., Hornnes, P., Pedersen, O. and Hansen, T. (2004). Studies of the Ala/Val98 polymorphism of the hepatocyte nuclear factor-1alpha gene and the relationship to beta-cell function during an OGTT in glucose-tolerant women with and without previous gestational diabetes mellitus. *Diabet Med*, **21**(12): 1310-5.

Le Roith, D., Hendricks, S. A., Lesniak, M. A., Rishi, S., Becker, K. L., Havrankova, J., Rosenzweig, J. L., Brownstein, M. J. and Roth, J. (1983). Insulin in brain and other extrapancreatic tissues of vertebrates and nonvertebrates. *Adv Metab Disord*, **10**: 303-40.

Lee, J. H., Jo, J., Hardikar, A. A., Periwal, V. and Rane, S. G. (2010). Cdk4 regulates recruitment of quiescent beta-cells and ductal epithelial progenitors to reconstitute beta-cell mass. *PLoS One*, **5**(1): e8653.

Leipold, H., Knoefler, M., Gruber, C., Klein, K., Haslinger, P. and Worda, C. (2006). Plasminogen activator inhibitor 1 gene polymorphism and gestational diabetes mellitus. *Obstet Gynecol*, **107**(3): 651-6.

Leipold, H., Knofler, M., Gruber, C., Haslinger, P., Bancher-Todesca, D. and Worda, C. (2004). Calpain-10 haplotype combination and association with gestational diabetes mellitus. *Obstet Gynecol*, **103**(6): 1235-40.

Leung, T. W., Lin, S. S., Tsang, A. C., Tong, C. S., Ching, J. C., Leung, W. Y., Gimlich, R., Wong, G. G. and Yao, K. M. (2001). Over-expression of FoxM1 stimulates cyclin B1 expression. *FEBS Lett*, **507**(1): 59-66.

Li, R., Chase, M., Jung, S. K., Smith, P. J. and Loeken, M. R. (2005a). Hypoxic stress in diabetic pregnancy contributes to impaired embryo gene expression and defective development by inducing oxidative stress. *Am J Physiol Endocrinol Metab*, **289**(4): E591-9.

Li, S. K., Smith, D. K., Leung, W. Y., Cheung, A. M., Lam, E. W., Dimri, G. P. and Yao, K. M. (2008). FoxM1c counteracts oxidative stress-induced senescence and stimulates Bmi-1 expression. *J Biol Chem*, **283**(24): 16545-53.

Li, Y., Cao, X., Li, L. X., Brubaker, P. L., Edlund, H. and Drucker, D. (2005b). Beta-Cell Pdx1 expression is essential for the glucoregulatory, proliferative, and cytoprotective actions of glucagon like peptide-1. *Diabetes*, **54**: 482-91.

Lindsay, R. S. (2009). Gestational diabetes: causes and consequences. *The British Journal of Diabetes & Vascular Disease*, **9**: 27-31.

Ling, Z., Wu, D., Zambre, Y., Flamez, D., Drucker, D. J. and Pipeleers, D. G. (2001). Glucagon-like peptide 1 receptor signaling influences topography of islet cells in mice. *Virchows Arch.* , **438**: 382-87.

Lingohr, M. K., Dickson, L. M., McCuaig, J. F., Hugl, S. R., Twardzik, D. R. and Rhodes, C. J. (2002). Activation of IRS-2-mediated signal transduction by IGF-1, but not

TGF-alpha or EGF, augments pancreatic beta-cell proliferation. *Diabetes*, **51**: 966-76.

Litou, H., Anastasiou, E., Thalassinou, L., Sarika, H. L., Philippou, G. and Alevizaki, M. (2007). Increased prevalence of VNTR III of the insulin gene in women with gestational diabetes mellitus (GDM). *Diabetes Res Clin Pract*, **76**(2): 223-8.

Liu, M., Dai, B., Kang, S. H., Ban, K., Huang, F. J., Lang, F. F., Aldape, K. D., Xie, T. X., Pelloski, C. E., Xie, K., Sawaya, R. and Huang, S. (2006). FoxM1B is overexpressed in human glioblastomas and critically regulates the tumorigenicity of glioma cells. *Cancer Res*, **66**(7): 3593-602.

Liu, Z. and Habener, J. F. (2008). Glucagon-like peptide-1 activation of TCF7L2-dependent Wnt signaling enhances pancreatic beta cell proliferation. *J Biol Chem*, **283**(13): 8723-35.

Livak, K. J. and Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods*, **25**(4): 402-8.

Long, Q., Shelton, K. D., Lindner, J., Jones, J. R. and Magnuson, M. A. (2004). Efficient DNA cassette exchange in mouse embryonic stem cells by staggered positive-negative selection. *Genesis*, **39**(4): 256-62.

Loos, R. J., Franks, P. W., Francis, R. W., Barroso, I., Gribble, F. M., Savage, D. B., Ong, K. K., O'Rahilly, S. and Wareham, N. J. (2007). TCF7L2 polymorphisms modulate proinsulin levels and beta-cell function in a British Europid population. *Diabetes*, **56**(7): 1943-7.

Love-Gregory, L. and Permutt, M. A. (2007). HNF4A genetic variants: role in diabetes. *Curr Opin Clin Nutr Metab Care*, **10**(4): 397-402.

Lucassen, A. M., Screaton, G. R., Julier, C., Elliott, T. J., Lathrop, M. and Bell, J. I. (1995). Regulation of insulin gene expression by the IDDM associated, insulin locus haplotype. *Hum Mol Genet*, **4**(4): 501-6.

Ludwig, C. U., Menke, A., Adler, G. and Lutz, M. P. (1999). Fibroblasts stimulate acinar cell proliferation through IGF-I during regeneration from acute pancreatitis. *Am J Physiol*, **276**(1 Pt 1): G193-8.

Luscher-Firzlaff, J. M., Westendorf, J. M., Zwicker, J., Burkhardt, H., Henriksson, M., Muller, R., Pirollet, F. and Luscher, B. (1999). Interaction of the fork head domain transcription factor MPP2 with the human papilloma virus 16 E7 protein: enhancement of transformation and transactivation. *Oncogene*, **18**(41): 5620-30.

Lyon, M. A., Ducruet, A. P., Wipf, P. and Lazo, J. S. (2002). Dual-specificity phosphatases as targets for antineoplastic agents. *Nat Rev Drug Discov*, **1**(12): 961-76.

Ma, R. Y., Tong, T. H., Cheung, A. M., Tsang, A. C., Leung, W. Y. and Yao, K. M. (2005). Raf/MEK/MAPK signaling stimulates the nuclear translocation and transactivating activity of FOXM1c. *J Cell Sci*, **118**(Pt 4): 795-806.

Madsen, H. O., Garred, P., Thiel, S., Kurtzhals, J. A., Lamm, L. U., Ryder, L. P. and Svejgaard, A. (1995). Interplay between promoter and structural gene variants control basal serum level of mannan-binding protein. *J Immunol*, **155**(6): 3013-20.

Madureira, P. A., Varshochi, R., Constantinidou, D., Francis, R. E., Coombes, R. C., Yao, K. M. and Lam, E. W. (2006). The Forkhead box M1 protein regulates the transcription of the estrogen receptor alpha in breast cancer cells. *J Biol Chem*, **281**(35): 25167-76.

Major, M. L., Lepe, R. and Costa, R. H. (2004). Forkhead box M1B transcriptional activity requires binding of Cdk-cyclin complexes for phosphorylation-dependent recruitment of p300/CBP coactivators. *Mol Cell Biol*, **24**(7): 2649-61.

Malecki, M. T., Jhala, U. S., Antonellis, A., Fields, L., Doria, A., Orban, T., Saad, M., Warram, J. H., Montminy, M. and Krolewski, A. S. (1999). Mutations in NEUROD1 are associated with the development of type 2 diabetes mellitus. *Nat Genet*, **23**(3): 323-8.

Malumbres, M. and Barbacid, M. (2009). Cell cycle, CDKs and cancer: a changing paradigm. *Nat Rev Cancer*, **9**(3): 153-66.

Martin, J., Hunt, S. L., Dubus, P., Sotillo, R., Nehme-Pelluard, F., Magnuson, M. A., Parlow, A. F., Malumbres, M., Ortega, S. and Barbacid, M. (2003). Genetic rescue of Cdk4 null mice restores pancreatic beta-cell proliferation but not homeostatic cell number. *Oncogene*, **22**(34): 5261-9.

Marzo, N., Mora, C., Fabregat, M. E., Martin, J., Usac, E. F., Franco, C., Barbacid, M. and Gomis, R. (2004). Pancreatic islets from cyclin-dependent kinase 4/R24C (Cdk4) knockin mice have significantly increased beta cell mass and are physiologically functional, indicating that Cdk4 is a potential target for pancreatic beta cell mass regeneration in Type 1 diabetes. *Diabetologia*, **47**(4): 686-94.

Matsumoto-Taniura, N., Pirollet, F., Monroe, R., Gerace, L. and Westendorf, J. M. (1996). Identification of novel M phase phosphoproteins by expression cloning. *Mol Biol Cell*, **7**(9): 1455-69.

McEvoy, R. C. and Madson, K. L. (1980). Pancreatic insulikn-, glucagon-, and somatostatin-positive islet cell populatins during the perinatal development of the rat. I. Morphometric quantitation. *Biol Neonate*, **38**(5-6): 248-54.

McGettrick, A. J., Feener, E. P. and Kahn, C. R. (2005). Human insulin receptor substrate-1 (IRS-1) polymorphism G972R causes IRS-1 to associate with the insulin receptor and inhibit receptor autophosphorylation. *J Biol Chem*, **280**(8): 6441-6.

McGovern, U. B., Francis, R. E., Peck, B., Guest, S. K., Wang, J., Myatt, S. S., Krol, J., Kwok, J. M., Polychronis, A., Coombes, R. C. and Lam, E. W. (2009). Gefitinib (Iressa) represses FOXM1 expression via FOXO3a in breast cancer. *Mol Cancer Ther*, **8**(3): 582-91.

Megia, A., Gallart, L., Fernandez-Real, J. M., Vendrell, J., Simon, I., Gutierrez, C. and Richart, C. (2004). Mannose-binding lectin gene polymorphisms are associated with gestational diabetes mellitus. *J Clin Endocrinol Metab*, **89**(10): 5081-7.

Meier, J. J. (2008a). Beta cell mass in diabetes: a realistic therapeutic target? *Diabetologia*, **51**(5): 703-13.

Meier, J. J., Butler, A. E., Saisho, Y., Monchamp, T., Galasso, R., Bhushan, A., Rizza, R. A. and Butler, P. C. (2008b). Beta-cell replication is the primary mechanism subserving the postnatal expansion of beta-cell mass in humans. *Diabetes*, **57**(6): 1584-94.

Mellone, B. G. and Allshire, R. C. (2003). Stretching it: putting the CEN(P-A) in centromere. *Curr Opin Genet Dev*, **13**(2): 191-8.

Meraldi, P., Honda, R. and Nigg, E. A. (2004). Aurora kinases link chromosome segregation and cell division to cancer susceptibility. *Curr Opin Genet Dev*, **14**(1): 29-36.

Minh, L. C., Galasso, R., Gurlo, T., Basu, R., Rizza, R., Butler, P. (2008). Three Fold Increase in Beta Cell Mass in Twenty Weeks in Human Pregnancy. *Diabetes*, **57 (S1)**: A1.

Montanya, E., Nacher, V., Biarnes, M. and Soler, J. (2000). Linear correlation between beta-cell mass and body weight throughout the lifespan in Lewis rats: role of beta-cell hyperplasia and hypertrophy. *Diabetes*, **49**(8): 1341-6.

Moses, A. C., Young, S. C., Morrow, L. A., O'Brien, M. and Clemmons, D. R. (1996). Recombinant human insulin-like growth factor I increases insulin sensitivity and improves glycemic control in type II diabetes. *Diabetes*, **45**(1): 91-100.

Munoz, J., Lok, K. H., Gower, B. A., Fernandez, J. R., Hunter, G. R., Lara-Castro, C., De Luca, M. and Garvey, W. T. (2006). Polymorphism in the transcription factor 7-like 2 (TCF7L2) gene is associated with reduced insulin secretion in nondiabetic women. *Diabetes*, **55**(12): 3630-4.

Nakamura, T., Teramoto, H. and Ichihara, A. (1986). Purification and characterization of a growth factor from rat platelets for mature parenchymal hepatocytes in primary cultures. *Proc. Natl. Acad. Sci.*, **83**.

Nasir, I., Kedees, M. H., Ehrlich, M. E. and Teitelman, G. (2005). The role of pregnancy hormones in the regulation of Pdx-1 expression. *Mol Cell Endocrinol*, **233**(1-2): 1-13.

Neve, B., Fernandez-Zapico, M. E., Ashkenazi-Katalan, V., Dina, C., Hamid, Y. H., Joly, E., Vaillant, E., Benmezroua, Y., Durand, E., Bakaher, N., Delannoy, V.,

Vaxillaire, M., Cook, T., Dallinga-Thie, G. M., Jansen, H., Charles, M. A., Clement, K., Galan, P., Hercberg, S., Helbecque, N., Charpentier, G., Prentki, M., Hansen, T., Pedersen, O., Urrutia, R., Melloul, D. and Froguel, P. (2005). Role of transcription factor KLF11 and its diabetes-associated gene variants in pancreatic beta cell function. *Proc Natl Acad Sci U S A*, **102**(13): 4807-12.

Nikaidou, N., Kamio, Y. and Izaki, K. (1992). Molecular cloning and nucleotide sequence of a pectin lyase gene from Pseudomonas marginalis N6301. *Biochem Biophys Res Commun*, **182**(1): 14-9.

Nilsson, I. and Hoffmann, I. (2000). Cell cycle regulation by the Cdc25 phosphatase family. *Prog Cell Cycle Res*, **4**: 107-14.

O'Brien, R. M. and Granner, D. K. (1996). Regulation of gene expression by insulin. *Physiol Rev*, **76**(4): 1109-61.

Ober, C., Xiang, K. S., Thisted, R. A., Indovina, K. A., Wason, C. J. and Dooley, S. (1989). Increased risk for gestational diabetes mellitus associated with insulin receptor and insulin-like growth factor II restriction fragment length polymorphisms. *Genet Epidemiol*, **6**(5): 559-69.

Ogren, L., Southard, J. N., Colosi, P., Linzer, D. I. and Talamantes, F. (1989). Mouse placental lactogen-I: RIA and gestational profile in maternal serum. *Endocrinology*, **125**(5): 2253-7.

Otonkoski, T., Beattie, G. M., Rubin, J. S., Lopez, A. D., Baird, A. and Hayek, A. (1994). Hepatocyte growth factor/scatter factor has insulinotropic activity in human fetal pancreatic cells. *Diabetes*, **43**(7): 947-53.

Otonkoski, T., Cirulli, V., Beattie, M., Mally, M. I., Soto, G. and Rubin, J. S. (1996). A role for hepatocyte growth factor/scatter factor in fetal mesenchyme-induced pancreatic beta-cell growth. *Endocrinology*, **137**: 3131–9.

Palmer, N. D., Lehtinen, A. B., Langefeld, C. D., Campbell, J. K., Haffner, S. M., Norris, J. M., Bergman, R. N., Goodarzi, M. O., Rotter, J. I. and Bowden, D. W. (2008). Association of TCF7L2 gene polymorphisms with reduced acute insulin response in Hispanic Americans. *J Clin Endocrinol Metab*, **93**(1): 304-9.

Park, H. J., Costa, R. H., Lau, L. F., Tyner, A. L. and Raychaudhuri, P. (2008a). Anaphase-promoting complex/cyclosome-CDH1-mediated proteolysis of the forkhead box M1 transcription factor is critical for regulated entry into S phase. *Mol Cell Biol*, **28**(17): 5162-71.

Park, H. J., Wang, Z., Costa, R. H., Tyner, A., Lau, L. F. and Raychaudhuri, P. (2008b). An N-terminal inhibitory domain modulates activity of FoxM1 during cell cycle. *Oncogene*, **27**(12): 1696-704.

Park, M. K., Kim, D. K. and Lee, H. J. (2003). Adenoviral mediated hepatocyte growth factor gene attenuates hyperglycemia and beta cell destruction in overt diabetic mice. *Exp Mol Med.*, **35**: 494–500.

Parsons, J. A., Brelje, T. C. and Sorenson, R. L. (1992). Adaptation of islets of Langerhans to pregnancy: increased islet cell proliferation and insulin secretion correlates with the onset of placental lactogen secretion. *Endocrinology*, **130**(3): 1459-66.

Pearson, T. A. and Manolio, T. A. (2008). How to interpret a genome-wide association study. *JAMA*, **299**(11): 1335-44.

Perfetti, R. and Hui, H. (2004). The role of GLP-1 in the life and death of pancreatic beta cells. *Hormone & Metabolic Research*, **36**: 804-10.

Perkins, M. J., Dunn, J. P. and Jagasia, S. M. (2007). Perspectives in Gestational Diabetes Mellitus: A Review of Screening, Diagnosis, and Treatment. *Clinical Diabetes*, **25**(2): 57-62.

Petrovic, V., Costa, R. H., Lau, L. F., Raychaudhuri, P. and Tyner, A. L. (2008). FoxM1 regulates growth factor-induced expression of kinase-interacting stathmin (KIS) to promote cell cycle progression. *J Biol Chem*, **283**(1): 453-60.

Phelan, S. A., Ito, M. and Loeken, M. R. (1997). Neural tube defects in embryos of diabetic mice: role of the Pax-3 gene and apoptosis. *Diabetes*, **46**(7): 1189-97.

Plengvidhya, N., Kooptiwut, S., Songtawee, N., Doi, A., Furuta, H., Nishi, M., Nanjo, K., Tantibhedhyangkul, W., Boonyasrisawat, W., Yenchitsomanus, P. T., Doria, A. and Banchuin, N. (2007). PAX4 mutations in Thais with maturity onset diabetes of the young. *J Clin Endocrinol Metab*, **92**(7): 2821-6.

Postic, C. and Magnuson, M. A. (2000). DNA excision in liver by an albumin-Cre transgene occurs progressively with age. *Genesis*, **26**(2): 149-50.

Quin, J. D., Miell, J. P., Smith, K., Gordon, D., Strachan, J., Dick, J. B. and MacCuish, A. C. (1994). Effect of insulin-like growth factor-I therapy on erythropoetin concentrations in extreme insulin resistance. *Diabetologia*, **37**(4): 439.

Rachdi, L., Balcazar, N., Elghazi, L., Barker, D. J., Krits, I., Kiyokawa, H. and Bernal-Mizrachi, E. (2006). Differential effects of p27 in regulation of beta-cell mass during development, neonatal period, and adult life. *Diabetes*, **55**(12): 3520-8.

Rached, M. T., Kode, A., Silva, B. C., Jung, D. Y., Gray, S., Ong, H., Paik, J. H., DePinho, R. A., Kim, J. K., Karsenty, G. and Kousteni, S. (2010). FoxO1 expression in osteoblasts regulates glucose homeostasis through regulation of osteocalcin in mice. *J Clin Invest*, **120**(1): 357-68.

Radhakrishnan, S. K., Bhat, U. G., Hughes, D. E., Wang, I. C., Costa, R. H. and Gartel, A. L. (2006). Identification of a chemical inhibitor of the oncogenic transcription factor forkhead box M1. *Cancer Res*, **66**(19): 9731-5.

Raeder, H., Johansson, S., Holm, P. I., Haldorsen, I. S., Mas, E., Sbarra, V., Nermoen, I., Eide, S. A., Grevle, L., Bjorkhaug, L., Sagen, J. V., Aksnes, L., Sovik, O., Lombardo, D., Molven, A. and Njolstad, P. R. (2006). Mutations in the CEL VNTR

cause a syndrome of diabetes and pancreatic exocrine dysfunction. *Nat Genet*, **38**(1): 54-62.

Ramakrishna, S., Kim, I. M., Petrovic, V., Malin, D., Wang, I. C., Kalin, T. V., Meliton, L., Zhao, Y. Y., Ackerson, T., Qin, Y., Malik, A. B., Costa, R. H. and Kalinichenko, V. V. (2007). Myocardium defects and ventricular hypoplasia in mice homozygous null for the Forkhead Box M1 transcription factor. *Dev Dyn*, **236**(4): 1000-13.

Rane, S. G., Dubus, P., Mettus, R. V., Galbreath, E. J., Boden, G., Reddy, E. P. and Barbacid, M. (1999). Loss of Cdk4 expression causes insulin-deficient diabetes and Cdk4 activation results in beta-islet cell hyperplasia. *Nat Genet*, **22**(1): 44-52.

Reece, E. A., Leguizamon, G. and Wiznitzer, A. (2009). *Gestational diabetes: the need for a common ground. *Lancet*, **373**(9677): 1789-97.

Rieck, S. and Kaestner, K. H. (2009). Expansion of beta-cell mass in response to pregnancy. *Trends Endocrinol Metab*.

Rieck, S., White, P., Schug, J., Fox, A. J., Smirnova, O., Gao, N., Gupta, R. K., Wang, Z. V., Scherer, P. E., Keller, M. P., Attie, A. D. and Kaestner, K. H. (2009b). The transcriptional response of the islet to pregnancy in mice. *Mol Endocrinol*, **23**(10): 1702-12.

Robitaille, J. and Grant, A. M. (2008). **The genetics of gestational diabetes mellitus: evidence for relationship with type 2 diabetes mellitus. *Genet Med*, **10**(4): 240-50.

Roccisana, J., Reddy, V., Vasavada, R. C., Gonzalez-Pertusa, J. A., Magnuson, M. A. and Garcia-Ocana, A. (2005). Targeted inactivation of hepatocyte growth factor receptor c-met in beta-cells leads to defective insulin secretion and GLUT-2 downregulation without alteration of beta-cell mass. *Diabetes*, **54**(7): 2090-102.

Ruchat, S. M., Elks, C. E., Loos, R. J., Vohl, M. C., Weisnagel, S. J., Rankinen, T., Bouchard, C. and Perusse, L. (2009). Association between insulin secretion, insulin sensitivity and type 2 diabetes susceptibility variants identified in genome-wide association studies. *Acta Diabetol*, **46**(3): 217-26.

Ryan, E. A., Imes, S., Liu, D., McManus, R., Finegood, D. T., Polonsky, K. S. and Sturis, J. (1995). Defects in insulin secretion and action in women with a history of gestational diabetes. *Diabetes*, **44**(5): 506-12.

Saido, T. C., Sorimachi, H. and Suzuki, K. (1994). Calpain: new perspectives in molecular diversity and physiological-pathological involvement. *FASEB J*, **8**(11): 814-22.

Scaglia, L., Cahill, C. J., Finegood, D. T. and Bonner-Weir, S. (1997). Apoptosis participates in the remodeling of the endocrine pancreas in the neonatal rat. *Endocrinology*, **138**(4): 1736-41.

Scaglia, L., Smith, F. E. and Bonner-Weir, S. (1995). Apoptosis contributes to the involution of beta cell mass in the post partum rat pancreas. *Endocrinology*, **136**(12): 5461-8.

Schaefer, U. M., Songster, G., Xiang, A., Berkowitz, K., Buchanan, T. A. and Kjos, S. L. (1997). Congenital malformations in offspring of women with hyperglycemia first detected during pregnancy. *Am J Obstet Gynecol*, **177**(5): 1165-71.

Schafer, S. A., Tschritter, O., Machicao, F., Thamer, C., Stefan, N., Gallwitz, B., Holst, J. J., Dekker, J. M., t Hart, L. M., Nijpels, G., van Haeften, T. W., Haring, H. U. and Fritsche, A. (2007). Impaired glucagon-like peptide-1-induced insulin secretion in carriers of transcription factor 7-like 2 (TCF7L2) gene polymorphisms. *Diabetologia*, **50**(12): 2443-50.

Schalch, D. S., Turman, N. J., Marcsisin, V. S., Heffernan, M. and Guler, H. P. (1993). Short-term effects of recombinant human insulin-like growth factor I on metabolic control of patients with type II diabetes mellitus. *J Clin Endocrinol Metab*, **77**(6): 1563-8.

Schwanstecher, C., Meyer, U. and Schwanstecher, M. (2002). K(IR)6.2 polymorphism predisposes to type 2 diabetes by inducing overactivity of pancreatic beta-cell ATP-sensitive K(+) channels. *Diabetes*, **51**(3): 875-9.

Schwarz, P. E., Govindarajalu, S., Towers, W., Schwanebeck, U., Fischer, S., Vasseur, F., Bornstein, S. R. and Schulze, J. (2006). Haplotypes in the promoter region of the ADIPOQ gene are associated with increased diabetes risk in a German Caucasian population. *Horm Metab Res*, **38**(7): 447-51.

Scrocchi, L. A., Brown, T. J., MaClusky, N., Brubaker, P. L., Auerbach, A. B. and Joyner, A. L. (1996). Glucose intolerance but normal satiety in mice with a null mutation in the glucagon-like peptide 1 receptor gene. *Nature Medicine*, **2**: 1254–8.

Shaat, N., Ekelund, M., Lernmark, A., Ivarsson, S., Almgren, P., Berntorp, K. and Groop, L. (2005). Association of the E23K polymorphism in the KCNJ11 gene with gestational diabetes mellitus. *Diabetologia*, **48**(12): 2544-51.

Shaat, N., Ekelund, M., Lernmark, A., Ivarsson, S., Nilsson, A., Perfekt, R., Berntorp, K. and Groop, L. (2004). Genotypic and phenotypic differences between Arabian and Scandinavian women with gestational diabetes mellitus. *Diabetologia*, **47**(5): 878-84.

Shaat, N., Karlsson, E., Lernmark, A., Ivarsson, S., Lynch, K., Parikh, H., Almgren, P., Berntorp, K. and Groop, L. (2006). Common variants in MODY genes increase the risk of gestational diabetes mellitus. *Diabetologia*, **49**(7): 1545-51.

Shaat, N., Lernmark, A., Karlsson, E., Ivarsson, S., Parikh, H., Berntorp, K. and Groop, L. (2007). A variant in the transcription factor 7-like 2 (TCF7L2) gene is associated with an increased risk of gestational diabetes mellitus. *Diabetologia*, **50**(5): 972-9.

Sherr, C. J. and Roberts, J. M. (1999). CDK inhibitors: positive and negative regulators of G1-phase progression. *Genes Dev*, **13**(12): 1501-12.

Sherr, C. J. and Roberts, J. M. (2004). Living with or without cyclins and cyclin-dependent kinases. *Genes Dev*, **18**(22): 2699-711.

Shu, L., Sauter, N. S., Schulthess, F. T., Matveyenko, A. V., Oberholzer, J. and Maedler, K. (2008). Transcription factor 7-like 2 regulates beta-cell survival and function in human pancreatic islets. *Diabetes*, **57**(3): 645-53.

Sieradzki, J., Fleck, H., Chatterjee, A. K. and Schatz, H. (1988). Stimulatory effect of insulin-like growth factor-I on [3H]thymidine incorporation, DNA content and insulin biosynthesis and secretion of isolated pancreatic rat islets. *J Endocrinol*, **117**(1): 59-62.

Smith, L. K., Rice, K. M. and Garner, C. W. (1996). The insulin-induced down-regulation of IRS-1 in 3T3-L1 adipocytes is mediated by a calcium-dependent thiol protease. *Mol Cell Endocrinol*, **122**(1): 81-92.

Sorenson, R. L. and Brelje, T. C. (1997). Adaptation of islets of Langerhans to pregnancy: beta-cell growth, enhanced insulin secretion and the role of lactogenic hormones. *Horm Metab Res*, **29**(6): 301-7.

Sorenson, R. L., Brelje, T. C. and Roth, C. (1993). Effects of steroid and lactogenic hormones on islets of Langerhans: a new hypothesis for the role of pregnancy steroids in the adaptation of islets to pregnancy. *Endocrinology*, **133**(5): 2227-34.

Stoffel, M., Bell, K. L., Blackburn, C. L., Powell, K. L., Seo, T. S., Takeda, J., Vionnet, N., Xiang, K. S., Gidh-Jain, M., Pilkis, S. J. and et al. (1993). Identification of glucokinase mutations in subjects with gestational diabetes mellitus. *Diabetes*, **42**(6): 937-40.

Stoffers, D. A., Ferrer, J., Clarke, W. L. and Habener, J. F. (1997). Early-onset type-II diabetes mellitus (MODY4) linked to IPF1. *Nat Genet*, **17**(2): 138-9.

Stoffers, D. A., Heller, R. S., Miller, C. P. and Habener, J. F. (1999). Developmental expression of the homeodomain protein IDX-1 in mice transgenic for an IDX-1 promoter/lacZ transcriptional reporter. *Endocrinology*, **140**(11): 5374-81.

Stone, L. M., Kahn, S. E., Fujimoto, W. Y., Deeb, S. S. and Porte, D., Jr. (1996). A variation at position -30 of the beta-cell glucokinase gene promoter is associated with reduced beta-cell function in middle-aged Japanese-American men. *Diabetes*, **45**(4): 422-8.

Sumiya, M., Super, M., Tabona, P., Levinsky, R. J., Arai, T., Turner, M. W. and Summerfield, J. A. (1991). Molecular basis of opsonic defect in immunodeficient children. *Lancet*, **337**(8757): 1569-70.

Swenne, I., Hill, D. J., Strain, A. J. and Milner, R. D. (1987). Growth hormone regulation of somatomedin C/insulin-like growth factor I production and DNA replication in fetal rat islets in tissue culture. *Diabetes*, **36**(3): 288-94.

Tan, Y., Raychaudhuri, P. and Costa, R. H. (2007). Chk2 mediates stabilization of the FoxM1 transcription factor to stimulate expression of DNA repair genes. *Mol Cell Biol*, **27**(3): 1007-16.

Tan, Y., Yoshida, Y., Hughes, D. E. and Costa, R. H. (2006). Increased expression of hepatocyte nuclear factor 6 stimulates hepatocyte proliferation during mouse liver regeneration. *Gastroenterology*, **130**(4): 1283-300.

Teh, M. T., Wong, S. T., Neill, G. W., Ghali, L. R., Philpott, M. P. and Quinn, A. G. (2002). FOXM1 is a downstream target of Gli1 in basal cell carcinomas. *Cancer Res*, **62**(16): 4773-80.

Teta, M., Long, S. Y., Wartschow, L. M., Rankin, M. M. and Kushner, J. A. (2005). Very slow turnover of beta-cells in aged adult mice. *Diabetes*, **54**(9): 2557-67.

Tschen, S. I., Dhawan, S., Gurlo, T. and Bhushan, A. (2009). Age-dependent decline in beta-cell proliferation restricts the capacity of beta-cell regeneration in mice. *Diabetes*, **58**(6): 1312-20.

Ukkola, O., Santaniemi, M., Rankinen, T., Leon, A. S., Skinner, J. S., Wilmore, J. H., Rao, D. C., Bergman, R., Kesaniemi, Y. A. and Bouchard, C. (2005). Adiponectin polymorphisms, adiposity and insulin metabolism: HERITAGE family study and Oulu diabetic study. *Ann Med*, **37**(2): 141-50.

Ustiyan, V., Wang, I. C., Ren, X., Zhang, Y., Snyder, J., Xu, Y., Wert, S. E., Lessard, J. L., Kalin, T. V. and Kalinichenko, V. V. (2009). Forkhead box M1 transcriptional factor is required for smooth muscle cells during embryonic development of blood vessels and esophagus. *Dev Biol*, **336**(2): 266-79.

van Haeften, T. W. and Twickler, T. B. (2004). Insulin-like growth factors and pancreas beta cells. *Eur J Clin Invest*, **34**(4): 249-55.

Vasavada, R. C., Cavaliere, C., D'Ercole, A. J., Dann, P., Burtis, W. J., Madlener, A. L., Zawalich, K., Zawalich, W., Philbrick, W. and Stewart, A. F. (1996). Overexpression of parathyroid hormone-related protein in the pancreatic islets of transgenic mice causes islet hyperplasia, hyperinsulinemia, and hypoglycemia. *J Biol Chem*, **271**(2): 1200-8.

Vasavada, R. C., Garcia-Ocana, A., Zawalich, W. S., Sorenson, R. L., Dann, P., Syed, M., Ogren, L., Talamantes, F. and Stewart, A. F. (2000). Targeted expression of placental lactogen in the beta cells of transgenic mice results in beta cell proliferation, islet mass augmentation, and hypoglycemia. *J Biol Chem*, **275**(20): 15399-406.

Vasavada, R. C., Gonzalez-Pertusa, J. A., Fujinaka, Y., Fiaschi-Taesch, N., Cozar-Castellano, I. and Garcia-Ocana, A. (2006). Growth factors and beta cell replication. *Int J Biochem Cell Biol*, **38**(5-6): 931-50.

Vasavada, R. C., Wang, L., Fujinaka, Y., Takane, K. K., Rosa, T. C., Mellado-Gil, J. M., Friedman, P. A. and Garcia-Ocana, A. (2007). Protein kinase C-zeta activation markedly enhances beta-cell proliferation: an essential role in growth factor mediated beta-cell mitogenesis. *Diabetes*, **56**(11): 2732-43.

Vatamaniuk, M. Z., Gupta, R. K., Lantz, K. A., Doliba, N. M., Matschinsky, F. M. and Kaestner, K. H. (2006). Foxa1-deficient mice exhibit impaired insulin secretion due to uncoupled oxidative phosphorylation. *Diabetes*, **55**(10): 2730-6.

Vestergaard, H., Rossen, M., Urhammer, S. A., Muller, J. and Pedersen, O. (1997). Short- and long-term metabolic effects of recombinant human IGF-I treatment in patients with severe insulin resistance and diabetes mellitus. *Eur J Endocrinol*, **136**(5): 475-82.

Villareal, D. T., Robertson, H., Bell, G. I., Patterson, B. W., Tran, H., Wice, B. and Polonsky, K. S. (2009). TCF7L2 Variant rs7903146 Affects the Risk of Type 2 Diabetes by Modulating Incretin Action. *Diabetes*.

Wang, I.-C., Chen, Y.-J., Hughes, D., Petrovic, V., Major, M. L., Park, H. J., Tan, Y., Ackerson, T. and Costa, R. H. (2005). Forkhead Box M1 regulates the transcriptional network of genes essential for mitotic progression and genes encoding the SCF (Skp2-Cks1) ubiquitin ligase. *Mol. Cell. Biol.*, **25**: 10875–94.

Wang, I. C., Chen, Y. J., Hughes, D. E., Ackerson, T., Major, M. L., Kalinichenko, V. V., Costa, R. H., Raychaudhuri, P., Tyner, A. L. and Lau, L. F. (2008a). FoxM1 regulates transcription of JNK1 to promote the G1/S transition and tumor cell invasiveness. *J Biol Chem*, **283**(30): 20770-8.

Wang, I. C., Meliton, L., Ren, X., Zhang, Y., Balli, D., Snyder, J., Whitsett, J. A., Kalinichenko, V. V. and Kalin, T. V. (2009a). Deletion of Forkhead Box M1 transcription factor from respiratory epithelial cells inhibits pulmonary tumorigenesis. *PLoS One*, **4**(8): e6609.

Wang, I. C., Meliton, L., Tretiakova, M., Costa, R. H., Kalinichenko, V. V. and Kalin, T. V. (2008b). Transgenic expression of the forkhead box M1 transcription factor induces formation of lung tumors. *Oncogene*, **27**(30): 4137-49.

Wang, Q. and Brubaker, P. L. (2002). Glucagon-like peptide-1 treatment delays the onset of diabetes in 8 week-old db/db mice. *Diabetologia*, **45**: 1263-73.

Wang, Q., Li, L., Xu, E., Wong, V., Rhodes, C. and Brubaker, P. L. (2004). Glucagon-like peptide-1 regulates proliferation and apoptosis via activation of protein kinase B in pancreatic INS-1 beta cells. *Diabetologia*, **47**: 478–87.

Wang, S., Jensen, J. N., Seymour, P. A., Hsu, W., Dor, Y., Sander, M., Magnuson, M. A., Serup, P. and Gu, G. (2009b). Sustained Neurog3 expression in hormone-expressing islet cells is required for endocrine maturation and function. *Proc Natl Acad Sci U S A*, **106**(24): 9715-20.

Wang, X., Hung, N. J. and Costa, R. H. (2001a). Earlier expression of the transcription factor HFH-11B diminishes induction of p21(CIP1/WAF1) levels and accelerates mouse hepatocyte entry into S-phase following carbon tetrachloride liver injury. *Hepatology*, **33**(6): 1404-14.

Wang, X., Kiyokawa, H., Dennewitz, M. B. and Costa, R. H. (2002). The Forkhead Box m1b transcription factor is essential for hepatocyte DNA replication and mitosis during mouse liver regeneration. *Proc Natl Acad Sci U S A*, **99**(26): 16881-6.

Wang, X., Krupczak-Hollis, K., Tan, Y., Dennewitz, M. B., Adami, G. R. and Costa, R. H. (2002). Increased hepatic Forkhead Box M1B (FoxM1B) levels in old-aged mice stimulated liver regeneration through diminished p27Kip1 protein levels and increased Cdc25B expression. *J. Biol. Chem.*, **277**: 44310–16.

Wang, X., Krupczak-Hollis, K., Tan, Y., Dennewitz, M. B., Adami, G. R. and Costa, R. H. (2002a). Increased hepatic Forkhead Box M1B (FoxM1B) levels in old-aged mice stimulated liver regeneration through diminished p27Kip1 protein levels and increased Cdc25B expression. *J Biol Chem*, **277**(46): 44310-6.

Wang, X., Quail, E., Hung, N. J., Tan, Y., Ye, H. and Costa, R. H. (2001b). Increased levels of forkhead box M1B transcription factor in transgenic mouse hepatocytes prevent age-related proliferation defects in regenerating liver. *Proc Natl Acad Sci U S A*, **98**(20): 11468-73.

Wang, Z., Ahmad, A., Li, Y., Banerjee, S., Kong, D. and Sarkar, F. H. (2009c). Forkhead box M1 transcription factor: A novel target for cancer therapy. *Cancer Treat Rev*.

Watanabe, R. M., Black, M. H., Xiang, A. H., Allayee, H., Lawrence, J. M. and Buchanan, T. A. (2007). **Genetics of gestational diabetes mellitus and type 2 diabetes (candidate genes). *Diabetes Care*, **30 Suppl 2**: S134-40.

Weigel, D., Jurgens, G., Kuttner, F., Seifert, E. and Jackle, H. (1989). The homeotic gene fork head encodes a nuclear protein and is expressed in the terminal regions of the Drosophila embryo. *Cell*, **57**(4): 645-58.

Weinhaus, A. J., Bhagroo, N. V., Brelje, T. C. and Sorenson, R. L. (2000). Dexamethasone counteracts the effect of prolactin on islet function: implications for islet regulation in late pregnancy. *Endocrinology*, **141**(4): 1384-93.

Weng, J., Ekelund, M., Lehto, M., Li, H., Ekberg, G., Frid, A., Aberg, A., Groop, L. C. and Berntorp, K. (2002). Screening for MODY mutations, GAD antibodies, and type 1 diabetes--associated HLA genotypes in women with gestational diabetes mellitus. *Diabetes Care*, **25**(1): 68-71.

Westendorf, J. M., Rao, P. N. and Gerace, L. (1994). Cloning of cDNAs for M-phase phosphoproteins recognized by the MPM2 monoclonal antibody and determination of the phosphorylated epitope. *Proc Natl Acad Sci*, **91**: 714–8.

Wierstra, I. and Alves, J. (2007). FOXM1, a typical proliferation-associated transcription factor. *Biol Chem*, **388**(12): 1257-74.

Williams, J. A., Bailey, A., Humbel, R. and Goldfine, I. D. (1984). Insulinlike growth factors bind to specific receptors in isolated pancreatic acini. *Am J Physiol*, **246**(1 Pt 1): G96-9.

Wonsey, D. R. and Follettie, M. T. (2005). Loss of the forkhead transcription factor FoxM1 causes centrosome amplification and mitotic catastrophe. *Cancer Res.*, **65**: 5181–9.

Xia, L. M., Huang, W. J., Wang, B., Liu, M., Zhang, Q., Yan, W., Zhu, Q., Luo, M., Zhou, Z. Z. and Tian, D. A. (2009a). Transcriptional up-regulation of FoxM1 in response to hypoxia is mediated by HIF-1. *J Cell Biochem*, **106**(2): 247-56.

Xu, X., D'Hoker, J., Stange, G., Bonne, S., De Leu, N., Xiao, X., Van de Casteele, M., Mellitzer, G., Ling, Z., Pipeleers, D., Bouwens, L., Scharfmann, R., Gradwohl, G. and Heimberg, H. (2008). Beta cells can be generated from endogenous progenitors in injured adult mouse pancreas. *Cell*, **132**(2): 197-207.

Yamagata, K., Furuta, H., Oda, N., Kaisaki, P. J., Menzel, S., Cox, N. J., Fajans, S. S., Signorini, S., Stoffel, M. and Bell, G. I. (1996). Mutations in the hepatocyte nuclear factor-4alpha gene in maturity-onset diabetes of the young (MODY1). *Nature*, **384**(6608): 458-60.

Yamagata, K., Oda, N., Kaisaki, P. J., Menzel, S., Furuta, H., Vaxillaire, M., Southam, L., Cox, R. D., Lathrop, G. M., Boriraj, V. V., Chen, X., Cox, N. J., Oda, Y., Yano, H., Le Beau, M. M., Yamada, S., Nishigori, H., Takeda, J., Fajans, S. S., Hattersley, A. T., Iwasaki, N., Hansen, T., Pedersen, O., Polonsky, K. S., Bell, G. I. and et al. (1996). Mutations in the hepatocyte nuclear factor-1alpha gene in maturity-onset diabetes of the young (MODY3). *Nature*, **384**(6608): 455-8.

Yao, K. M., Sha, M., Lu, Z. and Wong, G. G. (1997). Molecular analysis of a novel winged helix protein, WIN. Expression pattern, DNA binding property, and alternative splicing within theDNA binding domain. *J Biol Chem.*, **272**: 19827–36.

Ye, H., Holterman, A. X., Yoo, K. W., Franks, R. R. and Costa, R. H. (1999). Premature expression of the winged helix transcription factor HFH-11B in regenerating mouse liver accelerates hepatocyte entry into S phase. *Mol Cell Biol*, **19**(12): 8570-80.

Ye, H., Kelly, T. F., Samadani, U., Lim, L., Rubio, S., Overdier, D. G., Roebuck, K. A. and Costa, R. H. (1997). Hepatocyte nuclear factor 3/fork head homolog 11 is expressed in proliferating epithelial and mesenchymal cells of embryonic and adult tissues. *Mol Cell Biol*, **17**(3): 1626-41.

Yogev, Y. and Visser, G. H. (2009). Obesity, gestational diabetes and pregnancy outcome. *Semin Fetal Neonatal Med*, **14**(2): 77-84.

Yoshida, Y., Wang, I. C., Yoder, H. M., Davidson, N. O. and Costa, R. H. (2007). The forkhead box M1 transcription factor contributes to the development and growth of mouse colorectal cancer. *Gastroenterology*, **132**(4): 1420-31.

Zacharova, J., Chiasson, J. L. and Laakso, M. (2005). The common polymorphisms (single nucleotide polymorphism [SNP] +45 and SNP +276) of the adiponectin gene predict the conversion from impaired glucose tolerance to type 2 diabetes: the STOP-NIDDM trial. *Diabetes*, **54**(3): 893-9.

Zarnegar, R. and Michalopoulos, G. K. (1995). The many faces of hepatocyte growth factor: from hepatopoiesis to hematopoiesis. *J Cell Biol*, **129**(5): 1177-80.

Zhang, H., Ackermann, A. M., Gusarova, G. A., Lowe, D., Feng, X., Kopsombut, U. G., Costa, R. H. and Gannon, M. (2006b). The FoxM1 transcription factor is required to maintain pancreatic beta-cell mass. *Mol Endocrinol*, **20**(8): 1853-66.

Zhang, H., Zhang, J., Pope, C. F., Crawford, L. A., Vasavada, R. C., Jagasia, S. M. and Gannon, M. (2010). Gestational diabetes mellitus resulting from impaired beta-cell compensation in the absence of FoxM1, a novel downstream effector of placental lactogen. *Diabetes*, **59**(1): 143-52.

Zhang, W., Feng, D., Li, Y., Iida, K., McGrath, B. and Cavener, D. R. (2006a). PERK EIF2AK3 control of pancreatic beta cell differentiation and proliferation is required for postnatal glucose homeostasis. *Cell Metab*, **4**(6): 491-7.

Zhang, Y., Zhang, N., Dai, B., Liu, M., Sawaya, R., Xie, K. and Huang, S. (2008). FoxM1B transcriptionally regulates vascular endothelial growth factor expression and promotes the angiogenesis and growth of glioma cells. *Cancer Res*, **68**(21): 8733-42.

Zhao, Y. Y., Gao, X. P., Zhao, Y. D., Mirza, M. K., Frey, R. S., Kalinichenko, V. V., Wang, I. C., Costa, R. H. and Malik, A. B. (2006). Endothelial cell-restricted disruption of FoxM1 impairs endothelial repair following LPS-induced vascular injury. *J Clin Invest*, **116**(9): 2333-43.

Zurawek, M., Wender-Ozegowska, E., Januszkiewicz-Lewandowska, D., Zawiejska, A. and Nowak, J. (2007). GCK and HNF1alpha mutations and polymorphisms in Polish women with gestational diabetes. *Diabetes Res Clin Pract*, **76**(1): 157-8.