REGULATORS OF MOUSE AND HUMAN BETA CELL PROLIFERATION

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Dedication

To my mother and father for their unconditional love, support, and teaching me the value of hard work.

To my husband who has followed me on this journey and provided me with tireless love and support. Without him I would never have had the strength or courage to accomplish my doctoral study.

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ABSTRACT

REGULATORS OF MOUSE AND HUMAN BETA CELL PROLIFERATION

Yang Jiao

Klaus H. Kaestner

Diabetes mellitus is an increasingly prevalent metabolic disorder that is estimated to affect over 300 million people worldwide by 2025. Common to either type 1 or type 2 diabetes is a progressive inadequacy of functional beta-cell mass. Recent studies have shown that during times of prolonged metabolic demand for insulin, the endocrine pancreas can respond by increasing beta-cell mass by beta-cell proliferation. Advances that further our knowledge of the molecular factors that control beta-cell proliferation will be crucial for understanding the homeostasis of beta-cell mass during adulthood, and are pivotal for any attempt to use instructive cues to induce the proliferation of terminally differentiated fully functional insulin-producing beta-cells that are suitable for transplantation. In the first part of my thesis, I investigated the role of CISH on beta cell proliferation in pregnant mice by using a pancreas-specific *Cish*-ablation mouse model. My results demonstrate that CISH is not required for beta cell proliferation or glucose homeostasis before, during, and after pregnancy. *Socs2* expression is up-regulated in *Cish*-ablation islets at pregnant day 9.5, indicating that SOCS2 might be compensating for CISH deficiency. In the second part of my thesis, I studied the role of HNF-4α in beta cell proliferation during obesity by inducing beta-cell-specific *Hnf4α* ablation in *ob/ob* mice. My results indicated that HNF-4α is required for beta-cell proliferation during obesity. In the third part of my thesis, I investigated the role of betatrophin on human beta cell replication by transplanting human islets under kidney capsule of immunodeficient

mice and induce betatrophin overexpressing in the liver. In spite of the mitogenic role of betatrophin on mouse beta-cells, betatrophin does not promote replication in human beta cells. In summary, my thesis work furthered the understanding of the role of CISH, HNF-4α, and betatrophin on beta-cell proliferation.

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Chapter 1 Introduction

1. Blood glucose homeostasis

Glucose is an essential fuel for all mammalian cells. Blood glucose levels are tightly regulated as a part of metabolic homeostasis. The process of maintaining blood glucose at a steady-state level is called glucose homeostasis, which is accomplished by hormonal regulation of peripheral glucose uptake, hepatic glucose production, and carbohydrate uptake during ingestion (Bergman, 2007).

After a carbohydrate containing meal, blood glucose increases, which triggers insulin secretion from the beta cells in the pancreatic islets of Langerhans. Insulin secretion follows two phases: the first phase of secretion of 'docked' insulin granules, and the second phase of secretion of newly synthesized insulin. Circulating insulin acts on the liver, muscle, and adipose tissue to increase glucose uptake and reduce glucose output by increasing glycogenesis (synthesis of glycogen from glucose), and decreasing gluconeogenesis (generation of glucose from non-carbohydrate substrates) and glycogenolysis (breakdown of glycogen to glucose). As a result, blood glucose levels are down-regulated to counteract hyperglycemia. In contrast, during fasting, blood glucose levels decrease, which induces glucagon secretion from the alpha-cells in the pancreatic islets. Glucagon is the major counter regulatory hormone to insulin. Glucagon exclusively acts on the liver to increase glucose output by activating hepatic glycogenolysis, followed by gluconeogenesis, both of which leads to releasing of glucose into the blood stream. As a result, blood glucose levels are increased to prevent hypoglycemia (Fig 1.1) (Muhammad Z. Shrayyef, 2010).

The gastrointestinal tract also plays an important role in regulating blood glucose levels. Nutrient stimulated secretion of hormones from the gut enteroendocrine cells

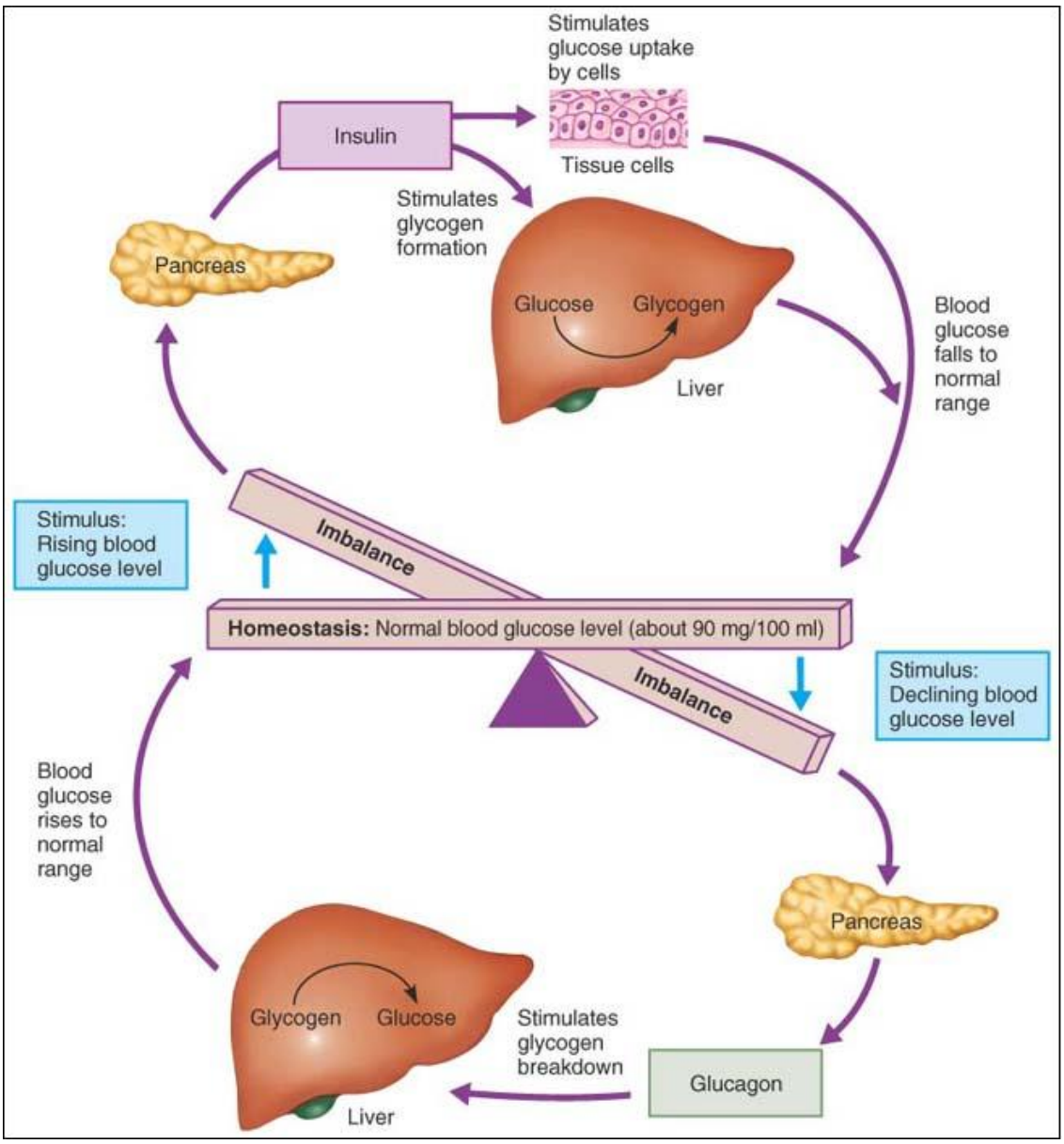


Figure 1.1 (Adapted from (Marieb E.N, 2005)) Blood glucose homeostasis. When blood glucose increases, pancreatic islet beta cells secrete insulin to decrease glucose output from liver and increase glucose uptake of liver, muscle and fat, so that blood glucose level is lowed to normal range. When blood glucose level is low, glucagon is secreted from pancreatic islet alpha cells, which induces hepatic glucose output and increases blood glucose level to normal range.

participates in postprandial glucose clearance by functioning through a number of metabolic processes, including stimulated secretion of insulin, inhibition of glucagon output, and reduced feeding. These gut hormones are called incretins, which are secreted from specialized gastrointestinal enteroendocrine cells to the blood stream. Intestinal L-cell secreted Glucagon-Like Peptide-1 (GLP-1) and K-cell secreted Glucose-Dependent Insulinotropic Polypeptide (GIP) are two well-characterized incretins. Both GLP-1 and GIP are secreted after food intake to stimulate glucose-dependent insulin secretion to lower blood glucose. Indeed, it is demonstrated that ingested food causes a more potent release of insulin than glucose infused intravenously. And this effect is termed the 'incretin effect' (Drucker, 2013).

2. Pancreas and beta cell function

2.1 Pancreas development

The pancreas is a glandular organ located in the upper abdomen, behind the stomach, nestling the duodenum, and ending at the spleen. During mouse embryogenesis, pancreas development is framed by two major transitions (Fig 1.2). The 'primary transition' from embryonic day 8.5 (E8.5) to E10.5 marks the initiation of pancreatic organ formation and proliferation of pancreas progenitor cells. Around E8.5, the initial expression of *Pdx1* marks the pre-pancreatic endoderm at the foregut/midgut junction. Another transcription factor, *Ptf1a*, also plays a crucial role in pancreas specification. *Pdx1* is expressed throughout the pancreatic epithelium from E11.5 to E15.5, and becomes restricted in beta cells afterwards. *Ptf1a*, however, becomes restricted to acinar progenitor cells at this later

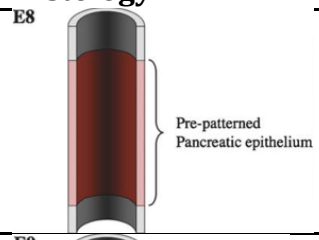
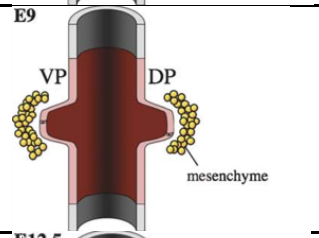
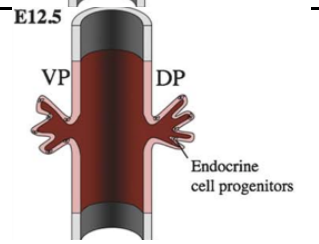
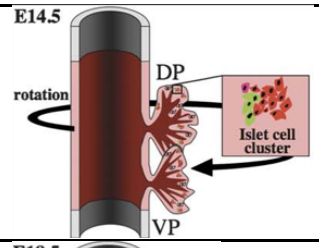
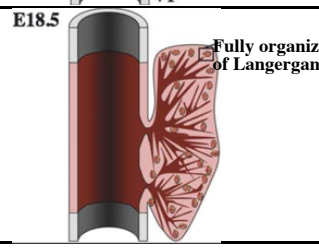
Embryonic stage	Key regulators	Histology
E8.0 Foregut patterning	*Foxa2* and *sox17* expression	
E8.5 Pancreas specification	*Pdx1* expression	
E9.0 Budding	*Ptf1a* expression	
E11.5 Branching	*Ngn3* expression Early gcg^{+} and ins^{+} cells	
E12.5 Endocrine progenitors	*Ngn3* expression	
E13.5-E14.5 Rotation and endocrine cell genesis	*Arx, Pax4, Nkx2.2, Nkx6.1,* and *Pax6* expression	
E18.5 Merge of two buds; fully organized islets		

Figure 1.2 (Adapted from (Collombat, Hecksher-Sorensen, Serup, & Mansouri, 2006)) Embryonic mouse pancreas morphogenesis. Pancreas development is operationally framed by two major transitions. The primary regulatory transition (E8.5-E10.5) marks the two pancreatic buds formation from foregut endoderm and proliferation of pancreas progenitor cells expressing *Pdx1* and *Ptf1a*. The secondary transition (E13.5-E14.5) is the major phase of endocrine islet development and acinar cell genesis, marked by proliferation of endocrine progenitor $Ngn3^{+}$ cells proliferation and clustering of islet cells. At later embryonic stage (E18), the two pancreatic buds merge and fully organized islets are formed.

stage. Around E9.5, two pancreatic buds emerge from the foregut endoderm, first on the dorsal (E9.5), and subsequently on the ventral gut tube (E9.75). Both pancreatic buds proliferate, thicken, branch and invade into the surrounding mesenchyme. The ventral bud rotates and eventually merges with the dorsal bud to form the intact pancreas around E18 (Collombat et al., 2006; Murry & Keller, 2008). The endocrine cells of the pancreas originate on E9.5 in the early gut endoderm and are marked by *Ngn3* expression in a small subset of pancreatic cells. Development of the endocrine pancreas is a complex process that requires orchestrated action of multiple transcription factors during different stages of development. *Ngn3* expressing cells are multipotent endocrine progenitor cells that give rise to all types of endocrine cells in the islet. Expression of additional transcription factors including *Nkx2.2*, *Nkx6.1*, and *Foxa2* further restrict cells into distinct endocrine lineages (C. S. Lee, Sund, Behr, Herrera, & Kaestner, 2005; Sander et al., 2000; Sussel et al., 1998). Notably, there are some early glucagon^{+} (E11.5) and insulin^{+} cells, occasionally cells co-expressing insulin and glucagon, but they do not contribute to later mature islet cells. The function of these early endocrine cells is unclear. The major phase of islet development occurs during the secondary transition (E13.5-E14.5), with a burst of endocrine and acinar cell genesis, leading to full differentiation and generation of mature beta cells and alpha cells. Delta cells emerge around E15.5, and pancreatic polypeptide (PP) cells appear around E18, when endocrine cells begin to cluster into islets. About 2 to 3 weeks after birth, maturely organized and functional islets are formed (Murtaugh, 2007; Oliver-Krasinski & Stoffers, 2008).

2.2 Exocrine and endocrine/islets function

The pancreas has two distinct functional portions (Fig 1.3a). The exocrine portions

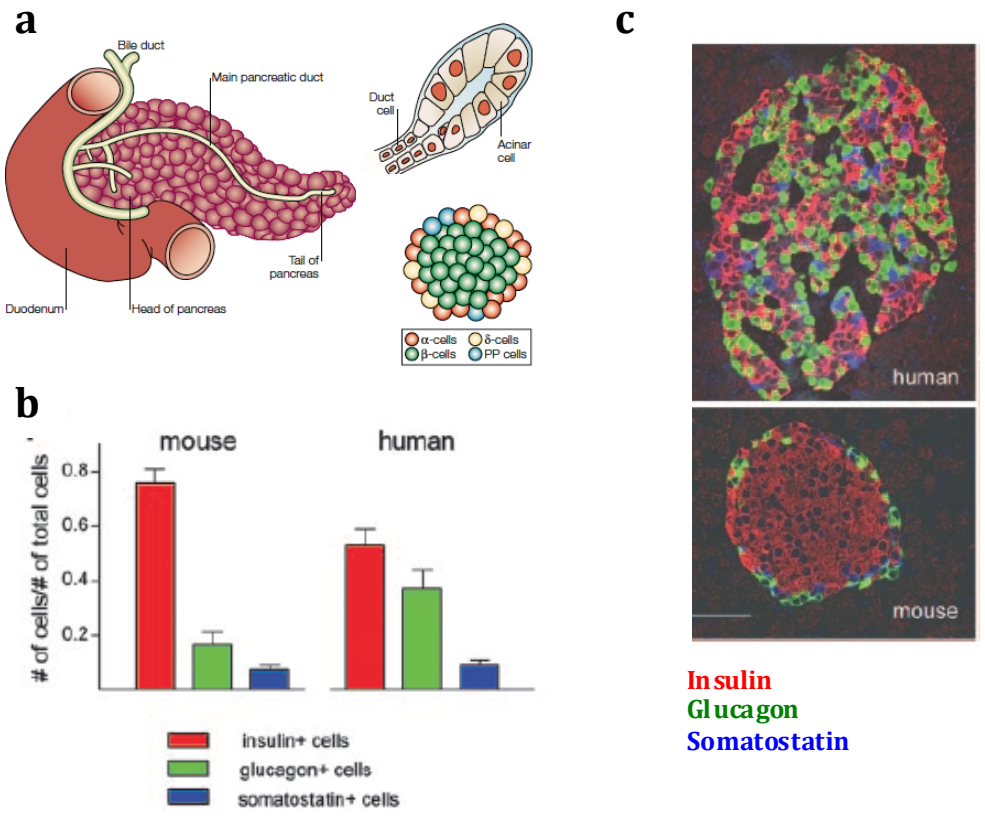


Figure 1.3 (Adapted from (Cabrera et al., 2006; Edlund, 2002)) Pancreas and islets structure a) Pancreas has two distinct functional portions. 90% are exocrine portions, which consist of acini and ducts. Acinar cells secret digesting enzymes to the gut for food digestion. 1-2% of the pancreas consists of endocrine portions, which are the islets of Langerhans. Each islet consists of four to five cell types: insulin-producing beta cells, glucagon-producing alpha cells, somatostatin-producing delta-cells, pancreatic polypeptide-producing PP cells, and ghrelin producing epsilon cells. b) Different cell composition of human islets and mouse islets. Human islets have lower percentage of beta cells and higher percentage of alpha cells comparing to mouse islets. c) Human islets have different construction than mouse islets, with all cells types mixed together. Mouse islets have the vast majority of beta cells clustering in the core of the islets, while other cell types locating in the periphery of the islets.

constitute more than 90%, while the endocrine portions make up of 1 to 2% of the pancreas mass. The exocrine portions consist of acini as functional units of pancreatic enzyme production and ducts for delivering digestive enzymes into duodenum. Acini have the architecture of blind-ended tubules surrounded by polarized acinar cells. These tubules are organized into grape-like lobules. Acinar cells synthesize, store, and secrete digestive enzymes into the lobular lumens, called the intercellular canaliculi, which are connected to intralobular ducts. Intralobular ducts drain into larger extralobular ducts, which in turn merge into the bile duct and empty into the duodenum. Acinar cells secrete three major types of digestive enzymes, alpha-amylases, lipases, and proteases, which facilitate food digestion by catalyzing the hydrolysis of carbohydrates, fats, and proteins, respectively (Leung & Ip, 2006).

The endocrine portions of the pancreas consist of islets of Langerhans as functional units. A healthy human adult has about 1 million islets dispersed throughout the pancreas, with sizes ranging from 50 to 300 micrometers in diameter. Islets are considered as micro-organs. Each islet consists of four to five cell types: insulin-producing beta cells, glucagon-producing alpha cells, somatostatin-producing delta-cells, pancreatic polypeptide- producing PP cells, and ghrelin producing epsilon cells (Fig 1.3a). The architecture and composition of pancreatic islets differs among species. The rodent islets are usually spherically-shaped, with the vast majority of beta cells clustering in the core of the islets, while other cell types, namely alpha cells, delta cells, and PP cells locating to the periphery of the islets. Human islets, however, are usually irregularly shaped, with all cell types mixed and scattered within the islets without segregating into distinct regions (Fig 1.3c). Most human beta cells (~70%) neighbor non beta cells (heterotypic

contacts), while most rodent beta cells (70%) appose beta cells (homotypic contacts). Furthermore, rodent islets consist of a higher proportion of beta cells (~77%) than human islets (~55%), and a lower proportion of alpha cells (~18%) than human islets (38%) (Fig 1.3b) (Cabrera et al., 2006).

2.3 Insulin secretion by the beta cell

Beta cells secrete a low basal level of insulin under substimulatary conditions. Glucose-stimulated insulin secretion (GSIS) is the main mechanism for stimulated insulin secretion. When blood glucose is high, glucose is rapidly transported across the beta cell membrane by the glucose transporter 2 (GLUT2) in mice, or GLUT1 and GLUT2 in humans (Fig 1.4). Intracellular glucose is phosphorylated into glucose-6-phospate by glucokinase, and metabolized via the glycolytic pathway and the tricarboxylic acid cycle (TCA cycle). Glucose metabolism produces ATP. The increased ATP/ADP ratio results in closure of ATP-sensitive K^+ channels in the plasma membrane, preventing K^+ ions from being transported from the cytoplasm into the extracellular space. Accumulation of K^+ ions in the beta cells provokes cell membrane depolarization. Voltage-gated Ca^{2+} channels are activated, which leads to influx of Ca^{2+} ions. Increasing Ca^{2+} ions in the beta cell trigger exocytosis of insulin. Insulin granules fuse with the plasma membrane, and insulin is secreted from the beta cells. There are two phases of insulin secretion. The rapid burst of first phase insulin secretion features the exocytosis of pre-packaged insulin granules docked at the plasma membrane, which happens 1 to 3 minutes after blood glucose increase. The sustained rising of second phase insulin secretion involves the release of both stored and newly synthesized insulin. Insulin mRNA is transcribed and translated into preproinsulin, which is cleaved into proinsulin by removal of its signal

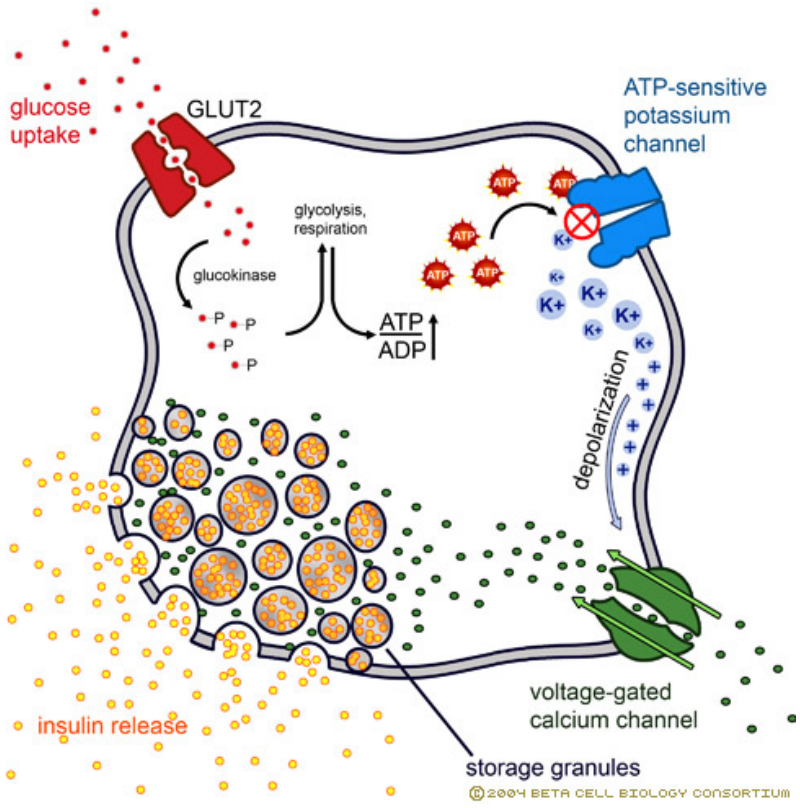


Figure 1.4 Glucose stimulated insulin secretion (from BCBC) When blood glucose is high, glucose is uptaken rapidly across the beta cell membrane by the high capacity, low affinity glucose transporter 2 (GLUT2). Intracellular glucose is phosphorylated into glucose-6-phospate by glucokinase, the rate-limiting enzyme for metabolizing glucose, and metabolized via the glycolytic pathway and the tricarboxylic acid cycle (TCA cycle). Glucose metabolism produces ATP. The increased ATP/ADP ratio causes ATP-sensitive K+ channels (K_{ATP} channels) in the plasma membrane to shut down, preventing K+ ions from being transported from the cytoplasm to the extracellular region. Accumulation of K+ ions in the beta cells provokes cell membrane depolarization. Voltage-gated Ca2+ channels are activated, which leads to influx of Ca2+ ions. Increasing Ca2+ ions in the beta cells triggers exocytosis of insulin. Insulin granules fuse with the cellular plasma, and insulin is secreted from the beta cells to the nearby blood vessels.

peptide upon insertion into the endoplasmic reticulum. Proinsulin is exposed to several endopeptidases in the endoplasmic reticulum and is cleaved into free C peptide and insulin. Free C peptide and insulin are packaged in the Golgi into secretary granules. Second phase insulin secretion is turned off when blood glucose levels decrease (Gregory & Moore, 2011).

3. Diabetes mellitus

Diabetes mellitus is a metabolic disorder characterized by chronic hyperglycemia (elevated blood glucose concentration), which leads to a series of classical symptoms including polyuria, polydipsia, and polyphagia. Diabetes can cause many complications, including cardiovascular disease, renal failure, and retinal damage. The world prevalence of diabetes was 6.4% among adults >20 years of age (285 million people) in 2010, and is estimated to increase to 7.7% (439 million) by the year 2030 (Shaw, Sicree, & Zimmet, 2010). Diabetes is an increasing international health burden both to the patients and to the society, which is worsened by ageing, urbanization, and increasing prevalence of obesity and physical inactivity. In many countries, about 5-10% of the total health care budget is used for diabetes. The total cost of diagnosed diabetes in the U.S. alone in 2012 was $245 billion. The major types of diabetes include Type 1 diabetes mellitus (T1DM), Type 2 diabetes mellitus (T2DM), gestational diabetes mellitus (GDM), and maturity onset diabetes of the young (MODY).

3.1 Type 1 diabetes mellitus

3.1.1 Pathogenesis of T1DM

T1DM is observed in approximately 10% of patients with diabetes mellitus. It was

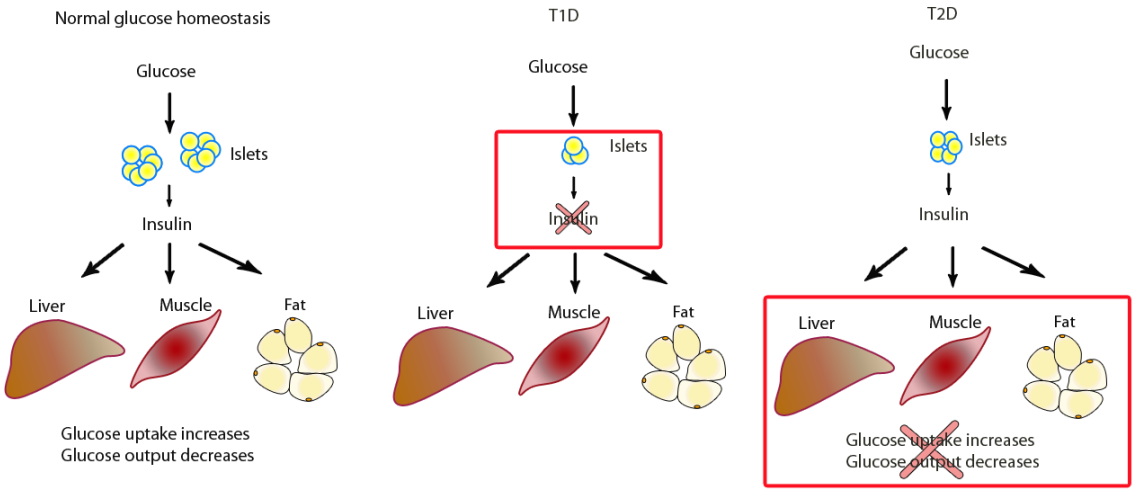


Figure 1.5 Pathogenesis of Type 1 diabetes and Type 2 diabetes. T1DM results from autoimmune progressive destruction of beta cells. Long standing T1DM patients have ~99% deficit in beta cell mass, which leads to hypoinsulinemia and hyperglycemia. T2DM develops from a combination of insulin resistance and relative insulin deficiency. In T2DM patients, insulin sensitivity decreases ~50% in the liver, muscle and fat. Beta cell mass also decreases by ~60%, which leads to failure of compensation for insulin resistance and development of T2DM.

previously known as juvenile or insulin-dependent diabetes. T1DM results from autoimmune progressive destruction of beta cells by $CD4^+$ and $CD8^+$ T cells and macrophages infiltrating the islets (Fig 1.5). The autoimmune-mediated depletion within the islets is beta cell-specific. In the non-obese diabetic (NOD) mouse model of T1DM, beta cell proliferation initially increases in response to beta cell loss, but beta cell mass is progressively reduced (Sreenan et al., 1999). These mice do not become hyperglycemic until residual beta cell mass is reduced to less than 30% of controls. Similarly in humans, destruction of beta cells precedes the clinical onset of T1DM, because impaired insulin secretion in T1DM patients can be detected several years before hyperglycemia. As a consequence, new-onset T1DM patients display a ~80-90% deficit in beta cell mass, while long-standing T1DM patients have lost ~99% of their beta cells (Meier, 2008; Sreenan et al., 1999). Impaired beta cell function is also suggested to be involved in the pathogenesis of T1DM in addition to beta cell mass deficiency, since the degree of beta cell dysfunction exceeds the percentage beta cell loss in T1DM patients. T1DM is a complex polygenic disorder with many different genes contributing to its onset, via either dominant, recessive, or other models of inheritance. To date, no single gene has been identified as either necessary or sufficient to predict the development of T1DM.

3.1.2 Current strategies for glycemic control in TIDM patients and their limitations

Insufficient beta cell number and insulin secretion, as well as unopposed glucagon secretion constitute the pathogenesis of T1DM. Therapeutic strategies to control normal blood glucose levels are based on two mechanisms: (1) increase insulin in the system by injecting exogenous insulin when needed, which has been in practice since the discovery of insulin in the 1920s; and (2) increase beta cell mass by islet transplantation, which

represents a relative new strategy substantially developed and progressed during the last three decades (Shapiro et al., 2006).

Both regimens have demonstrated considerable improvement for glycemic control in the T1DM patients; however, they also have side effects. Insulin therapy needs to be administrated in precise dosage and timing, which is often difficult to predict. The incorrect usage of insulin is associated with refractory hypoglycemic episodes, defined as blood glucose levels below 70 mg/dl and symptoms such as feeling shaky, sweaty, and pounding heart (1.3 severe hypoglycemic episodes per T1DM patient per year (McCall & Shapiro, 2012)), which present great risks for the patients.

Great progress has been made over the past two decades with islet transplantation strategy. During the first ten years of practice (1990-2000), only 8.2% of the 267 patients that received an islet allograft remained insulin independent for more than a year (Shapiro et al., 2000). This situation was much improved in 2000, when a glucocorticoid-free immunosuppressive regimen (known as the 'Edmonton protocol') was developed, in which all the seven islets recipients remained insulin independent after a year. From 2000 until 2012, >750 T1DM patients have received islet transplants world-wide, and recent data indicate that 50% of the recipients remained insulin-independent 3 years after the procedure (McCall & Shapiro, 2012). Although promising, islets transplantation faces the following obstacles: (1) the extreme scarcity of organ donors relative to the diabetic population, especially since often islets from multiple donors are required for one transplantation, and multiple rounds of islet transplantation are necessary for each recipient; (2) extremely high financial burden of the procedure with up to $150,000 per patient (McCall & Shapiro, 2012).

Therefore, it is tempting to develop alternative strategy for limitless expandable source of beta cells to restore beta cell mass, including stem cell or xenograft-derived insulin secreting cells, or beta cells obtained by proliferation.

3.2 Type 2 diabetes mellitus

Type 2 diabetes mellitus (T2DM) constitutes about 90% of all cases of diabetes mellitus. It was previously called 'non-insulin-dependent diabetes mellitus'. The pathogenesis of T2DM is not fully understood. Current theories favor that T2DM develops from a combination of insulin resistance and relative insulin deficiency (Fig 1.5).

3.2.1 Obesity, insulin resistance, and T2DM.

Obesity is associated with an increased risk of insulin resistance and T2DM. The risk of diabetes increases linearly with body mass index (BMI), from 2% in the 25-29.9 BMI group, to 8% in the 30-34.9 BMI group, and finally to 13% in people with a BMI exceeding 35 (Yaturu, 2011). In obesity, the initial triglycerides deposition occurs in subcutaneous adipose tissue, and BMI increases in parallel with subcutaneous adipose tissue accumulation. This provokes insulin resistance, and limits further subcutaneous lipid accumulation. Later on, triglycerides deposit into visceral fat (intra-abdominal fat) as well as ectopic sites, leading to an increase of waist circumference (WC), which is the prime determinant of insulin resistance in obese and overweight subjects (Yaturu, 2011).

Insulin resistance is characterized by a failure of peripheral tissues to respond to insulin, including a decreased ability of skeletal muscle and adipose tissue to take up

glucose upon insulin secretion, and a failure of the liver to reduce hepatic glucose output in the presence of insulin. Since skeletal muscle is responsible for ~75% of whole body insulin-stimulated glucose uptake, defects in this tissue have a major role in the impaired glucose homeostasis in T2DM patients (Kennedy et al., 1999). In skeletal muscle, insulin signals through insulin receptor/ insulin receptor substrate (IR/IRS)/PI3K/AKT, which activates multiple protein phosphatases and phospholipid phosphatases to modulate the strength and duration of insulin signaling, the biological output being translocation of glucose transporter 4 (GLUT4) to the plasma membrane and glucose uptake via GLUT4 (Zaid, Antonescu, Randhawa, & Klip, 2008; Zorzano, Palacin, & Guma, 2005) . T2DM patients have dysregulation of the IR/IRS pathway, impairment of PI3K activation, or defects in GLUT4 translocation in response to insulin in their muscles (Bjornholm & Zierath, 2005; Zierath, Krook, & Wallberg-Henriksson, 2000).

Adipose tissue exhibits decreased GLUT4 expression in T2DM patients (Shepherd & Kahn, 1999), but since skeletal muscle is the main glucose disposal site, down-regulation of GLUT4 in adipose tissue contributes less to insulin resistance and hyperglycemia in T2DM patients. Instead, adipose tissue generates multiple circulating factors, e.g., free fatty acids (FFA), adiponectin, and other adipokines, that serve in both endocrine and paracrine fashions, communicating with other organs and ultimately altering insulin sensitivity by regulating systemic insulin action and hepatic glucose production (Gimeno & Klaman, 2005; Lazar, 2005). Patients with T2DM have decreased oxidative capacity for fatty acids and high levels of circulating FFA (Blaak et al., 2000; Kelley & Simoneau, 1994). The latter promotes accumulation of lipid inside the skeleton muscle cells, causing insulin resistance by reducing glucose uptake (Boden, 1999), which is a key component

for skeleton muscle insulin resistance.

Liver is a main target of the anabolic hormone insulin and its catabolic counterpart glucagon. Hepatic glucose metabolism includes glycogenesis, gluconeogenesis, glycolysis, and glycogenolysis (Klover & Mooney, 2004). Liver is also the major organ for lipid metabolism, including fatty acid oxidation, *de novo* synthesis of fatty acids, cholesterol and bile acid synthesis, and lipoprotein assembly (Postic, Dentin, & Girard, 2004). These metabolic pathways are coordinately regulated to maintain glucose and lipid homeostasis (Raddatz & Ramadori, 2007). IRS-1 and IRS-2 are the key players in hepatic insulin signaling, as well as lipid metabolism (Fritsche, Weigert, Haring, & Lehmann, 2008). Defects in IRS signaling promotes insulin resistance by dysregulated insulin actin and increased hepatic glucose production, and is considered as a major component of the pathogenesis of liver insulin resistance and T2DM (Dong et al., 2006; Taniguchi, Ueki, & Kahn, 2005).

Most (80%) obese people do not develop T2DM, and not all subjects with T2DM are obese (80% T2DM patients are obese) (Lin & Sun, 2010; Venables & Jeukendrup, 2009). In obese and insulin-resistant subjects, failure of beta cells to compensate for insulin insensitivity ultimately leads to T2DM (Lin & Sun, 2010). In T2DM patients, beta cell mass is decreased by ~60% in both obese and lean subjects compared to their nondiabetic age- and weight-matched counterparts (Butler et al., 2003). The reduced beta cell mass in T2DM has been shown to result from a 3- to 10-fold increase of beta cell apoptosis, while islet neogenesis remains intact, as shown by analysis of human pancreas autopsy specimens (Butler et al., 2003). Several mechanisms have been proposed to trigger increased beta cell mass loss in T2DM. First, ‘glucose toxicity’ caused by chronic

hyperglycemia is capable of generating reactive oxygen species (ROS) in beta cells (R. P. Robertson, Harmon, Tran, & Poitout, 2004). In T2DM patients, excess and prolonged hyperglycemia accompanied by elevated ROS leads to chronic oxidative stress, with deleterious effects in the islets, which exhibit low levels of intrinsic antioxidant defenses. Among the adverse effects are decreased insulin gene expression, impaired insulin secretion, and increased apoptosis (R. P. Robertson, 2004), hence 'glucose toxicity'. Second, 'lipotoxicity' caused by non-esterified fatty acid (NEFA) accumulation can contribute to endoplasmatic reticulum (ER) stress (Laybutt et al., 2007) and induce beta cell death. Third, islet amyloid polypeptide oligomers (IAPP) aggregate in human islets, form islet amyloid deposits, become cytotoxic, and play a role in beta cell death (Janson, Ashley, Harrison, McIntyre, & Butler, 1999). Cytotoxicity of h-IAPP is suggested to result from dramatic disruption of beta cell membrane.

3.2.2 Genetics of T2DM

The etiology of human T2DM is multifactorial, the two critical components being genetic background and the afore-mentioned environmental factors like obesity and diet. Genetic predisposition is an important contributing factor for T2DM since (1) T2DM has a high prevalence in ethnic minorities and indigenous groups with low population admixture; (2) T2DM runs in families; and (3) monozygotic twins demonstrate a considerably higher disease concordance than dizygotic twins (70% vs. 10%) (Elbein, 1997). Importantly, T2DM does not follow simple Mendelian inheritance, indicating that it is a polygenic disease. During the last decade, tremendous effort and considerable progress have been made in the identification of T2DM risk genes by (1) candidate gene

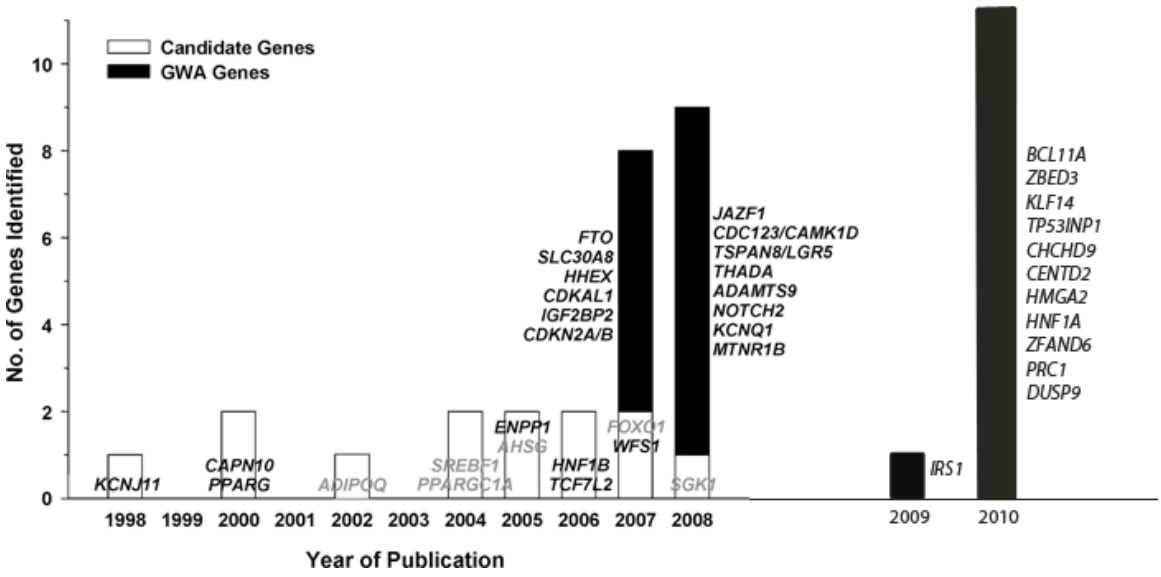


Figure 1.6 (Adapted from (Staiger, Machicao, Fritsche, & Haring, 2009; Voight et al., 2010)) Time-course of the discovery of 38 T2DM risk alleles up to date. At least 38 T2DM risk alleles have been identified. White bars indicate genes discovered during candidate research approach. Black bars indicate genes discovered by GWA studies. Confirmed risked genes are labeled in black and potential risk genes are listed in grey.

approaches and (2) hypothesis-free genome-wide association studies (GWAS) (Staiger et al., 2009). GWAS compare several hundred thousands Single nucleotide polymorphisms (SNPs) in large case-control cohorts to map and catalog SNPs. SNPs cover about 90% of the sequence variation within the human genome and are considered a major determinant of the individual predisposition for complex diseases. For example, a meta-analysis of GWAS studies derived data from a total of approximately 60,000 subjects in 2008, and identified six diabetic risk loci (Zeggini et al., 2008). Another meta-analysis in 2009 of 13 GWAS (~83,000 subjects) revealed one gene of increased risk for T2DM (Prokopenko et al., 2009). To date, GWAS has identified at least 38 (confirmed and potential) T2DM susceptibility genes (Watanabe, 2011) (Fig 1.6). Interestingly, the majority of T2DM risk genes are thought to affect beta cell function, which favors the theory that in T2DM, beta cell dysfunction primarily results from genetic causes, while insulin resistance predominantly results from environmental influences (Florez, 2008). Therefore, T2DM emerges when environmentally triggered insulin resistance happens in the context of genetically programmed beta cell dysfunction.

T2DM risk gene variants affect beta cell insulin secretion in several aspects. For example, *KCNJ11* encodes the pore-forming subunit Kir6.2 of the ATP-sensitive K channel of beta cells (Schwanstecher, Meyer, & Schwanstecher, 2002). *CAPN10* encodes calpain10, a Ca-dependent cysteine protease, which has been proposed to serve as a calcium sensor that triggers actin reorganization and stimulates exocytosis of insulin granules following cytosolic calcium accumulation. The *CAPN10* SNP is associated with glucose-stimulated insulin secretion (Turner, Cassell, & Hitman, 2005). *WFS1* encodes wolframin, a protein that regulates Ca transport in the ER (Florez et al., 2008). *SLC30A8*

encodes the ZnT-8 transporter responsible for insulin maturation and storage by transporting zinc into insulin secretory granules (Chimienti, Devergnas, Favier, & Seve, 2004). *PPARGC1A* encodes PGC1α, a central regulator of mitochondrial function, which is required for mitochondrial ATP formation for stimulus-secretion coupling in beta cells. The potential T2DM risk SNP in *PPARGC1A* produced the missense mutation G482S, which markedly reduces glucose-stimulated insulin secretion in human islets (Ling et al., 2008).

In addition to glucose-stimulated insulin secretion, T2DM risk genes also affect incretin-stimulated insulin secretion. *TCF7L2* encodes a component of the *TCF7L2* transcription factor complex that is involved in the Wnt signaling. Some experiments suggest that TCF7L2 affects GLP-1 stimulated insulin secretion (Florez, 2008). *MTNR1B* encodes a member of the melatonin receptor family, while *CRY2* encodes a member of the cryptochrome family that regulates the clock gene, pointing to the contribution of 'clock' genes and circadian rhythms to T2DM (Prokopenko et al., 2009). These latter two genes are likely to function in non-beta-cells (Gupta et al., 2005).

Furthermore, transcription factors like HNF4A, TCF1, TCF2, and HHEX encode transcription factors in the beta cells, which are implicated in pancreatic development. However, recent unpublished data from the Kaestner lab have shown that HHEX functions in somatostatin producing delta cells, not in beta cells as had been assumed. In addition to affecting insulin secretion, several T2DM risk genes also affect insulin sensitivity, glucagon secretion, and adiposity. Unfortunately, however, all T2DM risk alleles do not provide better disease prediction than simple, conventional clinical risk assessments (e.g., BMI, age, gender, family history, etc.), nor do they explain the

heritability of T2DM (Lango et al., 2008). It is possible that copy number variants, affecting gene dosage, could also contribute to the genetic component of T2DM. Furthermore, epigenetic inheritance might explain some of the familial clustering of T2DM.

3.2.3 Treatment and medication of T2DM

T2DM patients have more fluctuating and higher circulating blood glucose levels than normal subjects as a result of insulin resistance and impaired beta cell function. Management of T2DM focuses on lifestyle intervention, maintaining blood glucose levels in the normal range, and lowering cardiovascular risk factors. A healthy lifestyle includes proper diet and exercise. Some T2DM patients need insulin therapy as well. Bariatric surgery might also be considered, which is a surgery that removes a portion of the stomach to control body weight and blood sugar levels. On the molecular level, the ultimate goal of understanding the insulin signaling pathway and diabetes susceptibility genes is to (1) predict the development of the disease, and (2) identify manipulatable molecular targets to improve glucose uptake, lower hepatic glucose production, and prevent hyperglycemia (Bjornholm & Zierath, 2005).

Several currently used diabetes drugs have ill-defined mechanism of action. Metformin is the most commonly used anti-diabetic medicine. It inhibits hepatic glucose production and hepatic steatosis (Ballav & Gough, 2013). PPARγ agonists constitute another universally studied class of compounds to treat T2DM. They function as senitizers for insulin sensitivity. PPARγ agonists increase insulin sensitivity in peripheral adipocytes, resulting in lower plasma fatty acids and redistribution of muscle and liver lipid to adipocytes (Bajaj et al., 2007) (Bjornholm & Zierath, 2005). PPARγ agonists

have fallen out of favor because their adverse effects including weight increase, edema, and congestive cardiac failure (Bermudez et al., 2010). Another class of anti-diabetic drugs is insulin secretagogues, including sulfonylureas, GLP-1 agonists, and dipeptidyl peptidase 4 (DPP-4) inhibitors. GLP-1 agonists are the 'incretin mimetics', which prime insulin secretion and have a lower risk of causing hypoglycemia like sulfonylureas (Pratley, 2008). DPP-4 is the main enzyme responsible for GLP-1 degradation. DPP-4 inhibitors slow down the clearance of GLP-1 and lower glucose levels by prolonged incretin signaling (Karagiannis, Paschos, Paletas, Matthews, & Tsapas, 2012). Sulfonylureas bind to K_{ATP} channel on beta cells and increase insulin secretion by depolarizing the membrane and opening of Ca^{2+} channels ("Intensive blood-glucose control with sulphonylureas or insulin compared with conventional treatment and risk of complications in patients with type 2 diabetes (UKPDS 33). UK Prospective Diabetes Study (UKPDS) Group," 1998).

3.3 Maturity onset diabetes of the young (MODY)

MODY is a monogenic and autosomal dominant disorder that is characterized by a familial, young-onset non-insulin dependent form of diabetes with pancreatic beta cell dysfunction. MODY typically presents in lean children, adolescents, or young adults before 25 years old, and accounts for 2-5% of all diabetes cases (Shaat et al., 2006). MODY is different from T1DM in that there is no autoimmune destruction of beta cells. MODY is also different from T2DM in that the MODY patients are usually lean and not insulin resistant. Eleven MODY genes have been identified to date, including seven transcription factors and two metabolic enzymes, namely, hepatocyte nuclear factor 4α (HNF4α, MODY1), Glucokinase (GCK, MODY2), hepatocyte nuclear factor 1α

(HNF1α, MODY3), pancreatic and duodenal homeobox 1 (PDX1, MODY4), hepatocyte nuclear factor 1β (HNF1β, MODY5), Neurogenic differentiation 1 (NEUROD1, MODY6), kruppel-like factor 11 (KLF11, MODY7), bile salt dependent lipase (BSDL), also known as carboxyl ester lipase (CEL) (MODY8), paired box gene 4 (PAX4, MODY9), insulin (INS, MODY10), and B-lymphocyte kinase (BLK, MODY11). Some of the MODY genes have also been associated with increased risk of T2DM and increased risk of GDM (Shaat et al., 2006).

Mutations in different MODY genes result in a spectrum of clinical symptoms and require different treatment strategies. The frequency of specific MODY gene mutations varies in MODY families from different countries. Mutation in *Hnf4α* results in MODY1, which is characterized by a defect in glucose-stimulated insulin release. HNF4α is a transcription factor that is part of a complex regulatory network important for glucose homeostasis in the liver and pancreas. *Hnf4α* is under regulation of two promoters: the liver-specific P1 promoter and the pancreatic beta cell-specific P2 promoter. Loss of function of *Hnf4α* leads to impaired expression of genes involved in glucose transport and glycolysis in the liver (Stoffel & Duncan, 1997). MODY1 is typically treated by oral hypoglycemics (Watanabe, 2011). In the beta cells, HNF4α has been suggested to regulate glucose-stimulated insulin secretion (GSIS) in a Kir6.2 dependent manner (Gupta et al., 2005). *GCK* mutations cause MODY2, which is characterized by a lifelong mild fasting hyperglycemia due to reduced glucose sensing in the beta cell. GCK acts as a glucose sensor and stimulates beta cell insulin secretion. Glucokinase facilitates phosphorylation of glucose to glucose-6-phosphate, which is the first step of glycolysis. Glycosis produces ATP, which causes depolarization of membrane, influx of Ca^{2+}, and

eventually insulin secretion. MODY2 can be managed by lifestyle modification alone. MODY3, caused by mutations in the *Hnf1α* gene, is characterized by severely defective insulin secretion but retained sensitivity to sulfonylureas. MODY3 is the most common type of MODY in populations with European ancestry (Frayling et al., 2001). HNF1α is a transcription factor regulating cellular functions including glucose metabolism and lipid transport. MODY3 is also treated by oral hypoglycemics. MODY4-11 are very rare, some reported in fewer than 10 families (Watanabe, 2011).

4. Regulation of beta cell mass

4.1 Establishment of beta cell mass

Total beta cell mass is determined by beta cell neogenesis (which mainly happens during embryogenesis), beta cell replication, beta cell hypertrophy, and beta cell apoptosis. There is a rapid expansion of beta cell number in early childhood (Meier et al., 2008), from both beta cell replication (accounting for ~90% of beta cell mass expansion during the neonatal stage) and beta cell/islet neogenesis (contributing to ~10% of beta cell mass expansion after birth). During the perinatal stage, beta cells have a relatively high proliferation rate (~1% of -2 to 2 months old infants) with a peak wave of apoptosis (~1.3% during the same period). Both proliferation and apoptosis decrease rapidly from 2 to 6 months (0.6% and 0.2%, respectively) and after 6 months of age, only rare beta cells replicate (0.13%) (Kassem, Ariel, Thornton, Scheimberg, & Glaser, 2000). Similarly in mice, the postnatal period between birth and 2 weeks is also characterized by a massive increase in beta cell mass by beta cell replication (Georgia & Bhushan, 2004).

After early childhood, beta cell mass slightly increases during adolescence, in

proportion to the increase of body weight (Meier et al., 2008). Although the beta cell proliferation rate is extremely low (0.13% after 6 months of age (Kassem et al., 2000)), it has been suggested to be the primary mechanism of beta cell mass increase during adolescence, because islet number does not change after infancy (2 years old), while the mean islet size increases about two-fold, with no change in cell size (Meier et al., 2008).

4.2 The regenerative capacity of beta cells

Postnatal beta cells have an impressive regeneration capacity in situations of increased metabolic demand (Lysy, Weir, & Bonner-Weir, 2012) (Fig 1.7). Evidence for beta cell regeneration includes: **(1)** Beta cell mass linearly correlates with body weight in adult rats (Montanya, Nacher, Biarnes, & Soler, 2000); **(2)** In the obese mouse model (ob/ob), total islet mass increases 3.6 times without increasing total islet number, indicating that cells within the existing islets undergo hypertrophy or hyperplasia, or both (Bock, Pakkenberg, & Buschard, 2003). **(3)** In both nondiabetic subjects and T2DM subjects, beta cell mass increases with BMI (Willcox, Richardson, Bone, Foulis, & Morgan, 2010), indicating an adaptive capacity of beta cell regeneration. It is noteworthy that beta cell replication capacity observed in the obese rodents (5-10 fold increase) is more profound than the increase in obese humans (~1.5 fold), indicating that different mechanisms might be underlying beta cell replication among different species (Butler et al., 2003). **(4)** In rats following partial pancreatectomy, where 60% of the pancreas is removed, normal glycemia is maintained by a 3-fold increase of beta cell proliferation 5 days after surgery, accompanied by enhanced glucose metabolism in existing beta cells (Y. Q. Liu, Montanya, & Leahy, 2001). Similarly, in a beta cell injury model in mice,

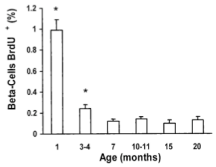
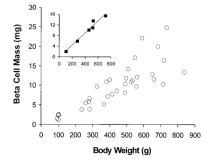
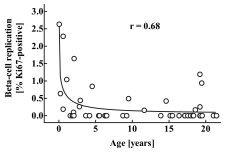
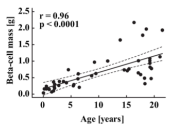
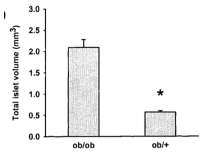
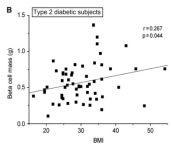
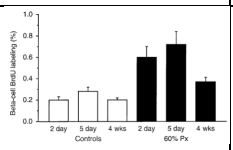
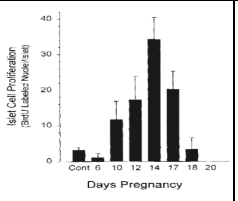
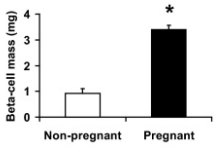
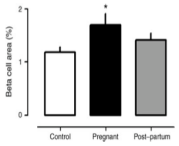
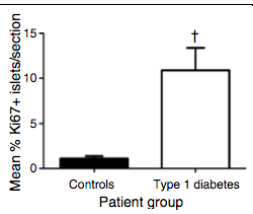
	Rodents		Human	
	Beta cell prolif	Beta cell mass	Beta cell prolif	Beta cell mass
Normal condition ((Meier et al., 2008; Montanya et al., 2000))				Young adults 
Increased metabolic demand ((Bock et al., 2003; Pick et al., 1998; Willcox et al., 2010))	Zucker Lean control vs. Zucker Diabetic Fatty mice % β-cell BrdU+/ 6 h 5–7 weeks 12 weeks ZLC 6 ± 0.14 0.50 ± 0.07 ZF 2 ± 0.04 0.68 ± 0.10 ZDF 5 ± 0.13* 0.92 ± 0.15*	Ob/Ob mice (5-10 fold) 	NA	T2D patients (~1.5 fold) 
Pancreas injury ((Y. Q. Liu et al., 2001))		NA	NA	NA
Pregnancy ((Butler et al., 2010; Rieck et al., 2009; Sorenson & Brelje, 1997))		P14.5 (2-4 fold) 	More small islets, more insulin-positive duct cells, and more schattered individual beta cells	mean of 24wk  gestation (1.4-fold)
T1D((Willcox et al., 2010))				1% left

Figure 1.7 Evidence for in vivo regeneration capacity of beta cells. Beta cells have a low basal proliferation rate after the boost of replication during perinatal stage, unless challenged by metabolic demands like pregnancy, obesity, and pancreas injury, during which beta cells adaptively proliferate and increase hypertrophy for beta cell expansion. Shown in this complex table here are the conditions during which beta cell proliferation increases and beta cell mass expanses. On the left are studies in rodents, and on the right are studies in humans.

where 70-80% beta cells are specifically ablated by diphthia toxin mediated cell ablation, significant regeneration of beta cell mass takes place, spontaneously normalizing blood glucose levels (Nir, Melton, & Dor, 2007). **(5)** During pregnancy, when the mother becomes insulin resistant, beta cell mass increases 3-4 fold in rodents by both increase of beta cell proliferation and beta cell hypertrophy. Interestingly, beta cell mass shrinks to pre-pregnancy levels within 10 days after delivery by cell apoptosis, supporting the intrinsic adaptive plasticity of beta cells (Karnik et al., 2007; Rieck & Kaestner, 2010). **(6)** In early onset T1DM patients, beta cell proliferation is detected with a 10-fold higher rate than in control subjects as an apparent attempt to compensate for the autoimmune mediated loss of beta cell mass, which contributes to the 'honeymoon period' of T1DM (Willcox et al., 2010). Remarkably, in one 89-year old patient with ongoing T1DM, proliferating beta cells were detected in the islets, indicating the capacity of beta cell proliferation even after aging (Willcox et al., 2010).

4.3 Proliferation is the primary source of new beta cells during adulthood

The origins of new beta cells that maintain beta cell homeostasis in adulthood and during regenerative repair has been controversial (Dor, Brown, Martinez, & Melton, 2004). Studies challenging beta cells adaptive regeneration capacity in different models have revealed several options as sources of new beta cells (Fig 1.8). **First**, neogenesis of islets or beta cells (i.e. the production of new beta cells from differentiation of progenitor cells) (Bonner-Weir & Weir, 2005a). Although it remains unclear whether there are 'facultative' stem cells or progenitor cells in the pancreas, neogenesis of beta cells from pancreatic duct cells has been extensively investigated (Xiao et al., 2013). The pancreatic

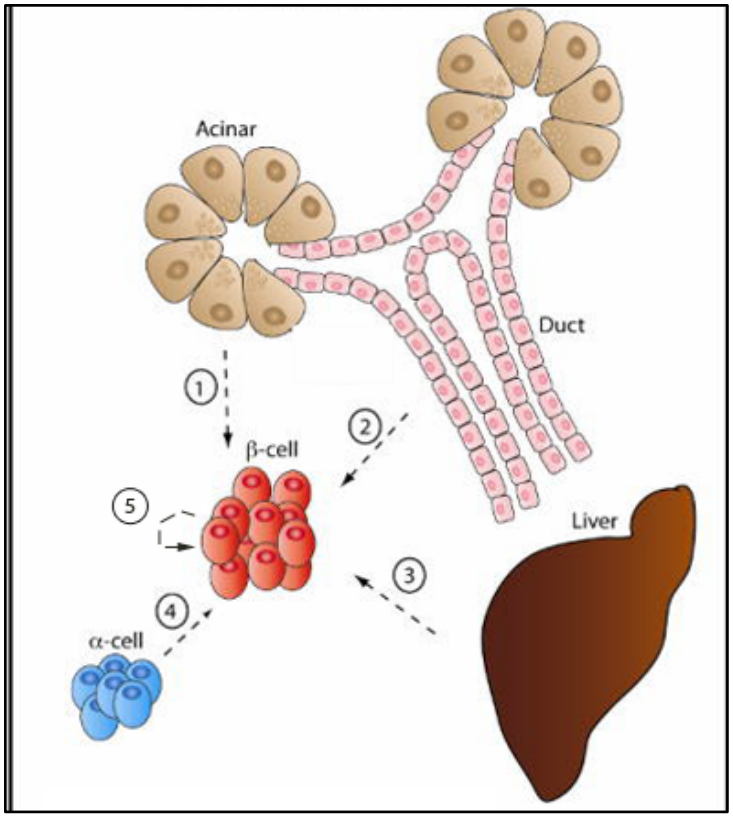


Figure 1.8 (Adapted from (Juhl, Bonner-Weir, & Sharma, 2010)) Different sources of new beta cells. 1) Transdifferentiating from acinar cells to beta cells. 2) Neogenesis of beta cells from facultative ductal progenitor cells. 3) Transdifferentiating from hepatocytes. 4) Transdifferentiating from alpha cells, and 5) Proliferating from beta cells.

ductal epithelium has been suggested to be a potential pool for beta cells progenitors (Bonner-Weir et al., 2004). Evidences for existence of ductal progenitor cells include the appearance of insulin^{+} cells near the duct (referred to as the 'budding new islets'), the appearance of Ngn3^{+} cells near the duct after pancreatic ductal ligation (PDL) (X. Xu et al., 2008), and the increase in the ratio of small to large islets in beta cell regeneration models such as obesity (Bonner-Weir et al., 2004). A recent study shows that 14% of the newly formed beta cells after PDL are attributed to beta cell neogenesis from Ngn3^{+} progenitor cells (Van de Casteele et al., 2013). It has also been suggested that beta cell neogenesis, rather than replication, predominates in newly diagnosed glucose intolerant diabetic patients (Yoneda et al., 2013). Controversially, genetic lineage tracing of ductal cells disproves the existence of progenitor cells in the duct epithelium (Collombat et al., 2009).

Second, transdifferentiation of beta cells from acinar cells, pancreatic alpha cells, or hepatocytes (Lysy et al., 2012). Other cells within the pancreas or other tissues have been investigated for their potential to transdifferentiate into beta cells (Lysy et al., 2012). Adult acinar cells have demonstrated the capacity to be converted into insulin-secreting beta cells *in vitro* (Mashima et al., 1996), but *in vivo* studies show controversial results (Desai et al., 2007; Zhou, Brown, Kanarek, Rajagopal, & Melton, 2008). In one study, acinar cells failed to convert into beta cells in several beta cell regeneration models (Desai et al., 2007). In another study, ectopic expression of *Ngn3, Pdx1* and *Mafa* successfully reprogrammed acinar cells into cells that resemble beta cells *in vivo* (Zhou et al., 2008). Islet alpha cells physiologically highly resemble beta cells, and several groups have demonstrated the capacity of reprogramming alpha cells to beta cells by ectopic

expression of *Pax4* or loss of *Arx* (Collombat et al., 2009; Yoneda et al., 2013). In an extreme beta cell ablation model (99% ablation), a fraction of alpha cells is shown to convert to beta cells by genetic lineage tracing (Chung, Hao, Piran, Keinan, & Levine, 2010; Thorel et al., 2010). Interestingly, many beta cell signature genes are actively marked in beta cells, while bivalently marked in alpha cells, indicating reprogramming plasticity of alpha cells to beta cells. Indeed, epigenetic manipulation of histone marks by a histone methyltransferase inhibitor successfully converts alpha cells to insulin producing cells in human islets, suggesting that epigenomic manipulation could provide a path for alpha-to-beta cell reprogramming (Bramswig et al., 2013). Hepatocytes are also on the list, with the rational that liver and pancreas arise from the common endodermal origin in the foregut (Kojima et al., 2003). Adult human liver cells can transdifferantiate into insulin^{+} cells after treated with PDX1 and soluble factors *in vitro* (Sapir et al., 2005). These studies all have had some success, but their ability to reproductively create abundant 'true' functional insulin producing beta cells that response to glucose has been limited.

Third, replication of pre-existing mature beta cells. One genetic lineage tracing study in mice shows that adult pancreatic beta cells are formed largely by self-replication, rather than stem cell differentiation under normal physiological conditions and even during the regenerative repair after partial pancreatectomy (Dor et al., 2004). Another genetic lineage tracing study made possible the detection of newly formed beta cells from non-beta cells, and found no evidence of such cell transition after PDL, suggesting that the replication of pre-existing beta cells is the major source of new beta cells in adulthood (Xiao et al., 2013). In summary, although there are several studies showing the possibility

of beta cell neogenesis from adult progenitor cells and transdifferentiation of other differentiated cell types to beta cells, the primary source of new beta cells during adulthood is proliferation of pre-existing beta cells. This is the case under normal physiology conditions, during pregnancy, obesity, and injury models like PDL. Islet neogenesis, if there is any, only plays a small role in the homeostasis of beta cell mass during adulthood.

4.4 Signals and mechanisms of beta cell proliferation

Since proliferation is the main mechanism of maintaining beta cell mass, it is critical to elucidate the external and internal regulators of beta cell proliferation. The ultimate goal will be to manipulate key regulators for beta cell proliferation and produce more beta cells *in vivo* as a therapeutic strategy for diabetes treatment. The current knowledge of mechanisms underlying adaptive beta cell proliferation relies heavily on experimental models of physiological stimuli of beta cell mass expansion, including insulin-resistance induced by obesity or pregnancy.

4.4.1 Intrinsic cell cycle regulators

The mammalian cell cycle machinery possesses two key checkpoints: the G_1/S transition and the G_2/M transition checkpoints. Regulatory proteins called **cyclins** associate with specific cyclin-dependent kinases (**CDKs**) and activate the catalytic activity of CDKs, which allows the cells to traverse cell cycle checkpoints. During the G_1/S phase of the cell cycle, cyclin D is up-regulated, which complexes with CDK4 and/or CDK6. The activated CDK4/6 complexes inactivate 'pocket proteins' (retinoblastoma protein (pRb), p107, and p130) by phosphorylation. Inactivation of the

pocket proteins releases the E2F family of transcription factors and facilitates the subsequent transcription of E2F target genes required for DNA synthesis during S phase, and cell cycle genes like *cyclin A* needed for the G_2/M transition. Later on, cyclin E-dependent CDK2 is induced. The cyclin E/CDK2 complex also phosphorylates non-Rb substrates to further power the cell through the G_1/S checkpoint. During G_2/M phase, cyclin A-dependent CDK1 is required for the onset of mitosis. Upon nuclear envelope breakdown, A-type cyclins are degraded, allowing the formation of the cyclin B/CDK1 complex, which facilitates the progression through mitosis (Malumbres & Barbacid, 2009) (Fig 1.9).

Cell cycle checkpoints are surveillance mechanisms that ensure the fidelity of cell division by verifying that each phase of cell cycle is accurately completed before progress into the next phase. Cell cycle arrest is achieved by cyclin-dependent kinase inhibitors (**CKIs**). There are two families of CKIs, the INK family (inhibitory kinases) and the CIP/KIP family (cyclin inhibitory proteins/kinase inhibitory proteins). The INK family has four members ($p16^{INK4a}$, $p15^{INK4b}$, $p18^{INK4c}$, and $p19^{INK4d}$), which bind to and inhibit cyclin D-dependent CDK4/6, causing cell cycle inhibition or arrest at the G_1/S checkpoint (Malumbres & Barbacid, 2009). The CIP/KIP group consists of three members ($p21^{CIP1}$, $p27^{KIP1}$, $p57^{KIP1}$), which have bifunctional effects on cell cycle. On the one hand, CIP/KIP family members inhibit the activity of cyclinE/CDK2 during the G_1/S transition and CDK2/1 during the G_2/M transition. On the other hand, p21 and p27 act as chaperones for cyclinD-dependent CDK4/6 to augment cell cycle progression during the G_1/S phase (Malumbres & Barbacid, 2009).

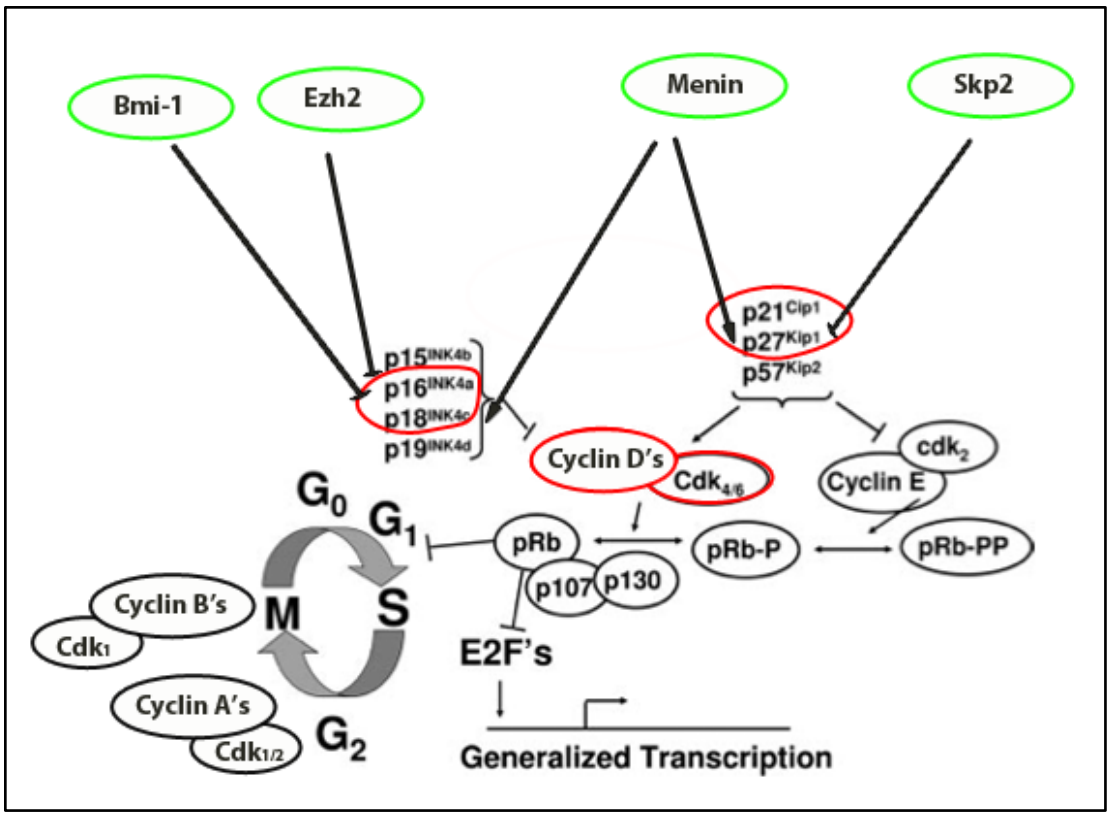


Figure 1.9 (Adapted from (Cozar-Castellano, Fiaschi-Taesch, et al., 2006)) Cell cycle machinery and its regulators. An overview of the proteins that control β-cell cycle with emphasis on the G_1/S-phase transition. Key regulators of beta cell proliferation include cyclins, cyclin-dependent kinases (CDKs), and cyclin-dependent kinase inhibitors (CKIs). Cell cycle regulators that have been shown to play important role in beta cell proliferation are circled in red. Cell cycle inhibitor regulators are circled in green.

In studies of mice with targeted global or cell-type-specific mutation of cell cycle regulators, it is revealed that CDKs have a cell type-specific pattern of expression. Therefore, it is essential to decipher the unique cell cycle regulators in beta cells. For example, islets are more susceptible to CDK4 deficiency than other tissues. Global ablation of CDK4 yields a milder phenotype than expected given the essential role of CDK4 during cell cycle progression (Rane et al., 1999). CDK4 mutant mice demonstrate abnormalities only in the ovary, the testis, and beta cells. CDK4 mutants are born euglycemic with normal beta cell mass, but develop diabetes during the first two months of life, due to a deficiency of postnatal beta cell replication. Other tissues are less susceptible to CDK4 deficiency, probably due to the compensation from CDK6. In islets, mRNA levels of CDK4 are two orders of magnitude higher than those of CDK6, explaining the lack of compensating from CDK6 (Martin et al., 2003). Later studies demonstrate that cell cycle machinery of beta cells could be accelerated by manipulating CDK4 levels. CDK4 knockin mice have significantly increased beta cell mass, and human islets expressing CDK4 cDNA exhibite higher beta cell proliferation (Marzo et al., 2004).

Interestingly, cyclin mutant mice exhibit variable phenotypes in different mouse genetic backgrounds. For example, during G_1 phase, cyclin D2 is the most abundant cyclin in mouse islets (Kushner et al., 2005). *Cyclin D2*$^{-/-}$ mice on the C57BL/6 background develop severe diabetes due to deficiency of beta cell proliferation at 3 months of age, indicating that cyclin D2 is the primary D-type cyclin for beta cell replication in these mice (Kushner, 2006). However, on a mixed B6/129 background, *cyclin D2*$^{-/-}$ mice develop diabetes at 12 months of age caused by decreased beta cell

proliferation. Removing one allele of *cyclin D1* in *cyclin D2*$^{-/-}$ mice accelerates the onset of diabetes from 12 months to 3 months (Kushner et al., 2005), indicating that both cyclin D1 and cyclin D2 levels need to be tightly regulated for proper beta cell mass expansion after birth. However, forced over-expression of stabilized *cyclin D2* fails to induce beta cell proliferation (He et al., 2009). Overexpression of *cyclin D1* in the beta cells also fails to stimulate beta cell proliferation at young age (3 months), but yields more beta cell proliferation in older mice (12 months) (X. Zhang et al., 2005). These studies emphasize the essential role of CDK4 inhibitors such as p15 and p16, and demonstrate that cell cycle arrest is difficult to overcome by overexpression of just one cyclin.

Intriguingly, human islets seem to utilize distinct cell cycle machinery. First, cyclin D3 is the most abundant D-type cyclin in human islets, while only variable and marginal quantities of cyclins D1 and D2 could be detected (Fiaschi-Taesch et al., 2010). In contrast, cyclin D2 is the most abundant D-type cyclin in mouse islets, as mentioned above. Second, CDK4 and CDK6 are easily detected in human islets at comparable levels, while mouse islets contain much more CDK4 than CDK6 (Fiaschi-Taesch et al., 2010). Overexpression of *cyclins D1/2/3* and/or CDK4/6 individually or in combination, all leads to variable increase in human beta cell replication, with cyclin D3 and CDK6 being the most potent ones (Fiaschi-Taesch et al., 2010). However, while yielding cell cycle entry, overexpression of these cell cycle regulators also caused widespread DNA damage and failure to complete the cell cycle (Rieck et al., 2012).

CKI proteins mainly function as brakes for cell cycle progression. Recent studies have elucidated CKIs as critical inhibitors of beta cell proliferation. For the **Cip/Kip** group of CKIs, $p21^{Cip1}$ and $p27^{Kip1}$ are expressed at far higher levels than $p57^{Kip2}$ in mice.

Studies of $p21^{Cip1}$ and $p27^{Kip1}$ have suggested that they might response to different metabolic or mitogenic cues for beta cell replication. Under normal physiological conditions, $p27^{-/-}$ mice are born with doubled beta cell mass due to excess beta cell proliferation during embryogenesis (Georgia & Bhushan, 2006). In insulin resistance mouse models, $p27^{kip1}$ accumulates in beta cell nuclei. Ablation of $p27^{kip1}$ in insulin resistant mice yielded compensatory beta cell proliferation, hyperinsulinemia, and prevented diabetes (Uchida et al., 2005). $P27^{-/-}$ mice treated with streptozotocin (STZ. It is transported into beta cells by GLUT2, and causes DNA damage and cell toxicity largely specific to beta cells) have better glycemic control than wild type mice due to higher beta cell proliferation rate (Georgia & Bhushan, 2006). Furthermore, gene silencing of $p27^{kip1}$ in islets results in more beta cell replication and shorter duration of hyperglycemia after transplantation into induced hyperglycemic mice (S. T. Chen, Fu, Hsu, Huang, & Hsu, 2012). $P21^{cip1}$ is up-regulated in HGF or placental lactogen overexpressing islets, and $p21^{cip1}$ null islets are released from growth inhibition and demonstrate markedly enhanced proliferation in response to mitogenic stimulation by HGF and prolactin (Cozar-Castellano, Weinstock, et al., 2006). These studies suggest that $p27^{kip}$ might be responsible for metabolic signals such as hyperglycemia/lipotoxicity, whereas $p21^{cip}$ may respond to mitogenic growth factors (Cozar-Castellano, Weinstock, et al., 2006). Although $p57^{Kip2}$ is the least abundant Cip/Kip protein among the three in mice, the loss of $p57^{Kip2}$ in humans is associated with hyperinsulinism of infancy, indicating an important cell cycle inhibition function of $p57^{Kip2}$ in human beta cells (Kassem et al., 2001). Indeed, suppression of $p57^{Kip2}$ by shRNA in human islets results in higher beta cell proliferation rate. Importantly, newly replicated cells demonstrated

normal glucose-induced calcium influx, indicating their functionality (Dana, unpublished data).

The **INK4 family** of cell cycle inhibitors consists of four members: $p16^{INK4a}$, $p15^{INK4b}$, $p18^{INK4c}$, and $p19^{INK4d}$. $P18^{-/-}$ mice have approximately 40% more islets than wild type controls (Pei, Bai, Tsutsui, Kiyokawa, & Xiong, 2004), suggesting the release of cell cycle arrest after ablation of this cell cycle inhibitor. However, loss of p18 in the context of CDK4 deficiency ($p18^{-/-}$; $CDK4^{-/-}$ mice) phenocopies $CDK4^{-/-}$ mice, which are diabetic with decreased beta cell mass. This finding suggests that releasing the brake (p18) without a functioning 'accelerator' leads to cell cycle arrest (Pei et al., 2004). Expression of p15 is downregulated during pancreatic endocrine tumorigenesis, indicating that p15 needs to be inactivated to promote islet hyperplasia (Karnik et al., 2005). Expression of p16, but not other CKIs tested (p15, p18, p19, p21, p27), increases markedly with ageing (Krishnamurthy et al., 2006), alongside the decline of beta cell proliferation. Overexpression of p16 in young mice results in reduced beta cell proliferation, suggesting that accumulation of p16 in old mice prevents beta cell proliferation. Indeed, inactivation of p16 in old mice rescues the age-induced decrease in proliferation (Krishnamurthy et al., 2006).

4.4.2 Transcriptional and epigenetic regulators of cell cycle inhibitors

Histone modifications of the genes encoding CKIs are dynamically regulated to control beta cell proliferation. *MEN1* is the gene responsible for the endocrine tumor syndrome termed multiple endocrine neoplasia type 1 (MEN1), which is characterized by pituitary, parythyroid, and pancreatic islet tumors. The protein product of *MEN1* is Menin (Stratakis & Marx, 2005), which associates with TrxG (trithorax group) histone

methyltransferases such as MLL, to promote the methylation of H3K4 (Karnik et al., 2005). In islets, Menin-dependent MLL targets H3K4 methylation of p27 and p18, making Menin a negative regulator of beta cell proliferation. Menin mutations in mice (*Men1*$^{+/-}$) result in decreased methylation of the p27 and p18 promoters, decreased expression of p27 and p18, increased CDK4 activity, and increased beta cell proliferation (Karnik et al., 2005; Schnepp et al., 2006). Furthermore, Menin expression is down-regulated during pregnancy in parallel with p27 and p18, permitting adaptive beta cell proliferation. Overexpression of *Men1* in beta cells results in gestational diabetes due to deficient beta cell replication (Karnik et al., 2007).

Bmi-1, a polycomb group protein (PcG), is a part of the polycomb-repressive complex 1 (PRC1), which possesses H2A ubiquitin E3 ligase activity. Together with PRC2, which possesses H3K27 methyltransferase activity mediated by Ezh2 of this complex, the Ink4a/Arf locus is repressed (H. Chen et al., 2009; Dhawan, Tschen, & Bhushan, 2009). Therefore, Bmi-1 and Ezh2 promote proliferation by down-regulating the cell cycle inhibitor p16. Bmi-1 plays an opposing role to MLL1 in controlling the Ink4a/Arf locus. In aging islets, Ezh2 and Bmi-1's binding to the p16 locus is decreased, leading to de-repression of the p16 locus and increased MLL1 recruitment. As a result, H3K27me3 decreases and H3K4me3 increases in the p16 promoter. These chromatin alterations increase expression of *p16*, which is partially responsible for the decreased cell replication in aging islets (Dhawan et al., 2009). *Bmi-1*$^{-/-}$ mice display glucose intolerance caused by a concomitant decrease in beta cell proliferation and beta cell mass. Beta-cell-specific *Ezh2* mutant mice have increased expression of p16 and p19, and also exhibit glucose intolerance with decreased beta cell proliferation (Dhawan et al., 2009).

Besides the above-mentioned epigenetic regulators of CKIs, the ubiquitin-proteasome pathway also plays a critical role in regulating beta cell replication. For example, the SCF ubiquitin ligase complex facilitates the degradation of p27 during the S phase of the cell cycle. Skp2 is a receptor component of SCF complex, and upon Skp2 ablation, mice exhibit nuclear accumulation of p27. In *Skp2*$^{-/-}$ pancreas, the increased p27 levels prevent mitosis and cause beta cell endoreduplication (cells undergo DNA synthesis without mitosis). As a result, *Skp2*$^{-/-}$ mice have larger beta cells but decreased beta cell mass, resulting in glucose intolerance for both normal conditions and HFD-induced insulin resistance mouse models (Zhong et al., 2007).

4.4.3 External mitogens: hormones, growth factors, and nutrients

A number of cell extrinsic stimuli can initiate beta cell replication, including hormones such as insulin, GLP-1, lactogens, and growth hormone; growth factors such as insulin-like growth factor (IGF), hepatocyte growth factor (HGF), epidermal growth factor (EGF), platelet-derived growth hormone (PDGF); and nutrients, including glucose and free fatty acids (FFA). The intracellular intermediates and pathways that link extracellular mitogens to the intracellular cell cycle machinery are not well understood. However, it is clear that the same intracellular signaling pathways (for example, PI3K/Akt pathway) are shared by different extrinsic stimuli, resulting in an orchestrated regulation of beta cell proliferation (Fig 1.10).

Ins/IGF→ IR/IGF-R→IRS1/2

Insulin and insulin-like growth factor (IGF) are two mitogens critical for beta cell proliferation. The receptors for these ligands are the tyrosine kinase receptors Insulin Receptor (IR) and IGF-1/2 Receptor (IGF-1/2R), respectively. Upon ligand binding, the

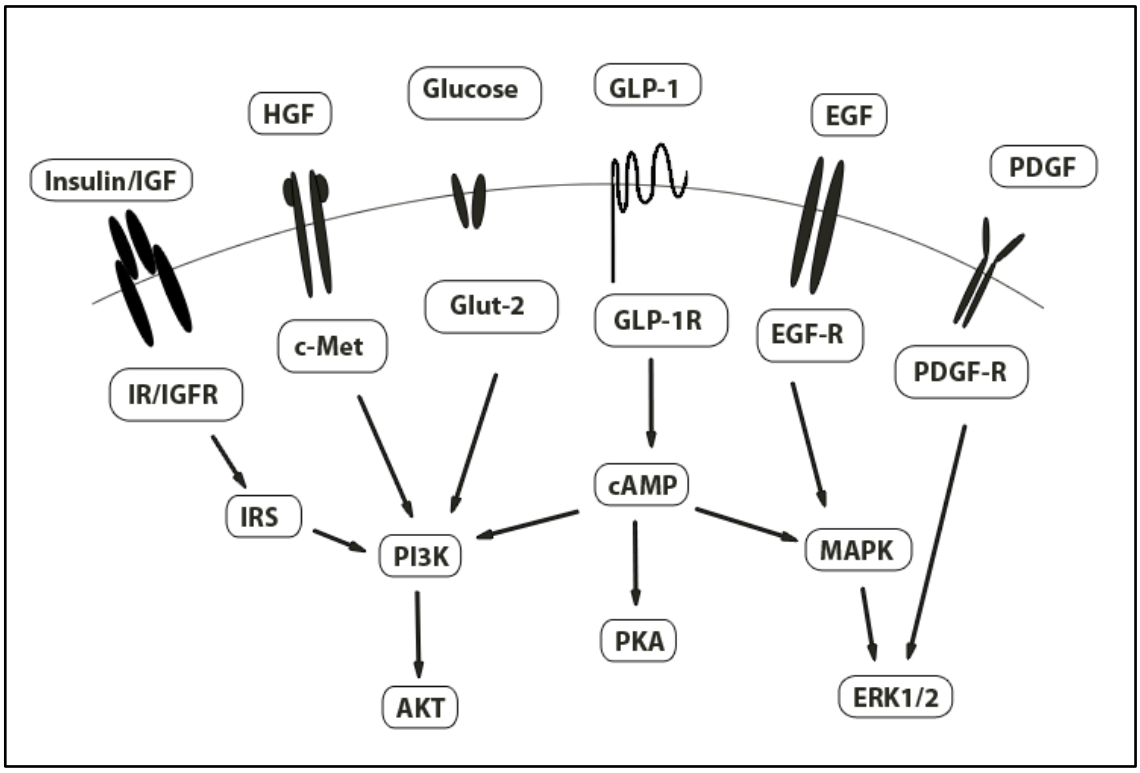


Figure 1.10 External mitogens of beta cells and and their intracellular signaling pathways. A number of extrinsic stimuli could trigger beta cell replication, including hormones like insulin, GLP-1, lactogens, growth hormone; growth factors like insulin-like growth factor (IGF), hepatocyte growth factor (HGF), epidermal growth factor (EGF), platelet-derived growth hormone (PDGF), and nutrients like glucose and free fatty acids (FFA). The intracellular intermediates and pathways that link these extracellular mitogens and the intracellular cell cycle machinery are not fully deciphered. But it is clear that same signaling pathways (for example, PI3K/Akt pathway) could be shared by different extrinsic stimuli, resulting in an orchestrated regulation of beta cell proliferation.

intracellular domains of insulin/IGF receptors become auto-phosphorylated, bind cellular scaffold proteins, and generate further intracellular signals. Mouse models that disrupt insulin or IGF1 signaling provide compelling evidence for insulin/IGF-mediated regulation of beta cell mass. Beta-cell-specific ablation of IR (βIRKO) in mice results in progressive glucose intolerance accompanied by defects in insulin secretion, reduced beta cell mass, and reduced expression of G*lut2* and *Glucokinase* (*Gck*) (Kulkarni et al., 1999; Otani et al., 2004). Beta cell-specific IGFR ablation (βIGFRKO) also causes glucose intolerance, but has no affect on

beta cell mass. Compound ablation of IR and IGFR in beta cells (βDKO) results in reduced beta cell mass and overt diabetes at 3 weeks of age (Ueki et al., 2006). Taken together, these results suggest that the IR pathway plays a more important role than IGFR in regulating beta cell mass (Kulkarni et al., 2002).

A recent study shows that overexpressing IR in the beta cells results in higher beta cell proliferation and helps ameliorate STZ-induced hyperglycemia (M. H. Kim, Hong, & Lee, 2013). However, it has been controversial whether IGF1 can also stimulate beta cell proliferation *in vivo*. One group reports that overexpressing IGF1 in beta cells causes increased replication after STZ treatment (George et al., 2002), while another study shows that IGF1 overexpression does not promote beta cell replication. This second study finds that IGF1 functions as an antiapoptotic factor after STZ treatment, indicating that IGF1 is not an *in vivo* islet growth factor (K. Robertson et al., 2008). Further studies are necessary to investigate the mitogenic effect of IGF1 on beta cells using different beta cell regenerative models.

Insulin receptor substrates (IRS) are essential intermediates for insulin/IGF signaling (M. F. White, 2002). Humans have three IRS proteins, IRS1, 2, and 4, while rodents express all four IRS proteins, including IRS-3 (M. F. White, 2002). IRS proteins activate PI3K and ERK phosphorylaiton cascades, which stimulate beta cell replication and promote survival. IRS1 and IRS2 are the two most important downstream effectors of Ins/IGF1 signaling, although IRS1 and IRS2 mutants display distinct phenotypes. $IRS1^{-/-}$ mice develop mild glucose intolerance accompanied with a two-fold increase of beta cell mass relative to wild type mice. In contrast, $IRS2^{-/-}$ mice become diabetic without beta cell mass expansion, indicating that IRS2 plays a more critical role in modulating beta cell mass expansion during periods of increased metabolic demand (Withers et al., 1998).

HGF→c-Met

HGF and its receptor c-Met play essential roles in regulating beta cell proliferation. Overexpression of HGF in beta cells causes increased beta cell replication and hypoglycemia (Garcia-Ocana et al., 2000). Beta-cell specific ablation of c-Met results in smaller islets, decreased *Glut-2* expression, impaired insulin secretion, and glucose intolerance (Dai, Huh, Thorgeirsson, & Liu, 2005). Surprisingly, beta cell mass and beta cell proliferation are not affected (Roccisana et al., 2005), indicating that HGF/c-Met signaling is not required for maintaining beta cell mass under normal conditions. Interestingly, pancreas-specific ablation of c-Met in mice causes increased susceptibility to STZ-induced diabetes (Mellado-Gil et al., 2011), indicating that HGF/c-Met signaling is critical for beta cell survival after injury. The same mouse model also results in gestational diabetes with impaired beta cell proliferation at pregnant day 15 and reduced

beta cell mass at pregnant day 19, suggesting that HGF signaling is required for maternal beta cell mass expansion during pregnancy (Demirci et al., 2012).

GLP-1/GIP

GLP-1 and GIP are secreted from the intestinal L-cells and K-cells, respectively, in response to carbohydrates and fats (Buteau, Foisy, Joly, & Prentki, 2003). GLP-1 and GIP account for almost the entire incretin effect (Brubaker & Drucker, 2004). Since T2DM is associated with resistance to GIP but not GLP-1, current therapeutic research is focused on GLP-1 (Brubaker & Drucker, 2004). GLP-1 is a potent treatment for diabetes because it acutely potentiates glucose-stimulated insulin secretion (Buteau et al., 2003), promotes beta cell proliferation, and displays antiapoptotic activity (Buteau et al., 2004). GLP-1 has a very short half life (2-6 mins) (G. Xu, Stoffers, Habener, & Bonner-Weir, 1999); therefore, it's long-lived analog, Exendin-4, has been popularly investigated on its role in beta cell function. Although sustained overexpression of Exendin-4 does not alter glucose homeostasis or beta cell mass (Baggio, Adatia, Bock, Brubaker, & Drucker, 2000), short term Exendin-4 treatment in mice results in increased beta cell proliferation and beta cell mass under both basal conditions and after partial pancreactomy (G. Xu et al., 1999). During metabolic challenges, GLP-1 or Exendin-4 infusion results in amelioration of diabetes in Zucker diabetic rats and prevents hyperglycemia in db/db mice, respectively (Farilla et al., 2002; Q. Wang & Brubaker, 2002).

The mechanism of GLP-1 activity in pancreatic beta cells has been extensively studied, and multiple signaling pathways are involved (Z. Liu & Habener, 2008). GLP-1 binds its high affinity receptor GLP-1R, which activates adenylate cyclase and increases intracellular cAMP levels. cAMP stimulates multiple downstream pathways, including

PKA, MAPK/ERK, and PI3K/Akt (Cornu et al., 2010). Studies have also shown that GLP-1 induces beta cell proliferation via trans-activation of EGFR and translational induction of IGF-1R. These proteins form an autocrine loop with IGF-2 and IGF-1R to activate the IRS2/Akt signaling pathway (Buteau et al., 2003; Cornu et al., 2010). Further downstream factors involved in GLP-1-induced beta cell proliferation include: SirT1, FoxO1 (SirT1-dependent), Skp2 (PI3K-dependent) (Tschen, Georgia, Dhawan, & Bhushan, 2011), Protein Kinase zeta (PI3K-dependent), mTOR (AMPK-dependent) (Miao et al., 2013), and pdx1 (Bastien-Dionne, Valenti, Kon, Gu, & Buteau, 2011; Brubaker & Drucker, 2004; Buteau et al., 2001; Buteau, Spatz, & Accili, 2006; X. Wang, Zhou, Doyle, & Egan, 2001). Cyclin A2 and cyclin D1 have also been suggested to mediate GLP-1 induced beta cell proliferation (Friedrichsen et al., 2006; Song et al., 2008).

EGF/EGFR, PDGF/PDGFR

Although EGFR can be trans-activated by GLP-1 (Buteau et al., 2003), the downstream factors of EGFR in beta cells are not well defined. $EGFR^{-/-}$ mice display failed beta cell replication and develop glucose intolerance both under basal conditions and upon the metabolic challenges of HFD or pregnancy, while maintained normal adaptive beta cell mass expansion after PDL (Hakonen et al., 2011; Miettinen et al., 2006).

PDGF (platelet-derived growth factor) acts through PDGF-R, which activates Ezh2 in an ERK1/2 dependent manner. Declining PDGF-R associates with down-regulation of Ezh2, increased p16, and decreased beta cell proliferation during aging. Ablation of PDGF-R in the young mice results in impaired glucose tolerance due to decreased beta

cell replication and halved beta cell mass. Overexpressing PDGF-R in old mice induces beta cell replication, highlighting the possibility of utilizing PDGF-R in beta cell replacement therapy (H. Chen et al., 2011). However, PDGF-R is not as highly expressed in human beta cells compared to mouse beta cells, making investigating its function in the human beta cells incredibly difficult.

Circulating factors: Glucose, betatrophin

Circulating factors such as glucose, fatty acids, and betatrophin also play an important role in regulating beta cell mass expansion. One main critique of insulin acting as beta cell mitogen is that insulin deficient neonatal mice actually have larger islets than their control littermates. In fact, the *insulin*$^{-/-}$ mice have increased beta cell proliferation (Duvillie et al., 1997; Duvillie et al., 2002), indicating that insulin is dispensable for beta cell development and might actually serve as a negative regulator of beta cell replication during embryogenesis.

In contrast to the observed effects of insulin, glucose infusion has a mitotic effect for beta cells both *in vitro* and *in vivo* (Levitt et al., 2011; Yi, Park, & Melton, 2013). Although the mechanism linking glucose and beta cell replication is not fully understood, it has been suggested that glucose sensing and glucose metabolism in beta cells is the key signal stimulating beta cell replication *in vivo,* rather than glucose level *per se*. Providing evidence for this theory is the beta-cell specific *Glucokinase* (*Gck*) mutant mouse model. In these *Gck* mutant mice, the first step of glycolysis in beta cells is ablated, resulting in uncoupled hyperglycemia with glucose sensing and metabolism. *Gck*$^{+/-}$ mutant mice develop hyperglycemia following HFD feeding and fail to expand beta cell mass compared to control mice (Terauchi et al., 2007). Furthermore, beta cell specific *Gck*$^{-/-}$

mutants exhibit reduced beta cell proliferation, increased beta cell apoptosis, and severe hyperglycemia (Porat et al., 2011), suggesting that glycolytic flux mediates beta cell proliferation. This GCK-induced beta cell replication is K_{ATP} channel-dependent and calcium-dependent, ultimately causing down-regulation of cyclin D2 during S-G2-M phase (Porat et al., 2011; Salpeter et al., 2011). Glucose has also been proposed to induce beta cell proliferation via calcium and cAMP dependent activation of the PI3K/Akt pathway, which leads to down-regulation of FoxO1, menin, and p27 (Martinez, Cras-Meneur, Bernal-Mizrachi, & Permutt, 2006; H. Zhang et al., 2012), but these effects might be secondary to insulin secretion and signaling.

Although glucose infusion has a mitotic effect on beta cells, chronic hyperglycemia has vicious effects on beta cell function partially due to glucotoxicity. The mechanism of glucotoxicity is not well understood. One study suggested that glucose promotes beta cell apoptosis in synergy with FFA due to impaired IRS signaling and activated GSK3b (Tanabe et al., 2011).

In summary, the roles of insulin and glucose in regulating beta cell proliferation may not be entirely separable. Both mitogens share common intracellular pathways including PI3K/Akt and cAMP. One microarray expression profiling dataset shows that glucose and insulin treatment of beta cells results in transcriptional regulation of a common set of genes, further supporting overlapping roles of glucose and insulin in beta cell regulation (Ohsugi et al., 2004).

In a recent study, a pharmacologically induced insulin resistance mouse model reveals a potential circulating mediator of beta cell proliferation termed betatrophin (Yi et al., 2013). Expression of betatrophin is up-regulated in liver and fat in several models of

beta cell regeneration including insulin resistant mice, *ob/ob* mice, and pregnant mice. Overexpression of betatrophin in the liver induces a rapid and specific increase of beta cell proliferation in mice, suggesting that betatrophin may be secreted by peripheral tissues and responsible for adaptive beta cell proliferation (Yi et al., 2013). However, the same insulin resistance model does not trigger human beta cell proliferation after human islets are transplanted into these insulin resistant mice, producing doubts about the importance of betatrophin in human islets. It is critical to decipher how and where betatrophin acts to determine if it is acting directly on beta cells. Also, betatrophin was previously shown to regulate fatty acid metabolism and serum triglyceride (TG) levels. It is crucial to decouple the indirect effects of FFA and TG on beta cell proliferation with the potential beta cell mitogenic function of betatrophin.

4.4.4 PI3K/Akt pathway

The intracellular signaling transmitters between extracellular mitogenic signals and the cell cycle machinery and transcriptional regulation of beta cell function are well defined. Many signal transduction pathways are involved, including PI3K/Akt, MAPK/ERK, cAMP/PKA pathway, and STAT5/JAK2 pathways (Burns, Squires, & Persaud, 2000). I will focus on PI3K/Akt pathway.

The PI3K/Akt signaling cascade is a well-defined pathway regulating beta cell replication (Tarabra, Pelengaris, & Khan, 2012) (Fig 1.11). It is essential for beta cell mitogenesis induced by glucose (Srinivasan, Bernal-Mizrachi, Ohsugi, & Permutt, 2002), Ins/IGF/IRS (Lingohr et al., 2003), HGF (Vasavada, Wang, et al., 2007), and GLP-1

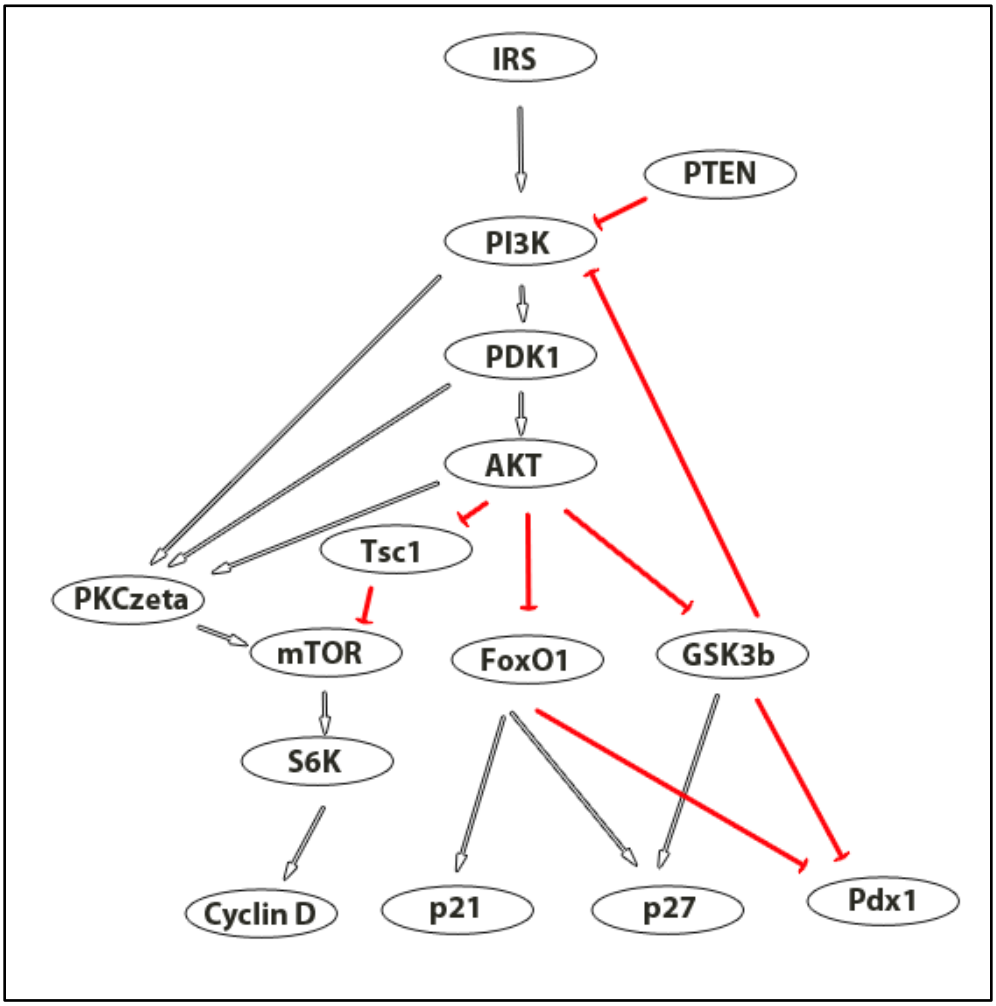


Figure 1.11 PI3K/Akt pathway. PI3K/Akt signaling cascade is one of the best-defined pathways regulating beta cell replication. It is essential for beta cell mitogenesis induced by glucose, Ins/IGF/IRS, HGF, and GLP-1. PI3K activates PDK1 which in turn activates Akt, and function through further downstream targets including mTOR, FoxO1, GSK3b, and PKCzeta.

(Cornu et al., 2010). It also plays an important role in anti-apoptosis. PI3K activates PI3K dependent protein kinase 1 (Pdk1), which in turn activates Akt, and functions through further downstream targets including mTOR, FoxO1, GSK3b, and PKCzeta. Beta cell specific ablation of *Pdk1* leads to progressive hyperglycemia as a result of reduced islet mass and decreased *Glut2* expression (Hashimoto et al., 2006). Beta cell specific ablation of phosphatase with tensin homology (PTEN), a negative regulator of PI3K, results in enhanced PI3K signaling. In turn, this model increases beta cell mass and maintains *Glut2* and *Pdx1* expression, acting to protect both HFD- and genetically-induced (*db/db*) hyperglycemic phenotypes (L. Wang et al., 2010). It is noteworthy that PTEN-ablated mice maintain normal beta cell function. In contrast, *Akt*-overexpressing mice exhibit augmented beta cell mass, compromised beta cell function (diminished relative quantity of insulin release), and hypoglycemia due to inability to autoregluate insulin secretion (Tuttle et al., 2001). This discrepancy illustrates the difference between removing a brake and adding an accelerator.

Downstream effectors of Akt in the beta cell are not fully mapped. Several Akt-controlled factors have shown critical function in both positive and negative regulation of beta cell proliferation, including FoxO1, GSK3b, and mTORC. For example, Akt down-regulates FoxO1 via phosphorylation-mediated translocation of FoxO1 from the nucleus to the cytoplasm, where it is ultimately degraded (Kitamura & Ido Kitamura, 2007). FoxO1 functions as a negative regulator of beta cell proliferation by (1) increasing expression of the cell cycle inhibitors p27 and p21 (Buteau & Accili, 2007); and (2) repressing *Pdx1* expression by competitively binding to its promoter with the *Pdx1* activator, FoxA2 (Kitamura & Ido Kitamura, 2007). Therefore, FoxO1 and Pdx1 exhibit

mutually exclusive nuclear localization in beta cells (Kitamura et al., 2002), and expansion of beta cell mass in response to insulin resistance requires nuclear exclusion of FoxO1 (Okamoto et al., 2006). Constitutively active FoxO1 in the nucleus accelerates the onset of diabetes due to impaired beta cell replication (Okamoto et al., 2006), while haplodeficiency of FoxO1 in an insulin resistance model reserves beta cell failure through partially restored beta cell proliferation and increased *Pdx1* expression (Kitamura et al., 2002).

Akt also negatively regulates GSK3b by phosphorylation, which leads to its eventual degradation (Y. Liu et al., 2010). GSK3b is suggested to form a negative feedback loop with the PI3K/Akt pathway to prevent the overshooting of the PI3K signaling. Overexpression of GSK3b in the beta cells results in impaired glucose tolerance, decreased beta cell mass, decreased beta cell proliferation, and decreased *Pdx1* expression (Z. Liu, Tanabe, Bernal-Mizrachi, & Permutt, 2008). In contrast, beta cell specific GSK3b ablation mice exhibit improved glucose tolerance, increased beta cell mass, and protection of diabetes induced by HFD (Y. Liu et al., 2010). *GSK3b*$^{-/-}$ mice have elevated IRS/PI3K/Akt protein levels and increased *Pdx1* expression, further suggesting that GSK3b forms a negative feedback loop with PI3K/Akt pathway. *In vitro* studies of human islets demonstrate that inhibition of GSK3b alone or with activated mTORC results in down-regulation of *p27* and increased beta cell proliferation (H. Liu et al., 2009; Stein, Milewski, Hara, Steiner, & Dey, 2011).

mTORC and its repressor Tsc1/2 also function downstream of PI3K/Akt, and integrate signals from nutrients and growth factors to mediate beta cell replication (Blandino-Rosano et al., 2012). mTOR is a serine/threonine kinase that is essential for

cell growth and proliferation. Akt induces mTOR/mTORC1 signaling by phosphorylating and inactivating its repressor Tsc2. mTORC1 promotes beta cell proliferation by (1) deactivating 4E-BP proteins and releasing elF4E, which leads to transcriptional up-regulation of its target genes; and (2) inducing tobosomal protein S6 kinase (S6K) and its targets. mTORC also forms feedback inhibition loops with the IRS/PI3K pathway. Overexpressing mTORC1 and inactivating Tsc2 both result in increased beta cell proliferation and beta cell mass (Balcazar et al., 2009; Rachdi et al., 2008). Overexpressing S6K in beta cells promotes more beta cells to enter the cell cycle, but there is no increase in the number of beta cells completing cell division. This discrepancy may be explained by increased p16 and p27 levels and partial inhibition of cyclin D/CDK4 and cyclin E/CDK2 (Elghazi et al., 2010). These mice also exhibit lower IRS1/2 levels and decreased p-GSK3b and p-FoxO1 by the feedback inhibition of S6K (Elghazi et al., 2010). A beta cell-specific S6K ablation mouse model will help elucidate its role in beta cell replication and function.

PKC zeta is an atypical isoform of PKC. It is found to be downstream of PI3K/PDK1 signaling and promotes cell proliferation in various cell types (Chou et al., 1998), but its role in beta cells is not well understood. GLP-1 induced beta cell replication *in vitro* is PKC zeta dependent (Buteau et al., 2001). After activation, PKC zeta translocates from the cytoplasm to the nucleus and modifies its target genes expression. Both HGF and PTHrP also induce beta cell replication in a PKC zeta dependent manner. Knockdown of PKC zeta blocks beta cell proliferation *in vitro* (Vasavada, Wang, et al., 2007), while overexpressing it increases beta cells replication. Transgenic mice with constitutively activate PKC zeta in beta cells exhibit increased beta cell proliferation, beta cell mass,

and insulin secretion (Velazquez-Garcia et al., 2011). The increased proliferation is associated with upregulation of cyclin Ds and downregulation of p21. mTOR is also suggested to be downstream of PKC zeta since rapamycin treatment abolishes the increase in beta cell proliferation and glucose tolerance in the PKC zeta overexpressing mice. Other potential targets of PKC zeta need to be investigated to fully understand its role in beta cell proliferation.

4.4.5 Transcription factors

In addition to kinases, transcription factors also play important roles as intracellular proteins that translate external cues into cell intrinsic responses. FoxM1 is a proliferation-specific member of the Fox family of transcription factors (H. Zhang et al., 2006). Pancreas-specific *FoxM1* ablation induces overt diabetes in male mice at 9 weeks of age (H. Zhang et al., 2006). In female mice, FoxM1 is up-regulated in islets during pregnancy, and ablation of *FoxM1* causes gestational diabetes due to the resulting deficiency in beta cell replication (H. Zhang et al., 2010). FoxM1 is also required for beta cell proliferation after partial pancreatectomy and obesity-induced beta cell mass expansion (Ackermann Misfeldt, Costa, & Gannon, 2008; Davis et al., 2010). Targets of FoxM1 include cyclin D for G_1/S phase, and CDK1 for G_2/M phase (Davis et al., 2010). But how FoxM1 regulates beta cell mass expansion under different physiological stimuli is not fully understood.

Hnf4α is one of the MODY genes. Mutations of *Hnf4α* in humans give rise to MODY1, which is characterized by impaired insulin secretion. Beta cell specific conditional ablation of *Hnf4α* in mice results in impaired glucose tolerance during pregnancy. The beta cell mass of mutant mice is approximately half of that of the control

mice due to decreased beta cell replication (Gupta et al., 2007). A direct transcriptional target of HNF4*α*, ST5, is suggested to link HNF4*α* to the ERK pathway to mediate beta cell replication. However, although overexpression of *Hnf4α* in the human islets increases cell cycle entry 300 folds, it is not sufficient for completion of the cell cycle due to DNA damage response and loss of beta cell lineage fidelity (Rieck et al., 2012).

Carbohydrate response element-binding protein (ChREBP) is a lipogenic glucose-sensing transcription factor. ChREBP translocates into the nucleus upon glucose stimulation and up-regulates fatty acid synthesis genes in a glucose-dependent manner. Although chREBP is known to play a lipogenic role in the liver, its role in beta cells has only been partially elucidated. ChREBP mutant mouse islets exhibit decreased glucose-stimulated proliferation and decreased expression of cell cycle accelerators, while overexpressing chREBP amplifies glucose-stimulated proliferation in rat and human beta cells (Metukuri et al., 2012). This study suggests that chREBP mediates glucose-stimulated beta cell proliferation; although how chREBP is regulated by glucose and how chREBP controls cell cycle machinery need more investigation.

The calcineurin/Nuclear Factor of Activated T cells (cn/NFAT) pathway regulates gene transcription to mediate proliferation, survival, and differentiation in various cell types (Wu, Peisley, Graef, & Crabtree, 2007). 10-30% of patients administrated with immunosuppressant drugs such as tacrolimus, a calcineurin inhibitor, develop diabetes, suggesting that calcineurin signaling is essential for adult beta cell function (Goodyer et al., 2012). A beta-cell specific ablation of the calcineurin subunit, calcineurin b1 (Cnb1) in mice results in age-dependent diabetes characterized by decreased beta cell replication and mass (Heit, Apelqvist, et al., 2006). This phenotype is accompanied by decreased

expression of essential beta cell factors, such as *Ins1*, *Pdx1*, and *Glut2*. Cell cycle regulators are also down-regulated, including *cyclin D2* (Heit, Apelqvist, et al., 2006), which may explain the decreased beta cell proliferation in the mutant mice. Interestingly, overexpressing NFAT is sufficient to induce beta cell proliferation and beta cell mass expansion *in vivo* (Heit, Apelqvist, et al., 2006). Later on, an Ngn3-Cre driven deletion of *Cnb1* reveals that cn/NFAT is required for neonatal beta cell replication as well. Neonatal mutant mice exhibit a 7-fold decrease in beta cell mass and a 4-fold decrease of beta cell proliferation at 26 days old (Goodyer et al., 2012). Cell cycle regulators cyclin A2, D2 and FoxM1 are direct targets of NFAT by ChIP assays on islets. Pathways that mediate cn/NFAT signaling are not well understood and require further investigation (Heit, 2007).

4.5 Reversible maternal beta cell mass expansion during pregnancy

4.5.1 Insulin resistance during pregnancy

During normal pregnancy, a complex endocrine-metabolic adaptation process occurs, which includes progressive insulin resistance, moderate increases in blood glucose levels, and changes in circulating FFA, TG, and cholesterol levels (Di Cianni, Miccoli, Volpe, Lencioni, & Del Prato, 2003). Insulin resistance begins around mid-gestation and progresses throughout the third trimester. Regardless of initial condition, during the third trimester insulin resistance reaches levels similar to those of T2DM patients (Buchanan & Xiang, 2005; Di Cianni et al., 2003). There is an estimated 47% reduction in insulin sensitivity in obese women and a 56% reduction of insulin sensitivity in lean women in the 3rd trimester of gestation. This insulin resistance results from a combination of increased maternal adiposity and insulin desensitization, induced by multiple hormones

and cytokines. Potential contributing factors include (1) progesterone, which causes glucose intolerance by impairing insulin-suppressed hepatic gluconeogenesis; (2) glucocorticoids, which in excess, impair insulin receptor phosphorylation and cause insulin resistance; (3) leptin, which is encoded by the *obese* (*ob*) gene, and secreted by the adipose tissue, and (4) lactogens (Lain & Catalano, 2007).

Gestational insulin resistance is not a pathological condition; rather, it presents a necessary adaptation to meet the energy demand of continually feeding and developing fetus (Burdge, Hanson, Slater-Jefferies, & Lillycrop, 2007; Lehnen, Zechner, & Haaf, 2013; Reece, Leguizamon, & Wiznitzer, 2009). The molecular mechanism of gestational insulin resistance involves impaired glucose transport and intracellular glucose metabolism in the skeletal muscle, and to a lesser extent, adipose tissue. Although it is normal to develop some degree of insulin resistance during pregnancy, these symptoms can worsen in pregnant women diagnosed with GDM. For example, phosphorylation of IRS1 is reduced in women with GDM by 41%, compared to a 28% reduction during a normal pregnancy. GLUT4 in adipose tissue is also reduced more in GDM subjects compared to normal pregnant women, indicating impaired insulin signaling in the periphery tissue. The gestational insulin resistance rapidly resolves after delivery.

4.5.2 Beta cell proliferation during pregnancy

In order to meet the higher demand of insulin secretion to maintain normal glucose homeostasis, beta cells mass increases 2-4 fold during pregnancy in rodents (Sorenson & Brelje, 1997), and 1.5-2 fold in humans (Butler et al., 2010). The change in islet size largely results from dramatically increased beta cell proliferation and beta cell hypertrophy, which provides a perfect example of the plasticity of beta cells.

Furthermore, to compensate for increased insulin resistance during pregnancy, beta cell function improves both by lowering the glucose-stimulated insulin secretion threshold and by increasing first phase insulin secretion. Multiple hormones help modify insulin secretion, including estrogens, progesterone, and glucocorticoids(Di Cianni et al., 2003). The peak of beta cell proliferation occurs about two-thirds into the gestation period, which roughly correlates with the peak of placental lactogen (PL), causing many researchers to speculate that PL regulates beta cell proliferation during pregnancy (Fig 1.12).

4.5.3 Molecular mechanisms for beta cell proliferation during pregnancy

β-cell proliferation during pregnancy is regulated by many factors, and has been only partly elucidated through previous studies (Rieck & Kaestner, 2010; Sorenson & Brelje, 1997). One of the most important hormonal factors during pregnancy is lactogen. There are two kinds of lactogens in mice and humans: prolactin (Prl) and placental lactogen(PL), both of which are induced during pregnancy. In mice, Prl peaks at day 6 of gestation and decreases when PL levels begin to rise. PL is expressed from approximately day 8 of gestation and peaks around day 12 during pregnancy (Brelje et al., 1993a). Both Prl and PL share a common receptor, prolactin receptor (Prl-R), which is a member of the cytokine receptor superfamily (Brelje et al., 1993a). Lactogens increase beta cell proliferation and insulin secretion both *in vitro* and *in vivo* (Fujinaka, Takane, Yamashita, & Vasavada, 2007). In fact, the onset of PL coincides with the earliest detection of increased beta cell proliferation during pregnancy, and culturing both human and rodent islets in medium containing PL induces beta cell proliferation and enhanced insulin

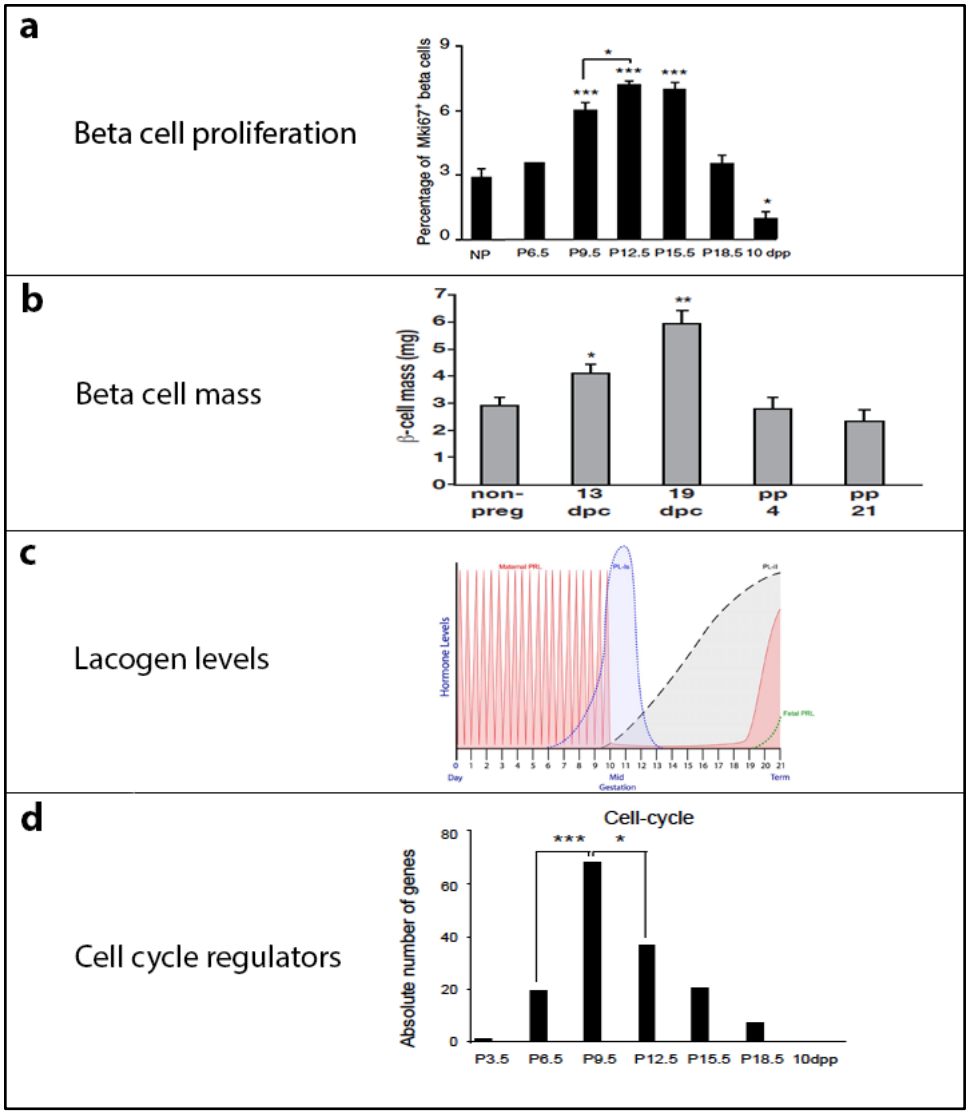

a
Beta cell proliferation
Percentage of Mki67+ beta cells
NP
P6.5
P9.5
P12.5
P15.5
P18.5
10 dpp
b
Beta cell mass
β-cell mass (mg)
non-preg
13 dpc
19 dpc
pp 4
pp 21
c
Lacogen levels
Hormone Levels
Day
Mid Gestation
Term
d
Cell-cycle
Absolute number of genes
P3.5
P6.5
P9.5
P12.5
P15.5
P18.5
10dpp

Figure1.12 (Adapted from (Karnik et al., 2007; Schraenen, de Faudeur, et al., 2010; Soares, 2004)) Beta cell proliferation and lactogen dynamics during pregnancy. a) Beta cell proliferation during pregnancy peaks at around 12 to 15 days during gestation, which rapidly decreases to pre-pregnancy level after delivery. b) Beta cell mass increases 2-4 times during pregnancy in rodents due to both beta cell proliferation and beta cell hypertrophy. It rapidly decreases to pre-pregnancy stage several days after delivery due to beta cell apoptosis. c) Prolactin exhibits a twice-daily surge during early pregnancy, produced by the maternal anterior pituitary. During mid-gestation, placental lactogen-I peaks at around 11 days of pregnancy. During the second half of gestation, placental lactogen-II dominates. The fetal pituitary begins producing prolactin at the end of pregnancy. d) Time-dependent changes in the number of up-regulated cell cycle regulator genes during pregnancy. Most of the cell cycle regulator genes peak at around 9.5 days of pregnancy, when beta cell proliferation begins to increase.

secretion (Brelje et al., 1993a). A transgenic mouse line overexpressing *Prl* exhibits protection against streptozotocin-induced beta cell specific injury via increased beta cell proliferation and hypertrophy (Fujinaka et al., 2007). Furthermore, *Prl-R* ablation mice show a 25-40% reduction in beta cell mass at non-pregnant stage (Freemark et al., 2002). Since *Prl-R*$^{-/-}$ females are sterile, *Prl-R* heterozygous mutants (*Prl-R*$^{+/-}$) are studied during pregnancy. *Prl-R*$^{+/-}$ pregnant mice exhibit decreased beta cell proliferation and impaired glucose tolerance. All of these studies validate the critical role of lactogen signaling in beta cell proliferation and function during gestation (Huang, Snider, & Cross, 2009).

Prl-R, like other cytokine receptors, acts through a complex signaling network, including IRSs/PI3K, MAP kinase, and most importantly, Janus kinase 2/signal transducer and activator of transcription 5 (JAK2/STAT5) (Amaral et al., 2004). Activation of the JAK2 kinase by Prl-R in turn phosphorylates Prl-R at specific tyrosine residues. STAT5 proteins are recruited to these phosphorylated tyrosine residues on Prl-R and are also phosphorylated by JAK2. Phosphorylated STAT5 then dimerizes and translocates into the nucleus, where it regulates gene expression as a transcription factor (Amaral et al., 2004). STAT5 is critical for several cytokine signaling pathways, including Prl/PL, growth hormone, IL2, and IL3 (X. Liu et al., 1997). STAT5 phosphorylation and nuclear translocation is up-regulated in islets during pregnancy by Prl/PL signaling (Friedrichsen et al., 2003). An *in vitro* study shows that STAT5 is sufficient to drive proliferation of rat beta cells by activating the expression of *Cyclin D2* (Friedrichsen et al., 2003). Furthermore, *Stat5a* ablation mice demonstrate deficiency in both mammary gland development and lactogenesis due to loss of prolactin signaling (X. Liu et al., 1997). Beta cell specific dominant-negative STAT5 mice display higher blood

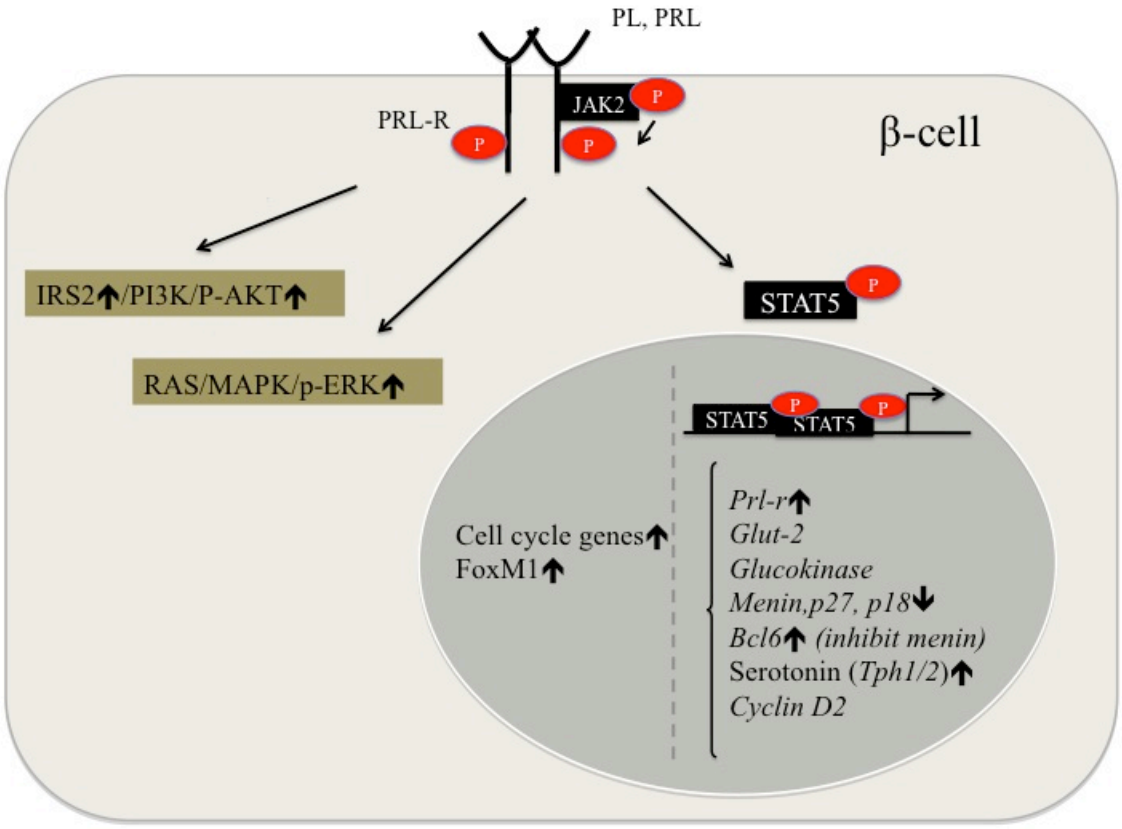


Figure 1.13 (Adapted from (Jiao, Rieck, Le Lay, & Kaestner, 2013)) Molecular mechanisms for beta cell proliferation during pregnancy. Latogens are key hormones regulating beta cell proliferation during pregnancy. After PL/PRL binds to PRL-R, the receptor dimerizes and triggers downstream signaling, including PI3K/Akt pathway, MAPK/ERK pathway, and JAK2/STAT5 pathway. STAT5 is recruited to phosphorylated PRL-R and is phosphorylated in turn by JAK2. Phosphorylated STAT5 then dimerizes and translocates into the nucleus to regulate gene expression as a transcription factor. A group of known targets of STAT5 are up-regulated during pregnancy, including *Prl-r, Glut-2, Glucokinase* and *Cyclin D2*, resulting in increased beta cell proliferation and insulin secretion.

glucose levels and lower beta cell proliferation rates compared to control mice after six months on HFD, which establishes STAT5 as a critical factor for beta cell proliferation and function *in vivo* (Jackerott et al., 2006).

Some targets of STAT5, such as menin and p27/p18, are down-regulated during pregnancy to allow beta cell proliferation (Hughes & Huang, 2011). In contrast, JAK2/STAT5 up-regulates anti-apoptotic protein Bcl-XL to promote beta cell survival during pregnancy (Fujinaka et al., 2007). *Cyclin D2* is a STAT5 target gene that leads to beta cell proliferation *in vitro* (Friedrichsen et al., 2003). Serotonin levels are also increased in beta cells during pregnancy in a JAK2/STAT5-dependent way, but its biological role needs to be further investigated (Schraenen, Lemaire, et al., 2010). Other regulators, such as FoxM1, are up-regulated in maternal islets during pregnancy. Deficiency in *FoxM1* results in decreased beta cell proliferation and GDM (H. Zhang et al., 2010). Glucokinase activity is also upregulated in the beta cells during pregnancy by prolactin, which partially explains the increased glucose-stimulated insulin secretion during pregnancy (Weinhaus, Stout, Bhagroo, Brelje, & Sorenson, 2007). Interestingly, previous study in our lab demonstrated that *Cytokine-induced SH2 domain-containing (Cish)* and *Suppressor of the cytokine signaling 2* (*Socs2)*, two targets and negative regulators of STAT5 signaling, are also upregulated during pregnancy.

mRNA expression analysis of cell cycle genes in islets of pregnant mice reveals an orchestrated peak of cell cycle genes at P9.5, after which beta cell replication increases, and peaks at between P12.5 and P14.5 (Schraenen, de Faudeur, et al., 2010). How this sharp peak of cyclin genes regulates a sustained cell replication peak for nearly a week is not fully understood.

5. Summary and specific aims

Previous studies have established beta cell proliferation as the main mechanism of maintaining beta cell mass during adulthood in rodents. The regenerative capacity of beta cells has been demonstrated in multiple experimental schemes including HFD, pregnancy, and pancreas injury. The extracellular mitogens and intrinsic cell cycle regulators and signaling pathways that regulate beta cell proliferation have been intensively investigated for better understanding the mechanisms underlying beta cell replication. The ultimate goal is to provide an unlimited source of beta cells for the regenerative therapy strategy for diabetes.

This dissertation describes our effort to investigate the function of several potential beta cell replication regulators in different models stimulating beta cell replication. Chapter 2 presents the data to answer the question of whether CISH and SOCS2 negatively regulate beta cell replication during pregnancy. Lactogens are the main hormone factor stimulating beta cell proliferation during pregnancy. STAT5 is the key downstream effector of lactogen signaling. CISH and SOCS2 are two negative regulators of STAT5 signaling, composing a negative feedback loop of the lactogen/STAT5 signaling. Expressions of *Cish* and *Socs2* are upregulated during pregnancy. Therefore, we hypothesized that ablation of *Cish* and/or *Socs2* may result in upregulated STAT5 signaling and increased beta cell proliferation during pregnancy. Chapter 3 describes results regarding the role of HNF-4α in regulating beta cell proliferation during obesity. Previous studies in our lab demonstrate that HNF-4α is required for beta cell replication during pregnancy. We hypothesized that HNF-4α is also required for beta cell proliferation during obesity, which is another insulin-resistant model and is more relevant

to human disease. Chapter 4 presents our progress in investigating the role of betatrophin on human beta cell replication. Betatrophin has been suggested to be a potent beta cell mitogen in mouse. We are curious about its role in human beta cells. Conclusions, implications and future directions for this dissertation are presented in Chapter 5. In addition, Appendices describes my effort to characterize epigenetic profiles for beta cell development during embryogenesis by performing FOXA2 and NKX6.1 chromatin immunoprecipitation on $Ngn3^{+}$ endocrine progenitor cells.

Chapter 2 CISH has no non-redundant functions in glucose homeostasis or beta cell proliferation during pregnancy in mice

Abstract

Beta cell proliferation increases during pregnancy in response to increased lactogen levels. This response is mediated by the Janus kinase 2/signal transducer and activator of transcription 5 (JAK2/STAT5) signaling pathway. Activation of the JAK2/STAT5 pathway leads to transcriptional up-regulation of *Cytokine-induced SH2 domain-containing (Cish)* and *Suppressor of the cytokine signaling 2* (*Socs2)*, two members of the "Suppressor of cytokine signaling" (SOCS) family of genes, which form a negative feedback loop. It is not known whether CISH and SOCS2 play key roles in regulating beta cell proliferation or beta cell function during pregnancy. Here, I examined whether conditional gene ablation of *Cish* in the mouse pancreas improved beta cell proliferation and beta cell function during pregnancy. Beta cell proliferation was quantified by Bromodeoxyuridine (BrdU) labeling. Glucose homeostasis was examined by glucose tolerance tests (GTT) and plasma insulin level measurements. Q-PCR was utilized to determine mRNA expressions of other *Socs* genes and target genes of p-STAT5 related to beta cell function and beta cell proliferation. There are no differences in beta cell proliferation or glucose homeostasis between the *Cish* mutant group and the control group. p-STAT5 protein levels are the same in the *Cish* mutant mice and the control mice. *Socs2* gene expression is higher in islets of the *Cish* mutant mice than control mice at pregnant day 9.5. Other *Socs* genes, *Socs3*-*Socs7*, are expressed at the same levels in control and mutant mice. Global $Socs2^{-/-}$ mice were also examined, and they exhibit increased beta cell mass during pregnancy. In summary, my results suggest that CISH has no non-redundant functions in beta cell proliferation or glucose homeostasis during pregnancy. *Socs2* might be compensating for the loss of *Cish* during pregnancy.

Introduction

An insufficient number of insulin-producing beta cells is a hallmark of both type 1 and type 2 diabetes. Therefore, one therapeutic strategy is to increase functional beta cell mass in order to overcome insulin deficiency (Bonner-Weir & Weir, 2005b). The majority of postnatal beta cell mass expansion in rodents is caused primarily by replication of preexisting beta cells, rather than by differentiation from progenitors or other cell types, thus making the pathways regulating beta cell proliferation an area of acute interest for research (Melton, Dor, Brown, & Martinez, 2004). However, one practical issue is that β-cells have a relatively long lifespan with very low proliferate rate (<1%), making accessing proliferating beta cells impractical (Bonner-Weir, 2001; Kaestner et al., 2007). However, beta cells have the capacity to expand by proliferation when metabolically challenged, such as during pregnancy, diet-induced insulin resistance, and experimental beta cell ablation, as shown in rodent models (Heit, Karnik, & Kim, 2006b).

Beta cell proliferation during pregnancy is regulated by many factors, chiefly the hormone signaling lactogens (placental lactogen (PL) and prolactin (PRL)) (Kaestner et al., 2009; Parsons, Brelje, & Sorenson, 1992). The essential mediator of lactogen signaling is the JAK2/STAT5 pathway (Boschero et al., 2004). Many known STAT5 target genes are up-regulated during pregnancy, including *PRL-R*, which forms a positive feedback loop with the PRL/PL signaling pathway (Fleenor & Freemark, 2001); *Glucose transporter-2* (*Glut-2*), which transports glucose into beta cells; and *cyclin D2*, which drives beta cell proliferation (Fleenor & Freemark, 2001; Galsgaard, Nielsen, & Moldrup, 1999; Glaser et al., 2011; Solimena et al., 2006; Sorenson, Weinhaus, Stout, Bhagroo, &

Brelje, 2007). As an apparent limit to unbridled replication, two of the negative feedback regulators of PRL/PL signaling, *Cish* and *Suppressors of the Cytokine Signaling 2* (*Socs2*), are also up-regulated during pregnancy (Kaestner et al., 2009; Laz, Sugathan, & Waxman, 2009; Matsumoto et al., 1997).

The "Suppressors of the Cytokine Signaling (SOCS)" gene family is composed of eight genes, all of which have similar protein structure and function as inhibitors of cytokine signaling (Dalpke, Heeg, Bartz, & Baetz, 2008). All family members, including CISH and SOCS2, contain a central SH2 domain for binding to phosphorylated tyrosines and a C-terminal 'SOCS-box' domain for directing targeted proteins to proteasomal degradation (Fig 2.1). SOCS1 and SOCS3 also contain an N-terminal kinase inhibitory region (KIR) domain, which is responsible for inhibition of cytokine receptor-associated JAKs (Dalpke et al., 2008). Cytokines are important mediators of multiple physiological responses, including survival, proliferation, differentiation and functional activities of immune cells and other cell types (Trengove & Ward, 2013). Cytokines and growth hormones bind to their specific receptors, activate JAKs, which in turn phosphorylate the receptors. Phosphorylated receptors provide intracellular
docking sites for SH2-containing signaling proteins, including STATs and SOCS proteins. For example, IFN-g activates JAK1/2, which mainly function through STAT1, whereas PRL/PL primarily activates JAK2 and STAT5 (Yoshimura, Nishinakamura, Matsumura, & Hanada, 2005). SOCS proteins serve as negative regulators for cytokine signaling to prevent sustained and/or excessive cytokine signaling, which can be harmful to organismal health.

SOCS proteins can be grouped into two categories reflecting their evolutionary

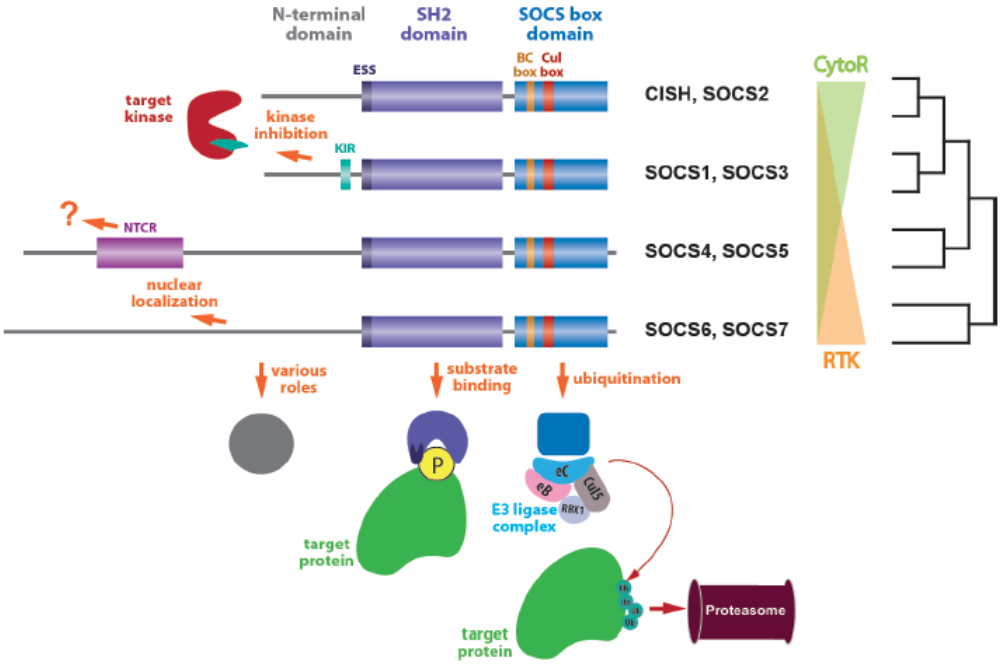


Fig. 2.1 (Adapted from (Trengove & Ward, 2013)) Structure and sub-grouping of SOCS proteins. There are eight mammalian SOCS family members; SOCS1-7 and CISH. SOCS1-3 and CISH are most often associated with regulation of cytokine receptor signaling through the JAK/STAT pathway, while SOCS4-7 predominantly regulate growth factor receptor signaling. Each SOCS protein contains three distinct domains: an N-terminal domain of low conservation, a conserved central SH2 domain, and a more highly conserved C-terminal SOCS box domain. The SH2 domain interacts with phosphorylated tyrosine residue on target proteins, including cell surface receptors. The SOCS box is comprised of two functional sub-domains; a BC box that recruits Elongin B and C, and a Cul box that mediates Cullin5 binding. Together, the BC box and Cul box activities lead to recruitment of the remaining components of an E3 ubiquitin ligase complex, and direct target protein degradation. CytoR: Cytokine receptor; RTK: Receptor tyrosine kinase; KIR: kinase inhibitory region; NTCR: N-terminal conserved region; ESS: extended SH2 sequence; eB: Elongin B; eC: Elongin C

history and signaling pathway preferences. CISH and SOCS1-3 are most often associated with cytokine receptor signaling through the JAK/STAT pathway, while SOCS4-7 primarily regulate growth factor receptor signaling through receptor tyrosine kinase (Trengove & Ward, 2013) (Fig 2.1). Of the first group, CISH and SOCS1-3 inhibit JAK/STAT signaling by various mechanisms. For example, SOCS1 binds to JAK2 and inhibits its phosphorylation of cytokine receptors and STAT5. SOCS2 and SOCS3 bind to phosphorylated tyrosine sites on growth-hormone receptors, which might also be STAT5-binding sites, thus inhibiting the activation of STAT5. In addition, CISH binds to the phosphorylated tyrosine site of PRL-R, which also results in blocking of STAT5 recruitment. Furthermore, all SOCS proteins may promote proteasomal degradation of their targets (Ahmed & Farquharson, 2010; Endo, Sasaki, Minoguchi, Joo, & Yoshimura, 2003; Ronn, Billestrup, & Mandrup-Poulsen, 2007).

Since SOCS proteins are responsible for a wide range of cytokine and growth hormone signaling repression, different *Socs* gene ablation mouse models and *Socs* transgenic mice have been generated. These mice exhibit various phenotypes depending on which particular cytokine signal is altered by *Socs* gene mutation or overexpression (Dalpke et al., 2008). For example, global *Socs2*$^{-/-}$ mice exhibit gigantism, with a 30-40% increase in body weight and proportional increase in bones and other organs, indicating an essential role for SOCS2 in negatively regulating growth hormone signaling (Alexander et al., 2000). SOCS2 has also been indicated to regulate insulin secretion. Mice with beta cell-specific overexpression of *Socs2* exhibit impaired insulin secretion and hyperglycemia (Lebrun et al., 2010). *Socs3* overexpressing mice have a 30% reduction in beta cell mass. Furthermore, islets from these mice exhibit reduced p-STAT5

levels in response to growth hormone *in vitro*, indicating negative regulation of SOCS3 on the STAT5 signaling pathway in islets (Lindberg K, 2005).

CISH was identified as an immediate-early gene induced in hematopoietic cells in response to cytokines such as erythropoietin (EPO), interleukin-2 (IL-2), and IL-3 (Yoshimura et al., 1995). Subsequent studies have revealed that *Cish* is also induced by growth hormone, prolactin (PRL), IL-9, and granulocyte colony-stimulating factor (G-CSF), each of which stimulates STAT5 (Helman et al., 1998; Hunter et al., 2004; Lejeune, Demoulin, & Renauld, 2001). *Stat5a/b*$^{-/-}$ mice have no detectable *Cish* expression, indicating the key role of STAT5 in *Cish* gene regulation (Moriggl et al., 1999; Teglund et al., 1998). Indeed, *Cish* has been shown to be a direct target gene of STAT5 (Moriggl et al., 2005; Yoshimura et al., 1995). CISH is known to negatively regulate signaling induced by EPO, GH, IL2, IL-3, and PRL (Aman et al., 1999; Dif, Saunier, Demeneix, Kelly, & Edery, 2001; Matsumoto et al., 1997; Pezet, Favre, Kelly, & Edery, 1999; Ram & Waxman, 1999; Yoshimura et al., 1995). CISH suppresses signaling by binding via its SH2 domain to phosphorylated tyrosine residues of cytokine receptors, leading to either a block to STAT5 docking or degradation of cytokine receptors (Hanada et al., 2003; J. A. Hansen, Lindberg, Hilton, Nielsen, & Billestrup, 1999; Piessevaux, De Ceuninck, Catteeuw, Peelman, & Tavernier, 2008; Ram & Waxman, 1999; Verdier et al., 1998). *Cish* transgenic mice exhibit impaired STAT5 signaling in liver, mammary glands and T cells (Matsumoto et al., 1999). However, the function of CISH and SOCS2 in regulating lactogen-dependent JAK2/STAT5 signaling in beta cells, as well as their potential function in beta cell proliferation during pregnancy, has not been previously studied.

Since lactogen-JAK2/STAT5 signaling is critical for beta cell proliferation and beta cell function during pregnancy, and *Cish*/*Socs2* are induced during pregnancy (Kaestner et al., 2009), I hypothesized that these two SOCS proteins negatively regulate beta cell proliferation and beta cell function by suppressing JAK2/STAT5 signaling. In order to test this hypothesis, I used a novel mouse model derived in our lab with conditional ablation of the *Cish* gene in the pancreas. I also investigated the role of SOCS2 in beta cell proliferation using global *Socs2*$^{-/-}$ mice.

Results

Derivation of pancreas-specific *Cish* ablation mice

A novel conditional allele of *Cish* was constructed by inserting two loxP sites flanking exon 2 of the *Cish* gene, which encodes the tyrosine binding SH2 domain. Deletion of exon 2 of the *Cish* gene results in a frameshift mutation and no CISH protein would be produced. Pancreas-specific *Cish*$^{-/-}$ mice were derived by breeding *Cish*$^{loxP/loxP}$ mice with *Pdx1-Cre*^{Early} transgenic mice (Gu, Dubauskaite, & Melton, 2002), in which expression of the Cre recombinase is driven by the *Pdx1* promoter (Fig. 2.2a). The resulting *Cish*$^{loxP/+}$; *Pdx1-Cre*^{Early} mice were bred to *Cish*$^{loxP/loxP}$ mice to obtain *Cish*$^{loxP/loxP}$*; Pdx1-Cre*^{Early} mutant mice and *Cish*$^{loxP/loxP}$ control mice. *Pdx1-Cre*^{Early} expression starts at approximately embryonic day 8.5 in all pancreatic progenitor cells. Therefore, *Cish* is ablated in the whole pancreas in *Cish*$^{loxP/loxP}$; *Pdx1-Cre*^{Early} mutant mice. PCR primers for differentiating *Cish* loxP allele and *Cish* wild type allele were designed upstream of the single loxP site and within exon 3 of the *Cish* gene, respectively (Fig. 2.2a). The primers amplify a 250bp product for the wild type allele and a 388bp

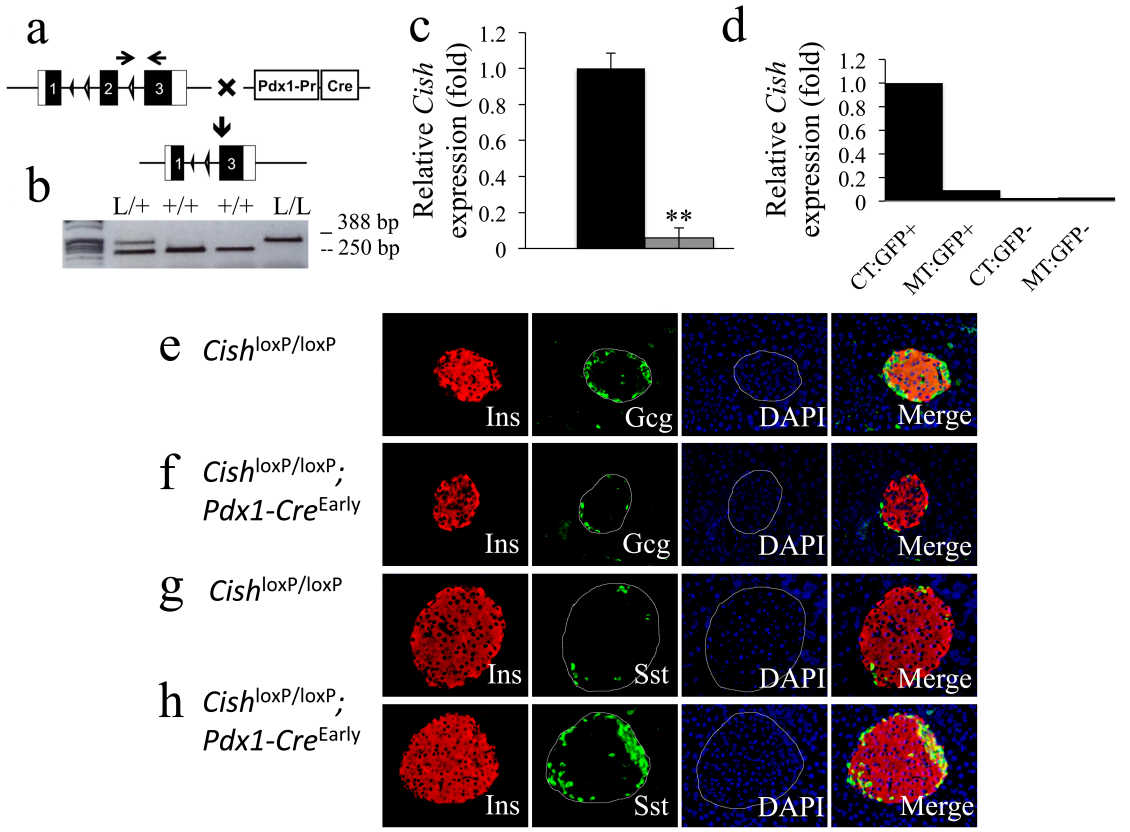


Fig. 2.2 *Cish* ablation in pancreatic islets. **a** *Cish*^{loxP} mice were crossed with *Pdx1-Cre*^{Early} mice to induce *Cish* ablation in the pancreas. PCR primers for genotyping of the *Cish* allele were designed upstream of the single loxP site and within exon 3 of the *Cish* gene, indicated by arrows. **b** Genotying for *Cish* alleles. The primers amplify a 250bp product in the wild type allele and a 388bp product in the loxP allele. **c** Quantitative RT-PCR showed dramatic reduction of *Cish* mRNA in *Cish*$^{loxP/loxP}$*; Pdx1-Cre*^{Early} islets. **p<0.01 (t-test). Black bars: *Cish*$^{loxP/loxP}$ controls; grey bars: *Cish*$^{loxP/loxP}$*; Pdx1-Cre*^{Early} mutants (n=5-6). **d** Quantitative RT-PCR showed enriched *Cish* mRNA expression in the sorted beta cells from virgin *Cish*$^{loxP/loxP}$*; Mip-GFP* (CT) mice and virgin *Cish*$^{loxP/loxP}$*; Mip-GFP ; Pdx1-Cre*^{Early} (MT) mice. GFP+ indicates the sorted GFP positive beta cells. GFP- indicates the sorted GFP negative islet non-beta islet cells. Pancreas sections from mice of indicated genotypes were co-stained with antibodies against insulin (red) and glucagon (green) (**e,f**), or alternatively, antibodies against insulin(red) and somatostatin (green) (**g,h**). All mice were sacrificed on day 14.5 of pregnancy except when virgin female mice were used (d). Ins: insulin; Gcg: glucagon; Sst: Somatostatin.

product for the loxP allele (Fig. 2.2b).

To evaluate the efficiency of *Cish* gene ablation, *Cish* mRNA expression levels in mouse islets were determined by qPCR using primers designed within exon 2 of the *Cish* gene. *Cish* mRNA levels were reduced by more than 90% in pancreatic islets isolated from $Cish^{loxP/loxP}$; $Pdx1\text{-}Cre^{Early}$ mice compared to islets from control $Cish^{loxP/loxP}$ mice (Fig. 2.2c). In order to ascertain that *Cish* was ablated in beta cells without a reliable CISH antibody, $Cish^{loxP/loxP}$; $Pdx1\text{-}Cre^{Early}$ mice were bred to Mip-GFP mice to allow for efficient fluorescence-activated cell sorting (FACS) of beta cells. Analysis of *Cish* mRNA levels in sorted islet cells provided new knowledge. First, in islets of control mice, *Cish* mRNA was highly enriched in beta cells (GFP^+ cells from $Cish^{loxP/loxP}$; Mip-GFP mice) compared to islet non-beta cells (GFP^- cells from $Cish^{loxP/loxP}$; Mip-GFP mice). Second, *Cish* mRNA expression was efficiently ablated in mutant beta cells (GFP^+ cells from $Cish^{loxP/loxP}$; $Pdx1\text{-}Cre^{Early}$ mice) relative to control beta cells (GFP^+ cells from $Cish^{loxP/loxP}$ mice) (Fig. 2.2d). Furthermore, mutant islets maintained normal morphology and hormone expression as shown by co-staining for insulin, glucagon, and somatostatin (Fig. 2.2 e-h). In summary, CISH is not required for maintenance of normal islet architecture.

CISH is not required for beta cell proliferation or glucose homeostasis in mice

Having established a mouse model for pancreas-specific ablation of *Cish*, I proceeded to investigate whether *Cish* ablation affects beta cell mass and/or glucose homeostasis. I found no differences in beta cell mass, body weight, resting insulin, fasting insulin, or glucose tolerance between control virgin mice and mutant virgin mice (Fig. 2.3a-d), although *Cish* was ablated in 90% of islet cells in these mice (Fig. 2.3e).

Therefore, CISH is not required for beta cell homeostasis or proliferation in non-pregnant mice. This result was expected because expression of *Cish* is upregulated during pregnancy, when we hypothesize that CISH functions as key repressor for PRL signaling to prevent overshooting of cell cycle entry. In other words, *Cish* is relatively lowly expressed under normal physiological condition, possibly with minimal functional activity. Therefore, deleting *Cish* in virgin mice did not have any effect on beta cell function or glucose homeostasis.

Next, I proceeded to investigate whether *Cish* ablation augmented beta cell proliferation during pregnancy, which was my original hypothesis. In order to measure beta cell proliferation, 24 hours prior to being sacrificed, day 13.5 pregnant mice were injected with bromodeoxyuridine (BrdU) intraperitoneally to label proliferating cells during S-phase. Immunofluorescence staining was performed on pancreas sections of control mice (n=9) and mutant mice (n=9). Since pregnancy is a robust model to induce beta cell proliferation, $BrdU^{+}$ proliferating beta cells were easily detected in islets (Fig. 2.4a-b). For each animal, more than 15 islets, or 1,000 beta cells, were quantified manually. The proliferation rate was about 1.5% in both $Cish^{loxP/loxP}$ mice and $Cish^{loxP/loxP}$*; Pdx1-Cre*^{Early} mice (Fig. 2.4c). Thus, *Cish* deficiency in beta cells is not sufficient to increase beta cell DNA replication during pregnancy. There also was no difference in beta cell mass between control and mutant mice, either (Fig. 2.4d).

Although I detected no difference in the rate of beta cell proliferation in *Cish*-deficient mice, it was possible that beta cell function was enhanced without affecting proliferation. To address this question, glucose tolerance tests (GTT) were performed on eleven $Cish^{loxP/loxP}$*; Pdx1-Cre*^{Early} mice and eleven $Cish^{loxP/loxP}$ mice, all on day 14.5 of

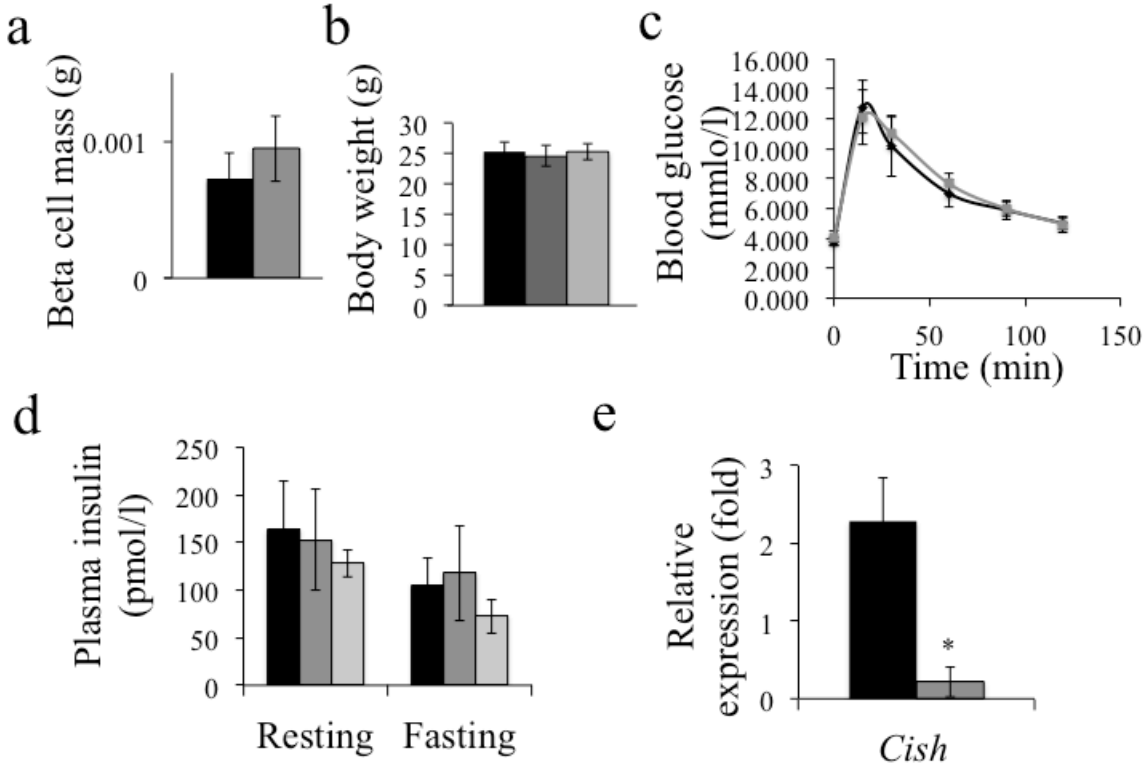


Fig 2.3 CISH is not required for beta cell proliferation or beta cell function in virgin mice. **a-d** Beta cell mass, body weight, glucose tolerance test, and resting and fasting plasma insulin levels of virgin mice. No difference was observed between control and mutant groups (t-test for **a**,**b**,**d**; MANOVA for **c**). **e** *Cish* mRNA levels are dramatically reduced in non-pregnant mutant mice (t-test, * $p<0.05$). Error bars show standard error of the mean. *Cish*$^{loxP/loxP}$ mice and *Pdx1-Cre*^{Early} mice were used as controls. **a**,**c**,**e** Black bars: *Cish*$^{loxP/loxP}$ controls; grey bars: *Cish*$^{loxP/loxP}$*; Pdx1-Cre*^{Early} mutants. **b**,**d** Black bars: *Cish*$^{loxP/loxP}$ controls; dark grey bars: *Pdx1-Cre*^{Early} controls; light grey bars: *Cish*$^{loxP/loxP}$*; Pdx1-Cre*^{Early} mutants (n=3-6).

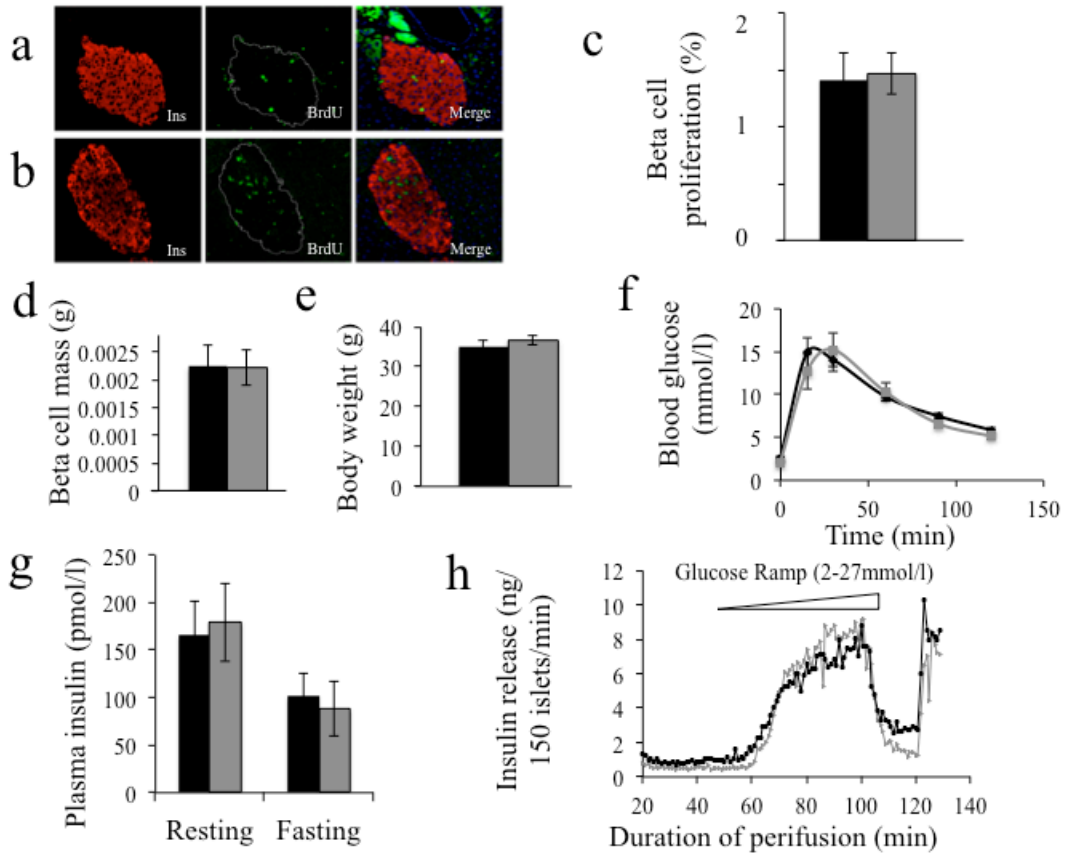


Fig 2.4 CISH is not required for beta cell proliferation or glucose homeostasis during pregnancy. BrdU (green) and insulin (red) co-staining of pancreata from day 14.5 pregnant (p14.5) *Cish*$^{loxP/loxP}$ (**a**); and *Cish*$^{loxP/loxP}$*; Pdx1-Cre*^{Early} (**b**) mice. **c** Percentage of beta cell nuclei staining positive for BrdU showed no difference between *Cish*$^{loxP/loxP}$ and *Cish*$^{loxP/loxP}$*; Pdx1-Cre*^{Early} mice (t-test). **d,e,f,g** Beta cell mass, body weight, blood glucose levels during glucose tolerance test, and resting and fasting plasma insulin levels, respectively, of pregnant day 14.5 mice. No difference was observed between control and mutant groups (t-test for **d**,**e**, and **g**; MANOVA for **f**). **h** Control (Black trace) and mutant (grey trace) islets from pregnant day 14.5 mice were perfused for a glucose-stimulated insulin secretion assay. No difference was detected between the two groups . Error bars show standard error of the mean. **c-g** Black bars: p14.5 *Cish*$^{loxP/loxP}$ controls; grey bars: p14.5 *Cish*$^{loxP/loxP}$*; Pdx1-Cre*^{Early} mutants (n=5-11).

pregnancy. No difference was observed in body weight or glucose tolerance between the control and mutant group (Fig. 2.4e-f). In addition, fed and fasted insulin level levels were the same in two groups (Fig. 2.4g). To exclude the beta cell non-autonomous effect of *Cish* ablation in the hypothalamus, where *Pdx1* is also expressed, I performed islet perifusion studies of insulin secretion on isolated islets from day 14.5 pregnant mice. There was no difference in glucose stimulated insulin secretion between the two groups (Fig. 2.4h). Thus, *Cish* deficiency in beta cells does not alter glucose tolerance or beta cell function during pregnancy.

After gestation, beta cell mass quickly returns to pre-pregnancy levels via decreased proliferation and increased apoptosis. Expression of *Cish* maintains elevated at later stages of pregnancy (P18.5), when the beta cell proliferating rate is reduced to pre-gestation levels. To test whether CISH is required for the cessation of the proliferative response of beta cells following pregnancy, mice five days post-partum (PP5) were analyzed. No statistically significant differences were observed in beta cell mass, body weight, resting insulin, fasting insulin, or glucose tolerance between control and mutant PP5 mice (Fig. 2.5a-d). Since there was a trend towards lower plasma insulin levels in *Cish*-deficient mice, I further tested islet function by perifusion assays. Islets from mice five days after birth were cultured, placed in a perifusion chamber, and subjected to a glucose ramp. I found no difference in glucose-stimulated-insulin secretion between control and mutant islets (Fig. 2.5e). In conclusion, *Cish* is not required for beta cell homeostasis in mice before, during, or after pregnancy.

Cish **ablation is not sufficient to increase activation of STAT5**

Since CISH competes with STAT5 to bind prolactin receptor, *Cish* ablation may

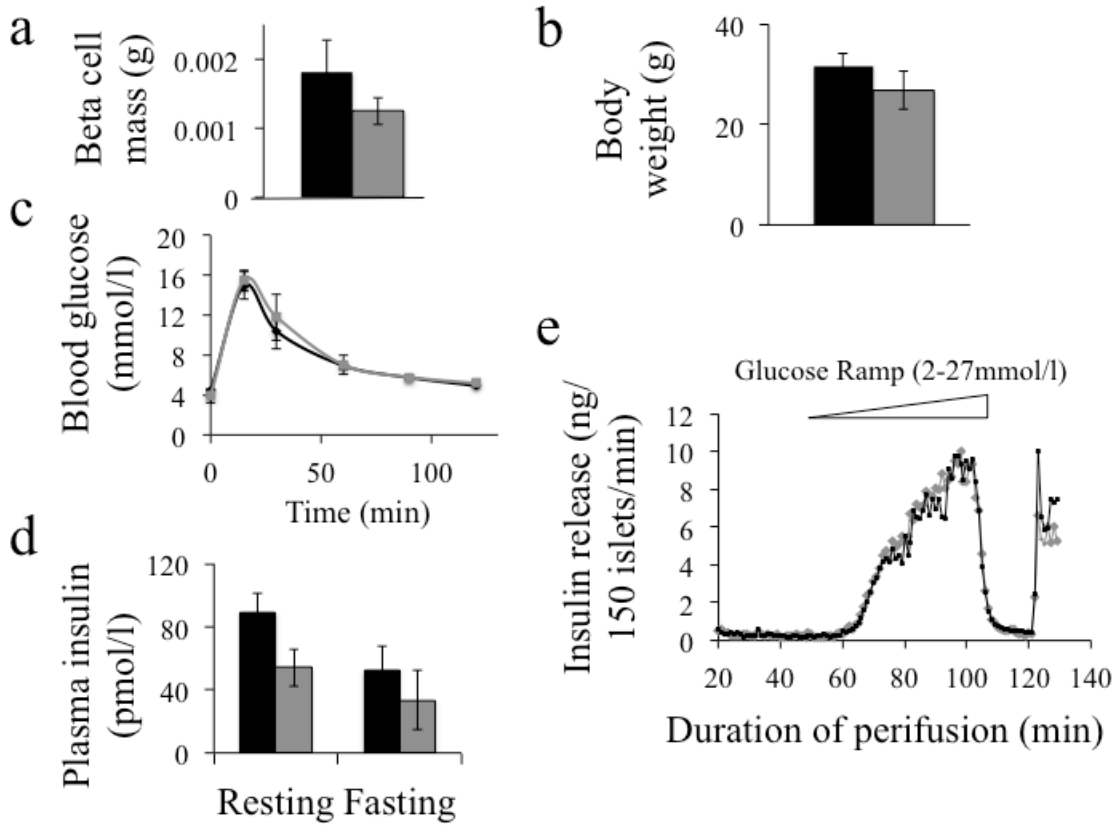


Fig 2.5 CISH is not required for beta cell proliferation or beta cell function in mice five days after pregnancy. **a-d** Beta cell mass, body weight, glucose tolerance test, and resting and fasting plasma insulin levels of mice five days after pregnancy. No statistically significant difference was observed between control and mutant mice, although there was a trend of lower resting insulin in the mutant mice (t-test for **a**, **b**, and **d**; MANOVA for **c**). **e** Control and mutant islets from PP5 mice were perifused for glucose-stimulated insulin secretion assay. No difference was detected between the two groups (Black trace: control; Grey trace: mutant). Error bars show standard error of the mean. **a-d** Black bars: *Cish*$^{loxP/loxP}$ controls; grey bars: *Cish*$^{loxP/loxP}$*; Pdx1-Cre*^{Early} mutants (n=4-6).

promote increased STAT5 binding to the receptor, which would result in elevated STAT5 phosphorylation levels. Islets from virgin mice and p14.5 mice were isolated and whole cell lysates were resolved using SDS-PAGE gel. P-STAT5 protein levels were detected using a specific antibody. P-STAT5 is undetectable in islets from non-pregnant mice, and is up-regulated with detectable bands in islets from p14.5 mice. However, no differences were observed in pSTAT5 levels between control and mutant pregnant mice (Fig. 2.6a), although there was some variability in phosphorylation status among animals with the same genotype. To further investigate the activity of the STAT5 pathway, I determined *Tph1, Tph2, Glut2, and Gck* mRNA levels in the islets, as these genes have been suggested to be downstream of JAK2/STAT5 pathway and regulate beta cell proliferation and beta cell function during pregnancy (Schraenen, Lemaire, et al., 2010; Sorenson et al., 2007). No differences were detected in mRNA levels of these genes between control and mutant islets (Fig. 2.6b), providing more evidence that STAT5 signaling was unperturbed by absence of CISH from beta cells. Therefore, ablation of *Cish* is not sufficient to induce elevated STAT5 activation.

Socs2 gene is up-regulated upon *Cish* ablation at pregnancy day 9.5

Since *Cish* ablation does not lead to elevated STAT5 activation, I hypothesized that other *Socs* family members might be up-regulated to compensate for the loss of *Cish*. Our lab had previously shown that *Socs2* is also up-regulated in islets during pregnancy, making it a prime candidate for the compensatory effector. qPCR were performed on cDNA from islets of control and mutant mice, both before and during pregnancy. At p14.5 in control mice, expressions of *Cish* and *Socs2* are increased, consistent with published work (Fig. 2.6c) (Kaestner et al., 2009). mRNA expression of other *Socs* genes

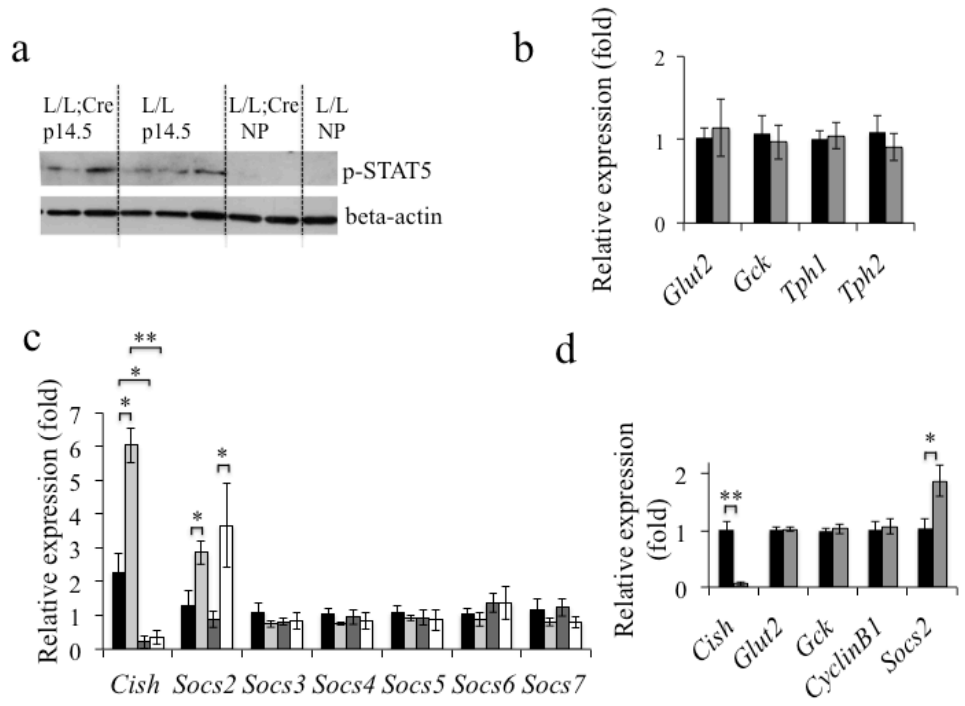


Fig 2.6 P-STAT5 western blot and quantitative RT-PCR of *Cish* family members in islets from non-pregnant mice, pregnant day 14.5 mice and pregnant day 9.5 (p9.5) mice **a** p-STAT5 was detected by western blot. p-STAT5 level is undetectable in the virgin mice, but highly induced during pregnancy. No difference between control pregnant mice and mutant pregnant mice p-STAT5 was detected. Beta-actin was used as the loading control. **b** mRNA levels of *Glut2, Gck, Tph1*, and *Tph2* were not affected in mutant mice at pregnant day 14.5. Black bars: p14.5 $Cish^{loxP/loxP}$ controls; grey bars: p14.5 $Cish^{loxP/loxP}$*; Pdx1-Cre*^{Early} mutants (n=5-6) (t-test).**c** *Cish* and *Socs2* are up-regulated during pregnancy (t-test). *Socs2* is not further up-regulated in the *Cish* mutant mice at pregnant day 14.5. Furthermore, none of the other family members (*Socs3-Socs7*) showed up-regulation at pregnant day 14.5 upon *Cish* ablation. Black bars: $Cish^{loxP/loxP}$ virgins; light grey bars: p14.5 $Cish^{loxP/loxP}$ mice; dark grey bars: $Cish^{loxP/loxP}$*; Pdx1-Cre*^{Early} virgins; white bars: p14.5 $Cish^{loxP/loxP}$*; Pdx1-Cre*^{Early} mice (n=5-6). (t-test) **d** mRNA expression of *Socs2* is higher in the *Cish* mutant mice than control mice at pregnant day 9.5. mRNA expression of *Glut2, Gck, CyclinB1* had no difference between control and mutant mice (t-test). Black bars: p9.5 $Cish^{loxP/loxP}$ mice; grey bars: p9.5 $Cish^{loxP/loxP}$*; Pdx1-Cre*^{Early} mutants (n=5). Error bars show standard error of the mean. * $p<0.05$; ** $p<0.01$ (t-test).

was also determined, and none of them are up-regulated significantly upon *Cish* ablation (Fig. 2.6c). The expression of *Socs1* is undetectable in islets. Interestingly, *Socs2* mRNA levels are higher in *Cish* mutant mice than control mice at p9.5 (Fig. 2.6d), suggesting that *Socs2* might be compensating for *Cish* ablation during this phase of pregnancy. *Glut2, Gck*, and *CyclinB1* mRNA levels are the same in *Cish* control and mutant mice at p9.5 (Fig. 2.6d), indicating normal glucose metabolism and beta cell proliferation.

Socs2 global ablation results in elevated beta cell proliferation during pregnancy.

Since *Socs2* is up-regulated in *Cish*-deficient islets during pregnancy, I hypothesized that *Socs2* compensates for *Cish* ablation and functions as a brake for beta cell proliferation. *Socs2* global ablation mice exhibit gigantism with normal glucose metabolism and adequate beta cell mass after normalizing to body weight (Alexander et al., 2000; Puff et al., 2010). But beta cell proliferation was not evaluated in these global mutant mice, nor the role of SOCS2 on beta cell proliferation during pregnancy.

In order to investigate the role of SOCS2 on beta cell proliferation during pregnancy, $Socs2^{+/-}$ mice were bred together to obtain $Socs2^{-/-}$ mutant mice and $Socs2^{+/+}$ littermates as controls. At day 13.5 of pregnancy, BrdU was intraperitoneally injected into both control and mutant mice to label proliferating cells. 24 hours later, day 14.5 pregnant mice were sacrificed, and pancreas sections from both $Socs2^{-/-}$ and $Socs2^{+/+}$ mice were immunostained for insulin and BrdU (Fig 2.7a). BrdU positive cells were quantified from four mice of each group manually. Interestingly, *Socs2* ablation resultes in doubled beta cell proliferation at p14.5 compared to control mice at p14.5 (Fig 2.7b), indicating that SOCS2 plays a key role in repressing beta cell proliferation during pregnancy.

Since $Socs2^{-/-}$ mice are gigantic with larger organs, including higher beta cell mass

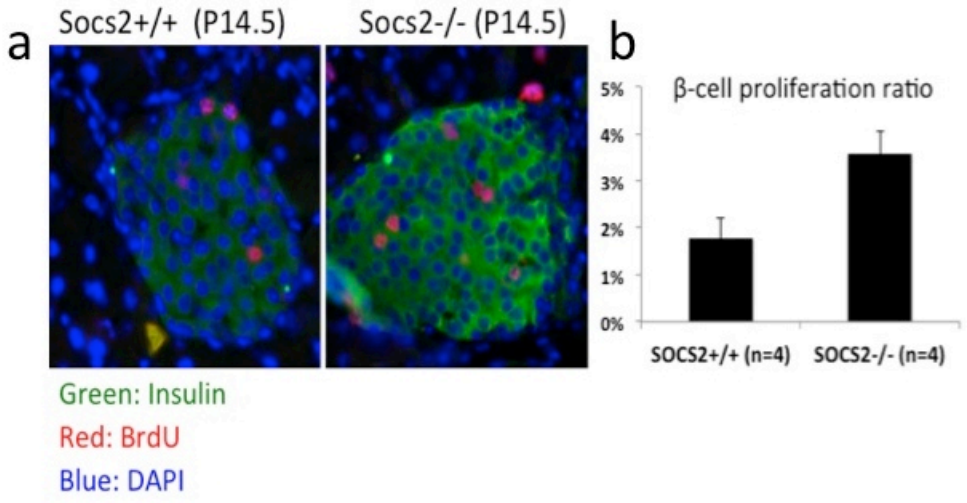


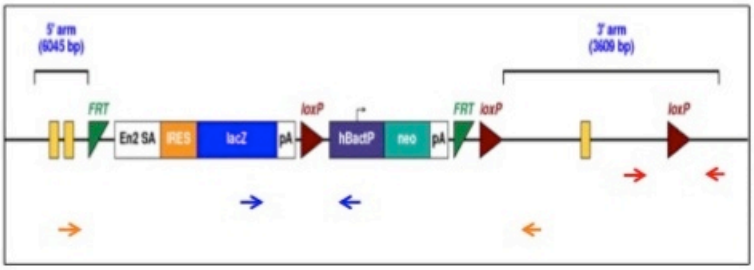
Fig 2.7 *Socs2* global mutants exhibit increased beta cell proliferation. **a** BrdU (red) and insulin (green) double staining of pancreata from *Socs2*$^{+/+}$ (left) and *Socs2*$^{-/-}$ (right) mice. **b** Quantification of proliferating cells. *Socs2*$^{-/-}$ mutant mice have a 2-fold increase in the ratio of beta cell nuclei stained positive for BrdU compared to *Socs2*$^{+/+}$ mice.

(30-40%) than *Socs2*$^{+/+}$ control mice, it is possible that the higher beta cell proliferation rate is an adaptive response for keeping the adaptive higher beta cell mass in line with higher body weight, to maintain normal blood glucose homeostasis. Further studies will require beta-cell specific *Socs2*$^{-/-}$ mice in which body weight is not a contributing factor for beta cell proliferation. Conditional *Socs2* ablation models will undoubtedly provide more knowledge on its role in beta cell proliferation.

Derivation of beta-cell-specific *Cish/Socs2* double ablation mice

An ES cell clone with heterozygous *Socs2* conditional ablation potential was obtained from EUCOMM. Exon 3 of *Socs2* is flanked by two loxP sites (Fig 2.8a). These JM8A3 agouti/brown ES cells were injected into 4-day-old Albino-B6 blastocysts and transferred into the uterine horn of pseudopregnant females. Male chimeric pups were backcrossed with Albino-B6 mice. Albino pups were removed while agouti and black pups were genotyped for the presence of *Socs2* loxP allele to determine germline transmission from the chimeric mice. *Socs2*$^{L/+}$ mice were bred with FLPe mice to excise the FRT-neo cassette from the original loxP construct. Two sets of FRT deletion primers were designed to confirm the excision of FRT-neo cassette by PCR (Fig 2.8b). *Socs2*$^{L/+}$;*Flp*$^{+}$ mice were then bred with *Cish*$^{L/+}$; *Pdx1-Cre*^{ER} mice to derive *Socs2*$^{L/+}$; *Cish*$^{L/+}$; *Pdx1-Cre*^{ER} mice. Subsequently, *Socs2*$^{L/+}$; *Cish*$^{L/+}$; *Pdx1-Cre*^{ER} mice were mated with *Socs2*$^{L/+}$; *Cish*$^{L/+}$ mice to generate *Socs2*$^{L/L}$; *Cish*$^{L/L}$; *Pdx1-Cre*^{ER} mice. Upon administration of tamoxifen to promote the nucleus translocation of the Cre^{ER} recombinase, these mice have beta-cell specific ablation of both *Cish* and *Socs2*.

a



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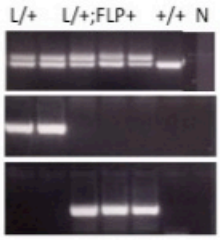


	Wild type	LoxP with FLP	LoxP w/o FLP
Genotyping primer	385bp	328bp	328bp
FRT deletion primers-1	No band	No band	479bp
FRT deletion primers-2	No band	474bp	No band (Too big)

Fig 2.8 Generation of *Socs2* conditional ablation mice **a** Construction of commercially available ES cells with *Socs2* loxP allele. Two loxP sites flank exon 3 of *Socs2* gene, which encodes the SH2 binding domain. Genotyping primers for the loxP allele and PCR primers for confirming FRT site excision are color-coded. **b** Genotyping of $Socs2^{loxP}$ allele and *Socs2* wild type allele. PCR validation of excision of the FRT flanked site were performed with FRT deletion primers.

Knockdown of *Cish* and *Socs2* in human islets by lentiviral shRNA

Replication incompetent lentivirus is widely used to enforce short hairpin RNA (shRNA) expression in cells, which triggers RNA interference and target mRNA suppression (Cockrell & Kafri, 2007; Kappes & Wu, 2001). shRNAs are short sequences of RNA transcribed by RNA PolIII and subsequently cleaved by Drosha and Dicer to produce small interfering RNAs (siRNA) (Rossi, 2008). siRNAs bind to target mRNAs and decrease gene expression by cleaving the mRNA or blocking the translation of the mRNA. The lentivirus construct contains both microRNA-adapted shRNA (shRNAmir) and GFP sequences (Silva et al., 2005) (Fig 2.9a).

To determine efficiency of the lentiviral shRNAs, ten clones of lentiviral shRNAmir targeting *Cish* or *Socs2* were purchased, respectively (C1~C10, S1~S10). A scrambled shRNA was also obtained and used as a negative control (referred to as CT shRNA afterwards). Human HEK293T cells were transfected with each clone, respectively (C1-C10, S1-S10, CT). Two days later, cells were run through fluorescence-activated cell sorting (FACS) to isolate GFP^+ cells, which were transfected by the lentivirus, from GFP^- cells, which were not transfected by the lentivirus. Figure 2.9b shows a representative FACS result with clear separation of GFP^+ and GFP^- cell populations. qPCRs of *Cish* and *Socs2* mRNA expression were performed. The four shRNA clones that produced the highest reduction of target gene expression from GFP^- to GFP^+ cells were selected for both *Cish* and *Socs2*, respectively (C1, C5, C7, C8, S4, S5, S7, S8) (Fig. 2.9c-d). The plasmids were sent to the Protein Expression and Libraries Facility at Wistar Institute to construct lentiviral vectors containing the respective *Cish* and *Socs2* shRNA sequences for stable transduction into human islets.

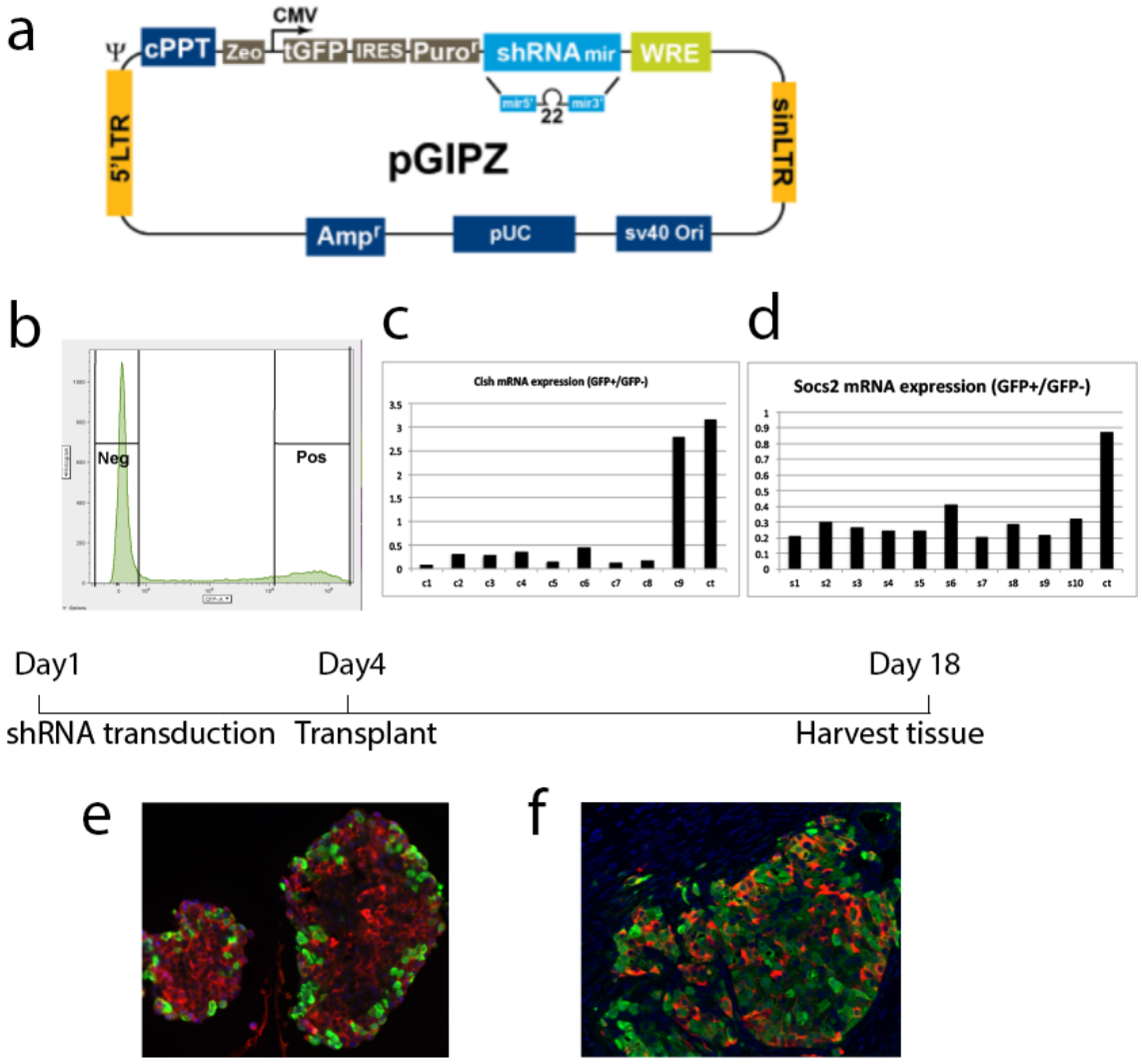


Fig 2.9 Validation of *Cish* and *Socs2* lentivirus on human cell line and human islets. **a** Structure of lentiviral constructs targeting *Cish* or *Socs2*, with GFP reporter sequence in backbone. **b** GFP^+ cells and GFP^- cells were differentiated by fluorescent-activated cell sorting (FACS). Ratio of mRNA levels of *Cish* (**c**) and *Socs2* (**d**) in sorted GFP^+ 293T cells compared to GFP^- 293T cells after transfection with lentiviral vectors. **e** Immunostaining of human islets before transplantation. **f** Immunostaining of transduced human islets under kidney capsule after 14 days of transplantation.

Human islets transduced with CT shRNA were transplanted into immunodeficient mice under a kidney capsule. The kidney capsule provides an environment enriched for capillaries, which makes it relatively easy for the islets to be recapillarized and remain functional (S. K. Kim et al., 2007). Before transplantation, only the cells on the periphery of the transduced human islets are GFP^+ (Fig 2.9e). Two weeks later, the kidney capsule was isolated, fixed, and sectioned. The GFP signal penetrated into the transplanted human islets (Fig 2.9f). There are several potential explanations for these results. It is feasible that at early stages post-transduction, the GFP protein concentration is simply too low for detection. Another possibility is that the GFP signal was transferred through the islet cells via gap junctions.

Discussion

Pregnant mouse islets provide a model for studying the signaling pathways that influence and regulate beta cell replication. Substantial evidences demonstrate that lactogens promote beta cell proliferation during pregnancy through the JAK2/STAT5 signaling pathway, at least in rodents (Boschero et al., 2004; Brelje et al., 1993b; Kaestner et al., 2009; Parsons et al., 1992; Vasavada, Fujinaka, Takane, & Yamashita, 2007). *Cish* and *Socs2* are also up-regulated during the same time period when beta cell proliferation peaks during pregnancy. Furthermore, CISH/SOCS2 bind to the prolactin receptor, block STAT5 docking, and phosphorylation of STAT5 by JAK2, suggesting a canonical negative feedback loop for repressing the JAK2/STAT5 pathway (Kaestner et al., 2009; Laz et al., 2009; Matsumoto et al., 1997). Based on these findings, I hypothesized that ablation of *Cish* would result in elevated JAK2/STAT5 signaling,

increased *cyclin D2* expression, and increased beta cell proliferation during pregnancy. To test this hypothesis, we generated mice with pancreas-specific *Cish* ablation. These mice represent the first reported mouse model for tissue-specific ablation of *Cish*. In this *Cish*$^{loxP/loxP}$*; Pdx1-Cre*^{Early} model, *Cish* expression is reduced by greater than 90% in the islets, demonstrating the efficiency of the system. FACS of control and mutant islets reveals that *Cish* is highly enriched in beta cells compared to other islet cell types, and is efficiently deleted in sorted *Cish*$^{loxP/loxP}$*; Pdx1-Cre*^{Early}; MIP-GFP islet beta cells. The reason for the remaining 5-10% *Cish* mRNA expression could be that Cre recombination does not occur in all beta cells. The efficacy of Cre mediated gene ablation observed for the *Cish* locus was comparable to that of other loxP flanked targets (Gao et al., 2008). The *Pdx1-Cre*^{Early} transgene is also expressed in a subset of cells in the hypothalamus, and because the hypothalamus provides central input to glucose homeostasis (Wicksteed et al., 2010b), I performed *ex vivo* experiments to exclude any potential beta cell non-autonomous effect. I did not observe any difference between control and mutant islets in the insulin secretion studies of the isolated islets, in which any potential neuronal input has been removed. Furthermore, in order to evaluate if the *Pdx1-Cre*^{Early} transgene itself might impact the phenotype of our mutant mice, I determined insulin levels in two control groups (*Cish*$^{loxP/loxP}$ mice and *Pdx1-Cre*^{Early} mice). No abnormal phenotypes in *Pdx1-Cre*^{Early} mice were observed, consistent with previous studies using the same mouse strain (J. Y. Lee et al., 2007).

Cish-deficient mice exhibit normal islet architecture, and normal glucose homeostasis before, during, and after pregnancy. Surprisingly, I found no difference in pregnancy-induced beta cell proliferation or glucose homeostasis in *Cish*-deficient females

compared to controls. *Cish* ablated islets also exhibit p-STAT5 levels comparable to those of control mice, and normal mRNA levels of *Tph1* and *Tph2*, further demonstrating that the STAT5 signaling pathway is not affected. mRNA expression levels of other *Socs* gene family members were determined, and *Socs2* mRNA levels in the absence of *Cish* are upregulated even compared to the already elevated levels in control mice on day 9.5 of pregnancy, suggesting that *Socs2* might be compensating for *Cish* ablation during pregnancy. Therefore, a mouse model with simultaneous, beta cell specific ablation of *Cish* and *Socs2* is necessary to uncover functions of these two proteins in beta cell proliferation and function.

To investigate possible compensatory effects of *Socs2* for *Cish*, global *Socs2*$^{-/-}$ mice were also analyzed during pregnancy. *Socs2*$^{-/-}$ mutants exhibit higher beta cell proliferation rates than control mice at 14.5 days of pregnancy, suggesting that SOCS2 plays a key role as a repressor of beta cell proliferation during pregnancy. But since global *Socs2*$^{-/-}$ mice are gigantic with proportional increases in the size of multiple organs, including bones, liver, and pancreas, the phenotype of increased beta cell proliferation might be an adaptive response to an increase in body weight. Therefore, a beta cell-specific ablation of *Socs2* would be essential to investigate the role of SOCS2 in beta cells independent of the effects of *Socs2* ablation on other organs. As mentioned above, it would also be helpful to study the effects of combined *Cish* and *Socs2* ablation in beta cells. I am currently working on generating beta cell-specific *Cish* and *Socs2* double-ablation mice. Furthermore, the lentiviral constructs containing shRNA for *Cish* and *Socs2* are being tested on human islets to examine the consequences of *Cish* and *Socs2* knockdown on human beta cell proliferation.

Alternatively, another explanation of lack of phenotype in pregnant *Cish*$^{loxP/loxP}$*; Pdx1-Cre*^{Early} mice is that STAT5 is maximally active during pregnancy, and thus its capacity for phosphorylation is already saturated. If this were true, then ablation of its negative regulator would not be able to increase STAT5 phosphorylation or affect beta cell function or proliferation. Furthermore, other mediators of the JAK2/STAT5 signaling pathway other than SOCS proteins might be compensating for the loss of *Cish*. Finally, it is possible that other signaling pathways limit the proliferative capacity of beta cells, such as the cell cycle inhibitor p16 (Pascoe et al.).

In summary, in the pancreas-specific *Cish*$^{-/-}$ mice, no difference is discovered in glucose homeostasis or beta cell function before, during, or after pregnancy. P-STAT5 levels are not altered in *Cish*-deficient mice, indicating that other mechanisms compensate in the regulation of the STAT5 pathway during pregnancy. *Socs2* mRNA is further up-regulated in *Cish*-deficient islets at 9.5 days of pregnancy, suggesting that *Socs2* might be compensating for the loss of *Cish* as a repressor of JAK2/STAT5 pathway and beta cell proliferation. Indeed, *Socs2* global ablation mice exhibit higher beta cell proliferation rate during pregnancy, indicating the SOCS2 does play a key role in regulating beta cell replication. To fully elucidate the role of Cish and Socs2 in beta cell replication, it will be necessary to generate and examine beta cell specific *Cish*$^{-/-}$; *Socs2*$^{-/-}$ mice.

Methods and Materials

Mice: Cish loxP mice were crossed with *Pdx1-Cre*^{Early} mice (kindly provided by Dr. D. A. Melton from Harvard University, Cambridge, MA, USA) (Gu et al., 2002) to induce

pancreas-specific *Cish* ablation. *Cish*$^{loxP/loxP}$ mice were used as controls and *Cish*$^{loxP/loxP}$*; Pdx1-Cre*^{Early} mice constituted the mutant group. *Pdx1-Cre*^{Early} mice were also used as controls in the virgin mice study in addition to the *Cish*$^{loxP/loxP}$ controls, and no difference was observed in the insulin levels among the two control groups and the mutant group, consistent with previous studies (J. Y. Lee et al., 2007), showing that the *Pdx1-Cre*^{Early} transgene has no impact on beta-cell proliferation or beta-cell function. Therefore, we used only *Cish*$^{loxP/loxP}$ mice as control mice in our further studies. In addition, *Cish*$^{loxP/loxP}$*; Pdx1-Cre*^{Early} mice were crossed with *Mip-GFP* mice (purchased from The Jackson Laboratory, Bar Harbor, Maine, USA) (Hara et al., 2003), a transgenic mouse with green fluorescent protein driven by mouse-insulin-promoter to label pancreatic beta cells. This mating generated *Cish*$^{loxP/loxP}$*; Pdx1-Cre*^{Early}; *Mip-GFP* and *Cish*$^{loxP/loxP}$*; Mip-GFP* mice, which were used for sorting of beta cells and non-beta cells from islets. Mice were analyzed between 3 and 5 months of age.

Socs2$^{L/+}$ mice were generated by injecting *Socs2*$^{L/+}$ targeted ES cells (purchased from EUCOMM) into blastocysts and transplantion into pseudopregnant mice. Germline transmission of the loxP allele in the chimeric pups was identified by crossing chimeras with albino C57BL/6J mice. Germline chimeras were then crossed to FLPe mice to excise the FRT-neo cassette. *Socs2*$^{L/+}$;*Flp*$^{+}$ mice were then bred with *Cish*$^{L/+}$; *Pdx1*-Cre^{ER} mice to derive *Socs2*$^{L/+}$; *Cish*$^{L/+}$; *Pdx1*-Cre^{ER} mice. Currently, *Socs2*$^{L/+}$; *Cish*$^{L/+}$; *Pdx1*-Cre^{ER} mice are mating with *Socs2*$^{L/+}$; *Cish*$^{L/+}$ mice to generate *Socs2*$^{L/L}$; *Cish*$^{L/L}$; *Pdx1*-Cre^{ER} mice.

Proliferation analysis: Twenty-four hours before euthanizing the mice, 1ml/100 g body weight of BrdU labeling reagent (Life Technologies, Grand Island, NY, USA) was intraperitoneally injected. Pancreata were dissected, flattened by forceps, fixed in 4% para-formaldehyde for 24 hours, and paraffin embedded so that tissues with the maximum pancreatic footprint were sectioned. Tissues were sectioned to 5μm thickness. Deparaffinized and rehydrated slides were subjected to antigen retrieval by pressure cooker in 10mmol/l pH 6.0 citric acid buffer. Simultaneous immunofluorescent staining was performed for BrdU and insulin. The primary antibodies used were guinea pig anti-insulin (1:1000 dilution, Dako North America, Inc., Carpinteria, CA, USA) and rat anti-BrdU (1:500 dilution, AbD Serotec; Raleigh, NC, USA). Secondary antibodies were Cy2-anti-guinea pig (1:200) and Cy3-anti-rat (1:200). The beta cell proliferation rate was quantified as BrdU/ insulin double positive cells divided by insulin positive cells.

Beta cell mass: Three sections (40 μm apart) from each animal were immunostained for insulin using the standard DAB method without counterstaining. The entire tissue section was scanned with Meyer PathScan Enabler IV Histology Slide Scanner and Silverfast Pathscan 6.6 software. The percentage of beta cell area relative to the total pancreatic area was measured and calculated using ImageJ. Beta cell mass was derived from the total pancreas weight multiplied by the percentage of beta cell area.

Glucose tolerance test and insulin assay: Animals were fasted overnight and fasting glucose levels determined by glucometer. 2g/kg body weight of glucose (Sigma-Aldrich Corp., St. Louis, MO, USA) was injected intraperitoneally. Glucose levels were

measured at 15, 30, 60, 90, 120 minutes postinjection. Glucose levels were measured by Glucometer Breeze2 (Bayer AG, Leverkusen, Germany). To determine plasma insulin levels during glucose tolerance tests, blood was collected from the tail vein of mice before and after overnight fasting. Plasma insulin concentration was measured by ELISA.

Islet isolation and real-time PCR: Islets were isolated using standard collagenase procedures followed by purification through a Ficoll gradient (Sigma-Aldrich Corp, Ficoll PM 400) as described preciously (Kaestner et al., 2007). Islets were handpicked under a light microscope. Total RNA was isolated in Trizol (Life Technologies) and reversely transcribed using 1μg oligo (dT) primer, Superscript II Reverse Transcriptase, and accompanying reagents (Life Technologies). PCR reaction mixes were assembled using the Brilliant III SYBR Green QPCR Master Mix (Agilent Technologies, Inc., Santa Clara CA, USA). PCR reactions were performed on an Mx4000 Multiplex Quantitative PCR System (Agilent Technologies). All reactions were performed in triplicate with reference dye normalization. Median cycling threshold values were used for analysis.

Islet perifusion: One hundred and fifty islets were hand picked under a light microscope and placed into a perifusion chamber (EMD Millipore, Billerica, MA, USA). A computer-controlled fast-performance HPLC system (625 LC System, Waters Corporation, Huntingdon Valley, PA, USA) allowed programmable rates of flow and concentration of the appropriate solutions held in a 37^{o}C water bath. Islets were perifused with Krebs bicarbonate buffer (2.2 mmol/l Ca^{2+}, 0.25% bovine serum albumin, 10 mmol/l HEPES, and 95% O_2/5% CO_2 equilibration [pH 7.35]) plus 2mmol/l glucose and 4mmol/l

AAM-19 and glutamine to reach baseline hormone secretion values before the addition of the appropriate secretagogues. Samples were collected at regular intervals with a fraction collector (Waters Corporation). Insulin content was determined using a radioimmunoassay.

Western Blotting: For western blot, islets were isolated and lysed in lysis buffer containing 50mmol/l Tris (pH 8.0), 5mmol/l EDTA, 150mmol/l NaCl, 1% Triton, 1% SDS, 0.5% sodium deoxycholic acid and Complete Protease Inhibitor Cocktail Tablets (Genentech Roche, Newtown, PA, USA). Protein concentrations were measured by Bradford Assay using SpectraMax Plus 384 (Molecular Devices). 6µg total lysate were heated at 95º C for 10 min and loaded on 4-12% Bis-Tris gel (Novex, Wadsworth, OH, USA). Proteins were transferred to PVDF membranes by iBlot Dry Blotting System (Life Technologies) and detected by antibodies against p-STAT5 (Santa Cruz Biotechnology, Inc., Dallas TX, USA) and rat anti-beta-actin (Cell Signaling). The ECL-Plus detection system (GE Healthcare Biosciences, Pittsburgh, PA, USA) was used to detect the signal.

FAC-sorting: Islets from two *Cish*$^{loxP/loxP}$*; Pdx1-Cre*^{Early}; *Mip-GFP* mice and two *Cish*$^{loxP/loxP}$*; Mip-GFP* mice were isolated and pooled by the same genotype. Dissociated cells from isolated islets were sorted into GFP^{+} cell fractions, which were highly enriched for beta cells, and GFP^{-} cell fractions, which were enriched for non-beta islet cells. Total RNA was extracted from sorted cells and subjected to further analysis.

Lentiviral transduction and xenotransplantation of human cadaveric islets: Ten clones of

lentiviral shRNAmir targeting *Cish* and *Socs2* respectively were purchased (C1~C10, S1~S10) from Open Biosystems. A scrambled shRNA was obtained from the same company as the negative control. Human HEK293T cells were transfected with each clone of plasmids separately (C1-C10, S1-S10, CT). Two days later, cells were collected and run through fluorescence-activated cell sorting (FACS) to isolate GFP^+ cells from GFP^- cells. Four clones of both *Cish* and *Socs2* shRNAs with highest knockdown efficiency of the respective target gene were chosen, and the plasmids were sent to the Protein Expression and Libraries Facility at Wistar Institute for packaging lentivirus for stable transduction (C1, C5, C7, C8, S4, S5, S7, S8). Cadaveric human islets were transduced with packaged CT lentivirus and transplanted under the kidney capsule of immunodeficient mice. Mice were given bromodeoxyuridine (BrdU) in the drinking water for the whole 14 days and the grafts were retrieved after 14 days of *in vivo* incubation and processed for immunostaining using standard methods.

Statistical analysis: Data are presented as mean±SEM. The statistical significance of differences was determined by Student's *t* tests or MANOVA. $P<0.05$ was considered statistically significant.

Chapter 3 HNF-4α is required for beta cell proliferation during obesity

Abstract

Hepatocyte nuclear factor 4 alpha (HNF-4α) regulates insulin secretion and beta cell proliferation during the metabolic stress of pregnancy. But it is not known whether HNF4α also plays a role in beta cell replication during obesity. Therefore, I investigated the role of HNF4α in obesity by using two obese mouse models.

In the first model, *Hnf4α* was ablated in pancreatic beta cells on the *ob/ob* mice background via tamoxifen pellet implantation in *Hnf4α*$^{L/L}$*; Mip-Cre*^{ER}*; ob/ob* mice. Beta cell proliferation rates were halved in *Hnf4α* mutant mice. However, glucose homeostasis was not affected, indicating that although HNF-4α is required for adaptive beta cell proliferation in response to obesity, three weeks of *Hnf4α* ablation is not enough to promote beta cell mass change or a physiological phenotype. In the second model, *Tre-Hnf4α; Rip-rtTA* mice were put on high-fat-diet to trigger murine obesity, and *Hnf4α* overexpression in beta cells was induced by doxycycline. *Hnf4α* mRNA expression increased 9-folds in beta cells of the overexpressing group. However, no difference in glucose homeostasis was observed between the control and the *Hnf4α* overexpressing groups, indicating that overexpressing *Hnf4α* is not sufficient to improve glucose tolerance in high-fat-diet induced obese mice.

I also investigated the function of ST5, a transcriptional target of HNF-4α, on beta cell proliferation. ST5 is a novel regulator of the Ras/ERK pathway in beta cells. Overexpression of *St5* in beta cells was induced by doxycycline in *Tre-St5; Rip-rtTA* mice. *St5* mRNA expression was up-regulated over 300-folds in beta cells of the overexpressing group. However, no statistically significant differences in glucose tolerance or beta cell proliferation were observed in the *St5* overexpressing group

compared to the control group. In summary, my results suggest that HNF-4α is required for beta cell proliferation in obese mice. However, overexpression of neither *Hnf4α* nor its downstream target *St5* is sufficient to promote protective effects on glucose homeostasis.

Introduction

Maturity onset diabetes of the young (MODY) is a group of monogenic autosomal dominant disorders characterized by a familial, early-onset, non-insulin dependent form of diabetes (Froguel & Velho, 1999). Using the best-characterized MODY pedigree, the RW family, DNA polymorphism of chr. 20q is linked to MODY1, and *Hnf4α* identified as the MODY1 gene (Bell et al., 1991; Stoffel et al., 1996; Yamagata et al., 1996). MODY1 patients with *Hnf4α* mutation exhibit diminished insulin secretion in response to glucose while retaining normal insulin sensitivity, indicating that beta cell dysfunction is the primary etiology of MODY1 (Byrne et al., 1995; Herman et al., 1997).

HNF-4α is the most abundant DNA-binding protein in the liver, regulating genes largely involved in hepatic gluconeogenesis and lipid metabolism (Chandra et al., 2013; Costa, Grayson, & Darnell, 1989). HNF-4α is also a master regulator in the pancreatic islets, controlling an estimated 11% of islet genes (Odom et al., 2004). Cloning of the *Hnf4α* gene and purification of its protein product suggest that HNF-4α is a member of the steroid hormone receptor superfamily (Sladek, Zhong, Lai, & Darnell, 1990). HNF-4α contains a zinc-finger DNA binding domain and a ligand-binding domain (Fig 3.1a). HNF-4α dimerizes at its hinge site and subsequently binds DNA at its consensus sequence, which consists of two direct repeats separated by one or two nucleotides

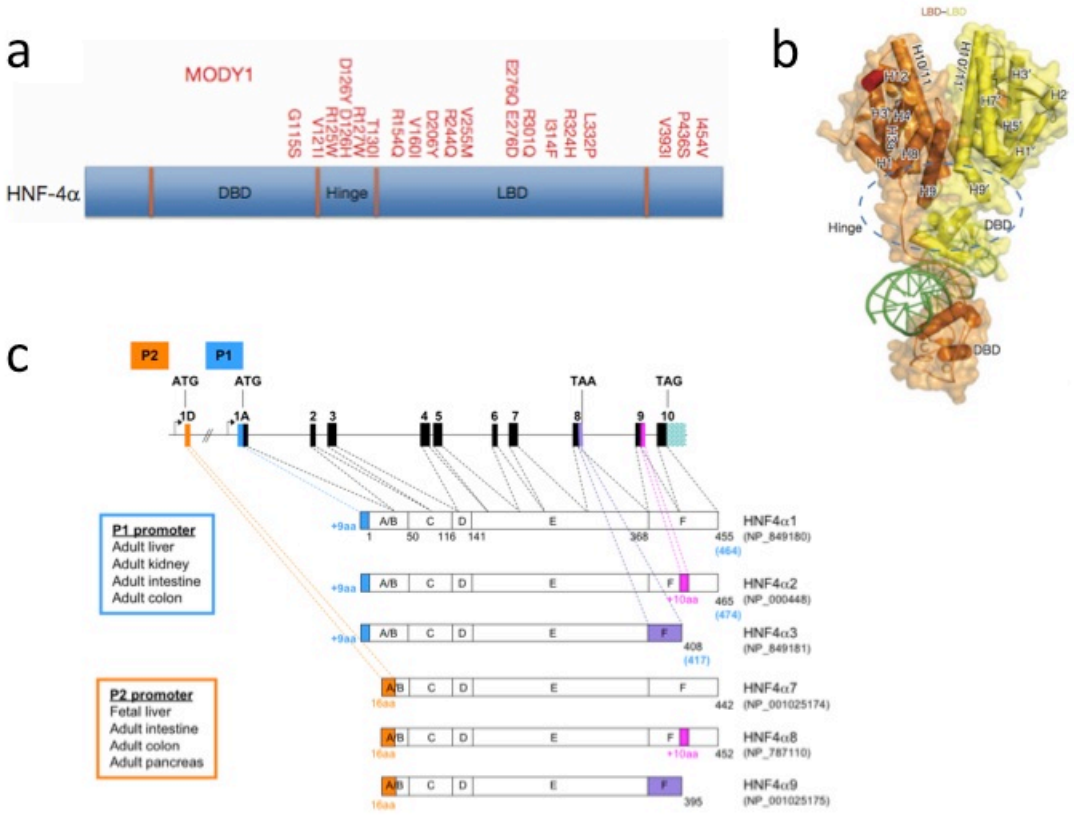


Fig 3.1 Structure of HNF-4α protein and its isoforms (adapted from (Chandra et al., 2013; Eugene Bolotin, 2010)) **a** Linear depiction of the HNF-4α protein domains and its MODY1-linked point mutations identified in human populations. DBD: conserved zinc-finger DNA binding domain; LBD: ligand binding domain. **b** Crystal structure of an HNF-4α homodimer. **c** HNF-4α isoforms and their functional domains. At least 6 isoforms of HNF-4α are generated by differential use of the promoters and alternative splicing events specific to the C-terminus. Isoforms 1-3 are derived from the P1 promoter and expressed in the adult liver, kidney, intestine, and colon. Isoforms 7-9 are derived from the P2 promoter and expressed in the fetal liver, adult intestine, adult colon and adult pancreas. A/B: amino-terminal transactivation domain; C: DNA binding domain, D: hinge domain; E: ligand binding domain; F: inhibitory carboxy-terminal domain

(Bolotin et al., 2010; Sladek et al., 1990) (Fig 3.1b). Point mutations of *Hnf4α* linked to MODY1 are positioned in the interjunctional surface, where two HNF-4α proteins bind to each other (Chandra et al., 2013) (Fig 3.1a). Mutations in this hinge site disrupt the interaction between two HNF-4α proteins and lead to failure of recognizing and binding their successive DNA half-sites (Chandra et al., 2013).

Hnf4α has tissue specific expression driven by either its proximal promoter P1 (in adult liver, kidney, intestine) or an alternative promoter P2 (in fetal liver, adult colon, and adult pancreas), which is located 46kb upstream of P1 (Tanaka et al., 2006). At least six splice variants are expressed; variants *Hnf4α*1-3 are driven by P1 and variants *Hnf4α*7-9 are driven by P2 (Fig 3.1c) (Eugene Bolotin, 2010). *Hnf4α* is highly expressed in the liver, kidney, and the intestines, and has detectable expression in pancreatic beta cells and the stomach (Duda, Chi, & Shoelson, 2004).

How HNF-4α becomes activated to regulate transcription of target genes is not entirely understood. One study resolves the crystal structure of the HNF-4α ligand-binding domain and suggests that fatty acids are endogenous ligands of HNF-4α (Dhe-Paganon, Duda, Iwamoto, Chi, & Shoelson, 2002). However, another study shows that coactivator rather than ligand binding leads to the active conformation of HNF-4α (Duda et al., 2004). Although the mechanism underlying HNF-4α transcriptional activity is still controversial, its important role in regulating metabolic processes such as glucose and lipid homeostasis has been of great interest (Hayhurst, Lee, Lambert, Ward, & Gonzalez, 2001).

Since beta cell dysfunction is the primary pathology of MODY1, several mouse models have focused on the role of HNF-4α in beta cells. First, HNF-4α has been

suggested to regulate glucose-stimulated insulin secretion (GSIS) in a Kir6.2 dependent manner. Kir6.2 is a potassium ion channel subunit in K_{ATP}-channel dependent insulin secretion (Gupta et al., 2005). Beta cell specific *Hnf4α* ablation in mice results in diminished first-phase insulin secretory response and impaired glucose tolerance (Gupta et al., 2005). Mechanistic study suggests that this impaired insulin secretion is due to downregulation of *KCNJ11*, the gene encoding Kir6.2 (Gupta et al., 2005). However, it is puzzling that although these mice are glucose intolerant due to diminished insulin secretion, they also exhibit hyperinsulinemia and mild hypoglycemia at fasted and fed status. This might be due to the relatively early age of the mice being studied (3-5 months old) compared to human MODY1 patients (15-25 years old) (Gupta et al., 2005). Indeed, children born haploinsufficient for *Hnf4α* also exhibit hypoglycemia and elevated basal insulin levels, which later evolves into diabetes due to reduced insulin secretion (Pearson et al., 2007). A similar study of beta cell specific *Hnf4α* ablation also exhibits deficiency in GSIS, but shows no decrease of *KCNJ11* expression, suggesting that other targets of HNF-4α might also be involved in regulating GSIS (Miura et al., 2006).

Second, HNF-4α has been suggested to regulate beta cell proliferation in response to increased metabolic demand during pregnancy (Gupta et al., 2007). Beta cell specific ablation of *Hnf4α* results in fewer beta cell replications, reduced beta cell mass, and impaired glucose tolerance during pregnancy (Gupta et al., 2007). Specifically, HNF-4α is required to activate the Ras/ERK signaling cascade, which promotes the proliferative response in beta cells during pregnancy. HNF-4α directly activates *suppression of tumorigenicity 5* (*St5*), a novel positive regulator of ERK signaling in beta cells, to influence ERK signaling. Thus, the downregulation of *St5* caused by *Hnf4α* deficiency

contributes to the reduction in ERK activation (Gupta et al., 2007). Furthermore, a recent study demonstrated that overexpression of *Hnf4α* in human islets is sufficient to initiate cell cycle entry detected by BrdU incorporation (Rieck et al., 2012). However, these rapidly proliferating cells exhibit loss of beta cell lineage, increased cell cycle arrest, and increased cell apoptosis (Rieck et al., 2012).

Third, HNF-4α is at the center of the transcriptional network of beta cells (Gupta et al., 2007). A microarray study comparing gene expression in the normal mouse *Hnf4α* mutant islets relative to control islets revealed 128 significantly upregulated genes and 57 downregulated genes in the mutant islets (Gupta et al., 2007). Gene ontology analysis shows that a large percentage of HNF-4α regulated genes are related to metabolism, proliferation, and signal transduction. This study shows that HNF-4α might have extensive roles in regulating beta cell function, which requires further investigation.

Although the role of HNF-4α in beta cell function and proliferation has been investigated at normal physiological conditions and during pregnancy, it is also important to investigate whether HNF-4α plays a role in obesity-induced beta cell compensation, which is much more relevant to human T2D. Indeed, in addition to its role as a MODY gene, *Hnf4α* has also been suggested to be a T2D risk gene (Gupta & Kaestner, 2004; Silander et al., 2004). SNPs spanning the P1, P2, and exons 1-3 regions of *Hnf4α* have been associated with increased susceptibility to T2D in multiple ethnic groups (S. K. Hansen et al., 2005; Lehman et al., 2007; Love-Gregory et al., 2004; Silander et al., 2004). However, these results are still highly controversial. For example, one recent study demonstrated that in a European American population of 1270 T2D cases and 1017

controls, there is no association of *Hnf4α* variants with risk of T2D (Hellwege et al., 2011).

In order to study the role of HNF-4α in obesity-induced beta cell adaptation, I used *ob/ob* mice, a genetic model of murine obesity, which is caused by mutation of the leptin gene (Ingalls, Dickie, & Snell, 1950). Leptin is a hormone that restricts food intake and controls body weight by acting on hypothalamus, and its mutation causes unrestricted food consumption and decreased energy expenditure, which leads to obesity (Seufert, 2004). Different mouse strains have different susceptibility to the leptin mutation. For example, $BTBR^{ob/ob}$ mice are very susceptible and become obese, insulin resistant, and develop severe diabetes from a young age of 6 weeks (Clee, Nadler, & Attie, 2005). $B6^{ob/ob}$ mice also become obese and insulin resistant at 6 weeks of age. However, $B6^{ob/ob}$ mice remain diabetes-resistant up to 16 weeks of age, because they compensate for the obesity-induced insulin resistance via stimulation of beta cell proliferation and increased insulin secretion. Thus, the $B6^{ob/ob}$ mice is an optimal mouse model for studying beta cell proliferation in adaptation to obesity (Clee et al., 2005).

Interestingly, expression of *Hnf4α* is upregulated in islets of $B6^{ob/ob}$ mice at 10 weeks of age, when the mice exhibit adaptive beta cell proliferation and hyperinsulinemia (Keller et al., 2008). In contrast, expression of *Hnf4α* is not upregulated in the $BTBR^{ob/ob}$ mice at the same age, in which failure of adaptive beta cell proliferation leads to diabetes (29). Therefore, I hypothesize that upregulation of *Hnf4α* expression in $B6^{ob/ob}$ mice plays an essential role in regulating beta cell replication in adaptation to obesity. In addition, HNF-4α may regulate both insulin secretion and beta cell replication in an obese mouse model.

Results

Generating *Hnf4a*$^{L/L}$*; Pdx1-Cre*^{Late}*; ob/ob* mice with a congenic B6 background

In order to investigate the role of HNF-4α on beta-cell function in adaptation to obesity-induced insulin resistance, I bred *Hnf4α*^{loxP} mice on a B6 background, which has the maximal beta cell proliferation capacity (Clee et al., 2005). *Hnf4α*$^{L/+}$*; Pdx1-Cre*^{Late} mice, which were originally on a 129 genetic background, were backcrossed for six generations to B6 mice to produce B6 congenic mice. Congenic mice strains are conventionally derived by backcrossing a donor strain (129) that harbors a gene allele of interest (*Hnf4α*^{loxP}) with a recipient inbred strain (B6) for at least ten generations. According to Mendel's law, ten generations of backcrossing results in mice heterozygous for the locus of interest on a genome which is approximately 99.90% identical to that of the recipient genome. This traditional method, which takes 3 years to complete, has been advanced by a speedy and efficient genetic polymorphic marker-based breeding strategy (Markel et al., 1997). The optimal breeders of each generation are selected based on a screen for microsatellite markers that have different sizes in the donor strain and the recipient strain. The pups that have the least amount of donor markers are used for the next backcross (Fig 3.2a).

Hnf4α$^{L/+}$*; Pdx1-Cre*^{Late} mice were backcrossed with B6 mice for six generations. Pups of each backcrossing were genotyped for microsatellite markers flanking the *Hnf4α* locus. Pups with B6 genetic markers closest to *Hnf4α* locus were selected for the next backcross (Fig 3.2b). *Hnf4α*$^{L/+}$*; Pdx1-Cre*^{Late} mice with B6 congenic background were then bred with B6$^{ob/+}$ mice to finally generate control *Hnf4α*$^{L/L}$*; ob/ob* mice and mutant *Hnf4a*$^{L/L}$*; Pdx1-Cre*^{Late}; *ob/ob* mice. All genotypes were determined by PCR (Fig 3.2c).

The *Pdx1-Cre*^{Late} transgene is not expressed in early pancreatic progenitors, in pancreatic ducts, or acinar cells, like other *Pdx1-Cre* transgene (Herrera, 2000). Rather, it is specifically expressed and efficiently ablates loxP-flanked DNA sequences in mature beta cells, thus providing a beta-cell specific *Hnf4a* ablation model.

Pdx1-Cre^{Late} recombinase fails to excise *Hnf4α* loxP flanked site

After obtaining control *Hnf4a*$^{L/L}$*; ob/ob* mice and mutant *Hnf4a*$^{L/L}$*; Pdx1-Cre*^{Late}; *ob/ob* mice, I first determined whether *Hnf4α* was ablated in the beta cells. Since mouse islets are 80% beta cells, whole islets were isolated to measure *Hnf4α* expression. Surprisingly, q-PCR analysis of mRNA isolated from islets showed no ablation of *Hnf4α* in islets from mutant mice compared to islets from control mice (Fig 3.3a). I also measured mRNA expressions of several HNF-4α target genes, including *Hepatocyte nuclear factor 1 homeobox α* (*Hnf1α)*, *Live pyruvate kinase* (*LPK*), *Kir6.2*, and *Glucokinase* (*Gck*) (Boj, Parrizas, Maestro, & Ferrer, 2001). Their expressions also remain the same in mutant mice compared to control mice (Fig 3.3a). In addition, both western blot and immunostaining for HNF-4α in islets confirmed on the protein level that *Hnf4α* was not ablated in islets of *Hnf4a*$^{L/L}$*; Pdx1-Cre*^{Late}; *ob/ob* mutant mice (Fig 3.3b,c).

To troubleshoot the failure of gene ablation, *Pdx1-Cre*^{Late} transgenic mice were tested for the presence of Cre recombinase. First, genotyping primers were designed to make sure that *Pdx1-Cre*^{Late} transgenic mice were not confused with *Pdx1-Cre*^{ER} transgenic mice during breeding. Since the *Pdx1-Cre*^{Late} transgene differs from the *Pdx1-Cre*^{ER} transgene following the *Cre* sequence, specific primers were designed to differentiate these two *Cre* transgenes (Fig 3.4a). Mice within my colony, presumably with the *Pdx1-*

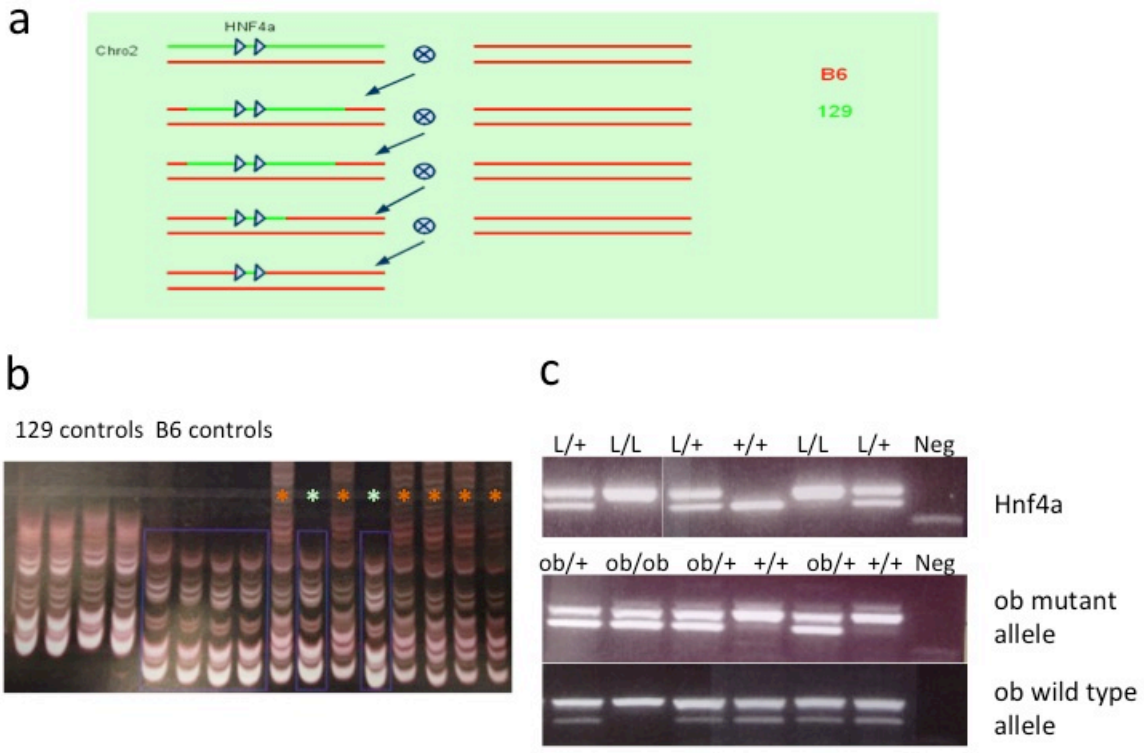


Fig 3.2 Generating congenic B6 *Hnf4a*$^{L/L}$*; Pdx1-Cre*^{Late}; *ob/ob* mice. **a** Backcrossing *Hnf4α*$^{L/+}$ 129 strain mice to B6 mice to derive congenic *Hnf4α*$^{L/+}$ B6 mice. **b** Genotyping of pups for microsatellite polymorphism markers in the donor 129 strain and the recipient B6 strain. From left to right are four 129 controls, four B6 control, and eight pups. Pups with B6 genetic markers are labeled with light green stars and pups with both 129 and B6 genetic markers are labeled with orange stars. Mice with B6 markers are chosen for the next generation of backcrossing. **c** Top panel: Genotyping for *Hnf4α* loxP allele and wild type allele. Lower panel: Genotyping for *ob* mutant allele and *ob* wild type allele.

Cre^{Late} transgene, and mice of *Pdx1-Cre*^{ER} colony were genotyped. Accurate sizes of PCR products from *Pdx1-Cre*^{Late} mice were produced, indicating that the correct *Cre* line was presented in my mouse colony (Fig 3.4b).

Next, I tested the recombinase activity of Pdx1-Cre^{Late}. *Pdx1-Cre*^{Late} mice were crossed with *Rosa-loxP-STOP-loxP-YFP* (shortened as *Rosa-YFP*) mice to generate *Pdx1-Cre*^{Late}*; Rosa-YFP* mice. Upon Cre recombination, a loxP flanked STOP codon is excised, which leads to expression of YFP in Cre^{+} cells and all progeny of Cre^{+} cells. This is a powerful system to visualize the expression pattern of specific Cre recombinases. Immunostaining of YFP demonstrates that *Pdx1-Cre*^{Late} is not active in any islet beta cells as expected (Fig 3.4c). One slide of liver tissue positive for YFP was used as control to verify that the YFP staining was effective (Fig 3.4c). In summary, the *Pdx1-Cre*^{Late} recombinase is silenced, which occasionally happens to transgenes for variable reasons (Bestor, 2000; Chevalier-Mariette et al., 2003; Garrick, Fiering, Martin, & Whitelaw, 1998). This explains the failure of *Hnf4α* ablation with this *Pdx1-Cre*^{Late} line.

Following these results, I went on to validate the *Hnf4α* loxP allele with another Cre recombinase. Adenovirus-associated virus packaged Cre (AAV-Cre) were injected retro-orbitally into *Hnf4α*$^{L/L}$*; ob/+* mice to induce *Hnf4α* ablation in the liver. AAV-GFP was also injected in a separate group of mice as a control. Eleven days after injection of either AAV-Cre or AAV-GFP, mice were sacrificed and liver tissues were collected. Western blot of liver lysis demonstrates efficient ablation of HNF-4α on AAV-Cre injected mouse comparing to AAV-GFP injected mice or mice without injection (Fig 3.5a). Furthermore, liver sections were immunostained for HNF-4α, and AAV-Cre injected mice exhibited

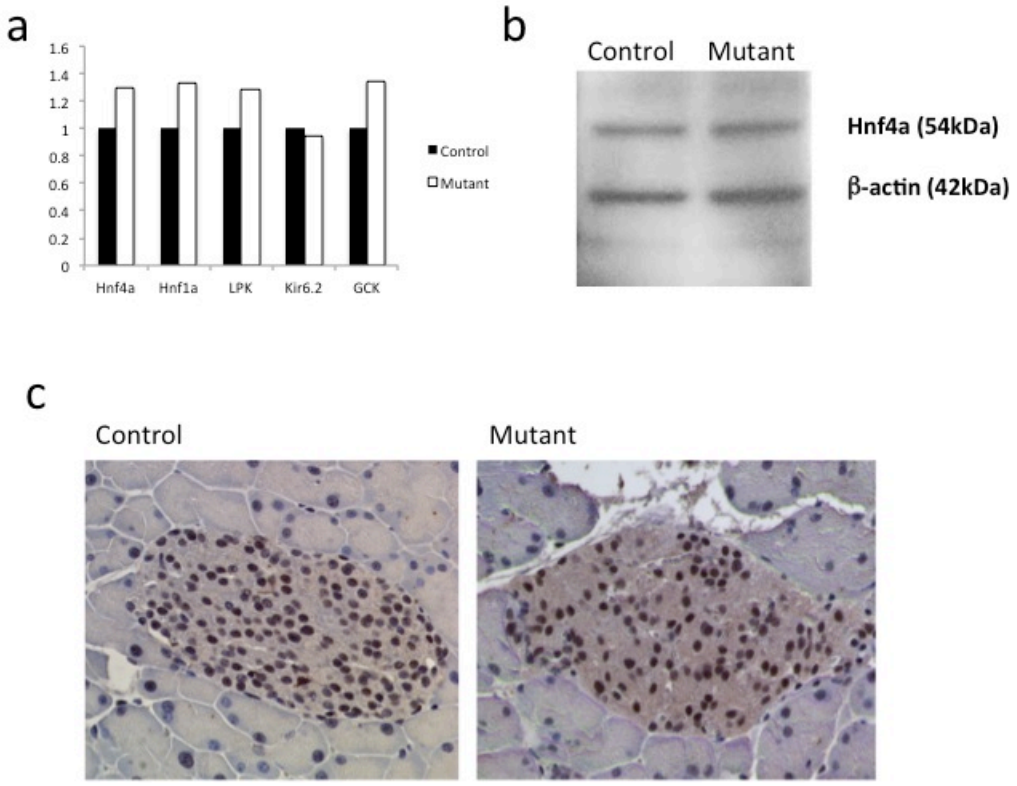


Fig 3.3 *Pdx1-Cre*^{Late} recombinase failed to excise *Hnf4α* loxP flanked site. **a** q-PCR analysis for mRNA expression of *Hnf4α* and its target genes. mRNA were isolated from islets of control *Hnf4α*$^{L/L}$*; ob/ob* mice and mutant *Hnf4a*$^{L/L}$*; Pdx1-Cre*^{Late}; *ob/ob* mice. No differences in gene expression were observed between control and mutant islets. **b** Western blot shows no difference of HNF-4α protein level between islets of control and mutant mice. **c** Immunostaining of pancreas sections shows no ablation of HNF-4α protein in islets of mutant mice.

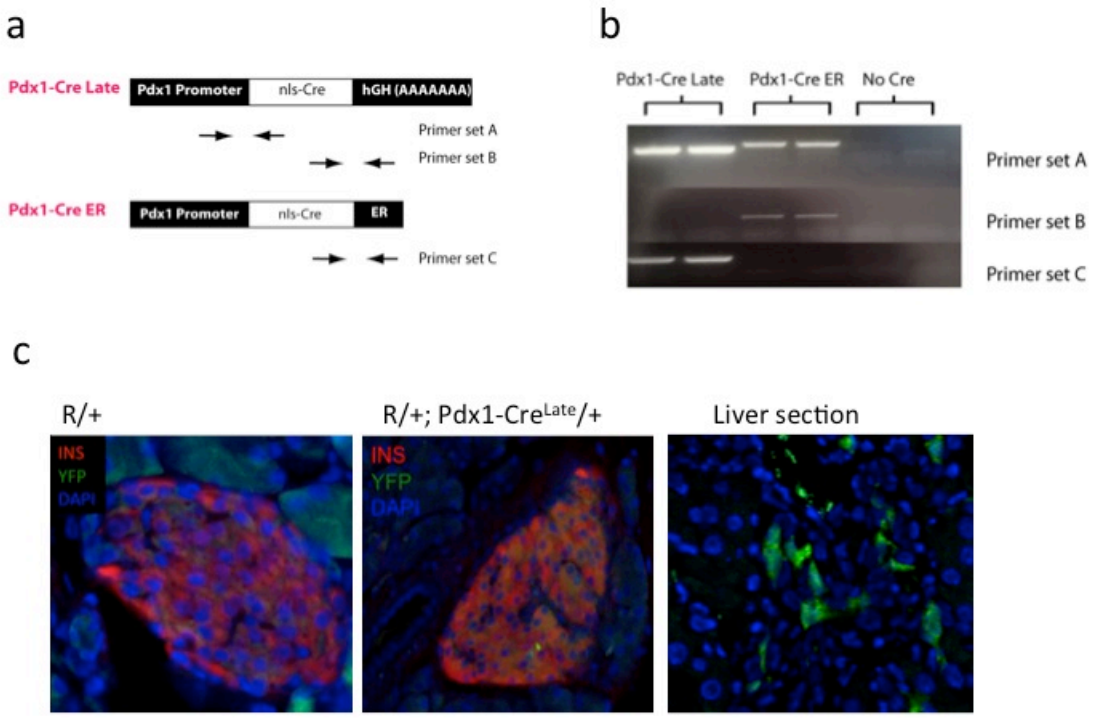


Fig 3.4 *Pdx1-Cre*^{Late} recombinase is silenced. **a** Comparison of the *Pdx1-Cre*^{Late} transgene and the *Pdx1-Cre*^{ER} transgene constructs, which differ following the *Cre* sequence. PCR primers were designed to differentiate between these two Cre lines. **b** Genotyping of *Pdx1-Cre*^{Late} mice and *Pdx1-Cre*^{ER} mice. *Pdx1-Cre*^{Late} gene has bands with primer sets A and C, while *Pdx1-Cre*^{ER} gene has bands with primer sets A and B. **c** *Pdx1-Cre*^{Late}; *Rosa-YFP* mice were generated and the expression pattern of *Pdx1-Cre*^{Late} recombinase was examined. The results show that *Pdx1-Cre*^{Late} recombinase was silenced because there was no YFP expression in beta cells. On the far right is a YFP^{+} liver section to validate the effectiveness of the YFP staining.

very efficient ablation of HNF-4α protein in more than 90% of the hepatocytes, indicating that *Hnf4α* loxP allele is susceptible to Cre recombination (Fig 3.5b).

Generating *Hnf4α*$^{L/L}$*; Mip-Cre*^{ER}*; ob/ob* mice

Since *Pdx1-Cre*^{Late} is silenced in my mouse colony, I bred *Mip-Cre*^{ER} mice with *Hnf4α*$^{L/L}$ mice and generated control *Hnf4a*$^{L/L}$*; ob/ob* mice and mutant *Hnf4α*$^{L/L}$*; Mip-Cre*^{ER}*; ob/ob* mice for further studies. *Mip-Cre*^{ER} is an inducible Cre line driven by the mouse insulin promoter (Wicksteed et al., 2010a). Cre^{ER} translocates into the nucleus and activates recombination only upon administration of tamoxifen, an estrogen receptor ligand.

First, I verified *Mip-Cre*^{ER} recombinase efficiency by crossing *Mip-Cre*^{ER} mice with *Rosa-YFP* mice. Tamoxifen was injected 2mg/day for three days into *Mip-Cre*^{ER}*; Rosa-YFP* mice. Three days after the last injection, mice were sacrificed and the pancreata were isolated, fixed, and sectioned. Pancreas sections were co-staining for insulin and YFP. *Mip-Cre*^{ER}*; Rosa-YFP* mice exhibit YFP positive staining in a large portion of their beta cells, indicating efficient Cre recombination (Fig 3.6a).

Next, I tested the *Hnf4α* ablation efficacy in *Hnf4α*$^{L/L}$*; Mip-Cre*^{ER}*; ob/ob* mice with *Hnf4a*$^{L/L}$*; ob/ob* mice as controls. To induce Cre recombination and *Hnf4α* ablation in beta cells, I injected 2mg/day tamoxifen for two successive days. Mice were sacrificed nine days after the last tamoxifen injection and pancreas tissues were harvested. Immunostaining of pancreas sections revealed a partial ablation of HNF-4α in mutant islets. This partial ablation could be caused by mosaic activity of the Cre^{ER} recombinase, or a relatively long half-life of the HNF-4α protein (Fig 3.6b).

To test the effects of prolonged tamoxifen administration, a tamoxifen pellet was

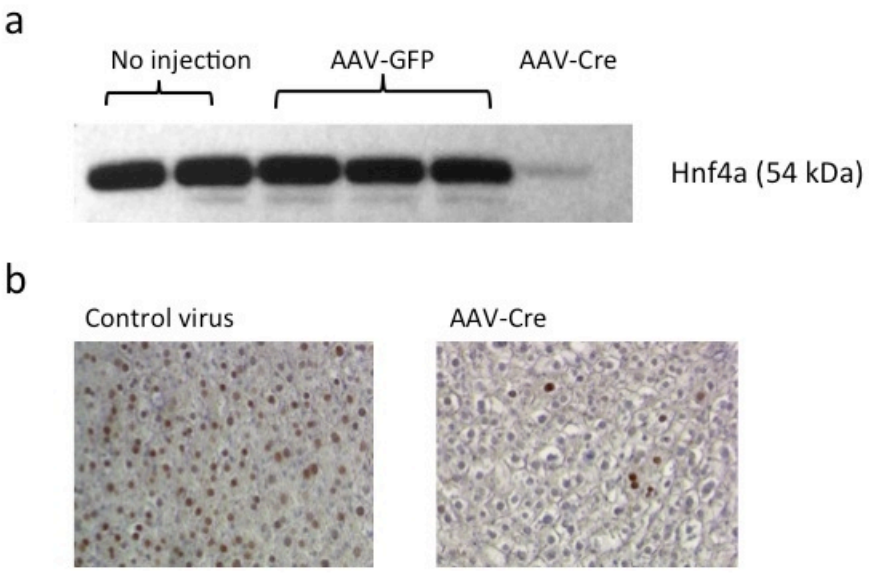


Fig 3.5 Validation of the *Hnf4α* loxP allele. **a** AAV-Cre were injected retro-orbitally into *Hnf4a*$^{L/L}$*; ob/+* mice to induce *Hnf4α* ablation in the liver. AAV-GFP was used as control virus. Western blot of lysed liver tissue showed efficient ablation of HNF-4α protein in AAV-Cre injected mouse. **b** Immunostaining of liver sections also proved efficient ablation of HNF-4α protein in >90% of hepatocyte supon AAV-Cre injection.

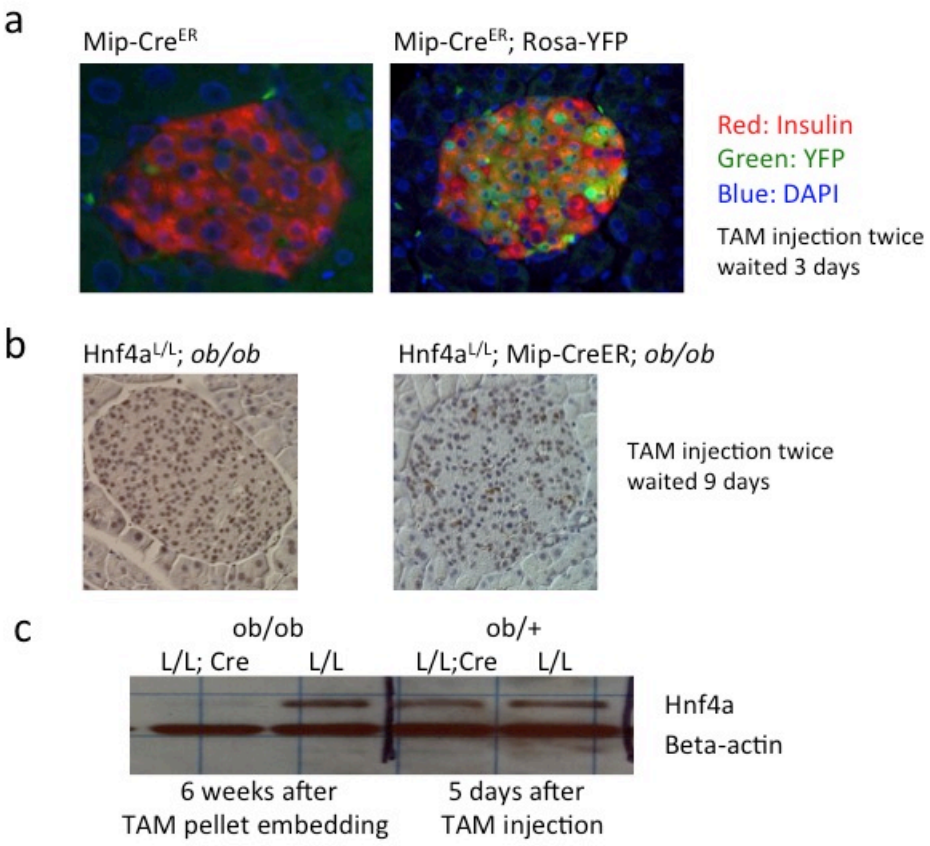


Fig 3.6 *Hnf4α* is ablated in islets of *Hnf4α*$^{L/L}$*; Mip-Cre*^{ER}*; ob/ob* mice. **a** *Mip-Cre*^{ER} mice were crossed with *Rosa-YFP* mice to evaluate Cre recombination efficiency. Three days after two injections of tamoxifen, mice were sacrificed. Immunostaining of pancreas sections reveal YFP expression in the majority of beta cells, suggesting that *Mip-Cre*^{ER} is effective in pancreatic beta cells. **b** *Hnf4a*$^{L/L}$*; ob/ob* mice and *Hnf4α*$^{L/L}$*; Mip-Cre*^{ER}*; ob/ob* mice were injected with tamoxifen to test *Hnf4α* ablation. Nine days after tamoxifen injection, mice were sacrificed. Partial ablation of HNF-4α protein is observed in pancreas sections of mutant mice compared to control mice. **c** Western blot of HNF-4α in islets of control and mutant mice. Six weeks after tamoxifen pellet embedding, HNF-4α protein is efficiently ablated in islets of mutant mice. In addition, partial ablation is observed in mutant mice five days after injection, indicating a relatively long half-life of HNF-4α protein. Furthermore, *ob/ob* mice have higher HNF-4α protein levels than *ob/+* mice, confirming that *Hnf4α* is upregulated upon obesity.

embedded under the skin of both control mice and mutant mice. These pellets allow 21 days of tamoxifen release, at the rate of about 1mg of tamoxifen per day. Six weeks after pellet embedding, islets were isolated for protein extraction. Western blot confirmed the ablation of HNF-4α protein in mutant mice (Fig 3.6c). In addition, control and mutant mice heterozygous for the *ob* mutation (*Hnf4α*$^{L/L}$*; ob/+*, and *Hnf4α*$^{L/L}$*; Mip-Cre*^{ER}*; ob/+*, respectively) were injected with tamoxifen and sacrificed five days later. Partial ablation of HNF-4α protein was observed in mutant mice compared to control mice, consistent with previous results that HNF-4α protein has a relatively long half-life. It is worth noting that there seems to be higher HNF-4α protein level in control *ob/ob* mice than in control *ob/+* mice, confirming that *Hnf4α* expression is positively correlated with increased body weight and increased metabolic demand for beta cell proliferation.

HNF-4α is required for beta cell proliferation in obese mouse

B6$^{ob/ob}$ mice have increased insulin secretion from 7 to 14 weeks of age, which helps prevent the severe hyperglycemia normally associated with obesity-induced insulin resistance (Clee et al., 2005). The most rapid upregulation of insulin secretion in male B6$^{ob/ob}$ mice occurs from 7 to 10 weeks of age. Interestingly, *Hnf4a* expression is also upregulated in islets of B6$^{ob/ob}$ mice at this timepoint (Keller et al., 2008). Therefore, I hypothesized that 7 to 10 weeks of age is the time window during which beta cell proliferation increases the most, in an HNF-4α-dependent manner, and leads to beta cell mass expansion. Indeed, control *ob/ob* mice possess huge islets and higher percentage of BrdU positive cells at 10 weeks of age compared to lean mice (data not shown). In order to investigate the role of HNF-4α in the adaptive beta cell proliferation in response to obesity, I induced *Hnf4α* ablation from 7-week old male *ob/ob* mice, and analyzed the

mice at 10 weeks of age.

Both control (*Hnf4a*$^{L/L}$*; ob/ob*) and mutant (*Hnf4α*$^{L/L}$*; Mip-Cre*^{ER}*; ob/ob*) 7 weeks old mice were embedded with tamoxifen pellets, which allow for 21 days of continuous tamoxifen release. At 10 weeks of age, mice were fasted overnight for glucose tolerance tests. Pancreas sections were immunostained for Ki67, a marker for proliferative cells (Fig 3.7a). Nine control and seven mutant mice with comparable body weight were analyzed. About 3000 beta cells were manually counted for each mouse. Beta cell proliferation rate was calculated as the ratio of Ki67/insulin double positive cells divided by the total number of insulin^{+} cells. Interestingly, mutant mice have a lower beta cell replication rate than control mice (Fig 3.7b). This result demonstrates for the first time that HNF-4α is required for beta cell proliferation in obesity. However, no difference in beta cell mass is observed between control and mutant mice (Fig 3.7c).

Since HNF-4α has been shown to regulate glucose tolerance by promoting insulin secretion, I measured fasting and fed glucose levels of both groups, and there are no significant differences. Glucose tolerance tests were performed following overnight fasting with 1mg/g body weight glucose injection. No difference in glucose tolerance is observed between control and mutant mice, indicating that the effects of decreased beta cell proliferation for three weeks are not severe enough to affect glucose homeostasis.

Overexpression of *Hnf4α* has no protective role in HFD induced hyperglycemia

HNF-4α is required not only for beta cell metabolic functions, but also for the proliferative response of the beta cell to increased metabolic demand (Gupta et al., 2007). I hypothesized that overexpressing *Hnf4α* would promote beta cell proliferation *in vivo*,

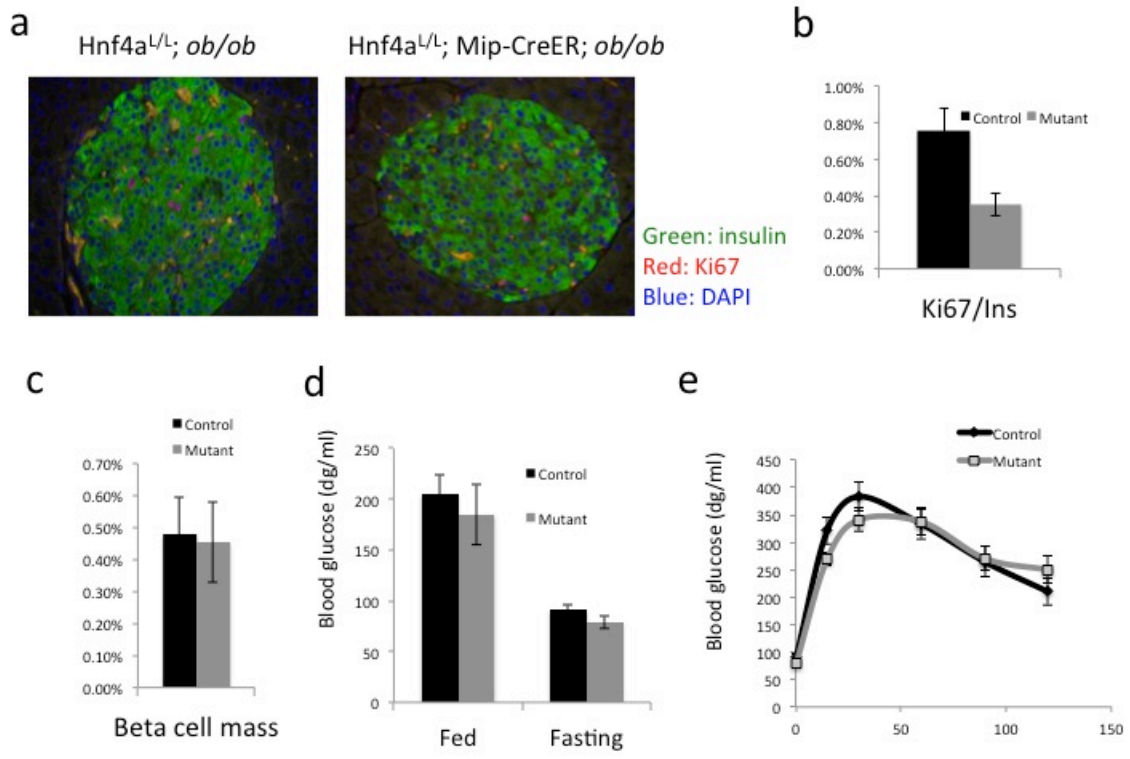
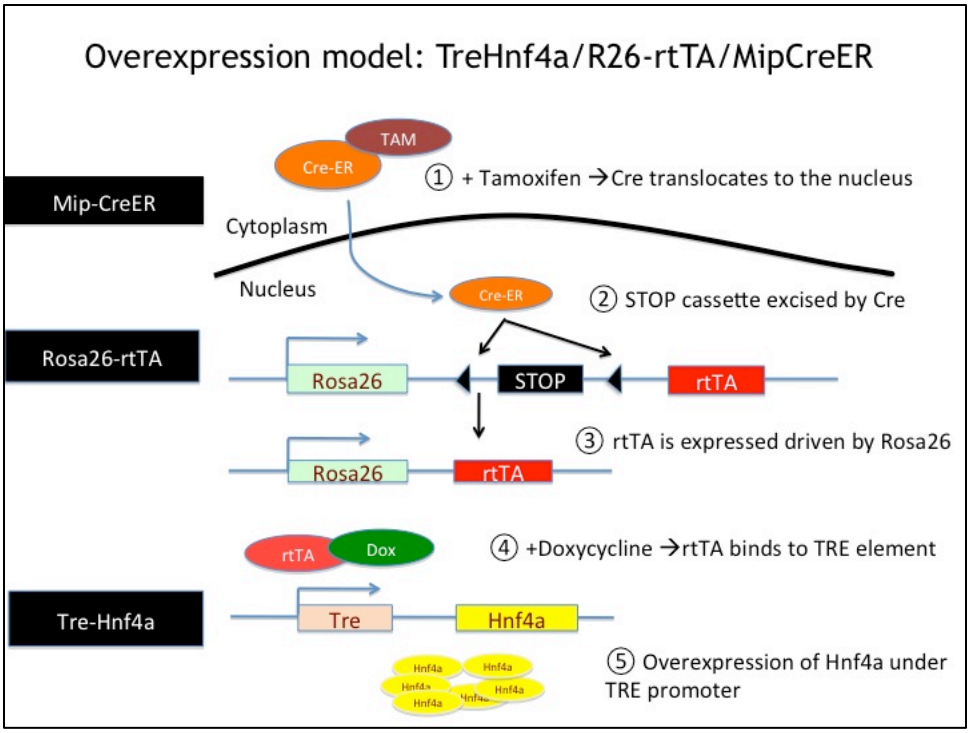


Fig 3.7 *Hnf4α* ablation in beta cells results in reduced beta cell proliferation. **a** Immunostaining of Ki67 and insulin in control and mutant *ob/ob* mice. Male mice were embedded with tamoxifen pellets at 7 weeks of age and sacrificed at 10 weeks of age. **b** Beta cell proliferation rates were calculated as the ratio of the number of $Ki67^{+}$/$Insulin^{+}$ cells divided by the total number of $insulin^{+}$ cells. 9 control mice and 7 mutant mice were analyzed. About 3000 beta cells were manually counted for each mouse. **c** Beta cell mass analysis reveals no difference between control and mutant mice. **d** Fed and fasting glucose levels of control and mutant mice are similar. **e** Glucose tolerance tests were performed on 10-weeks old control and mutant mice. 1mg/g body weight of glucose was injected after overnight fasting. No difference in glucose tolerance is observed between the two groups.

which may result in improved glucose tolerance. Two mouse models of overexpressing *Hnf4α* were designed. First, I bred triple transgenic lines of *Tre-Hnf4α/R26-rtTA/MipCre*^{ER} mice (Fig 3.8a). The *Mip-Cre*^{ER} recombinase is activated specifically in beta cells by administration of tamoxifen as mentioned previously, and results in excision of the stop cassette from the *Rosa26-rtTA* locus. Once the stop cassette is excised, the *Rosa26* promoter induces the expression of rtTA (reverse tetracycline transcriptional activator) solely in beta cells. Treatment with doxycycline activates rtTA, promotes its binding to the TRE element, and turns on expression of the *Tre-Hnf4α* transgene specifically in the beta cells (Balcazar et al., 2009; Rachdi et al., 2006). Unfortunately, no triple transgenic pups were born. Therefore, I dropped this line and proceeded with the second overexpression model, the *Tre-Hnf4α/Rip-rtTA* mouse line.

In *Tre-Hnf4α/Rip-rtTA* mice, *rtTA* is expressed specifically in the beta cells under the *rat-insulin-promoter* (*Rip*). Upon administration of doxycycline, rtTA binds to TRE element, which in turn promotes *Hnf4α* overexpression solely in beta cells (Fig 3.8b). Mice were given 2mg/ml doxycycline in drinking water when they were 12 weeks old. Four weeks later, islets of both control mice (*Tre-Hnf4α*) and overexpression mice (*Tre-Hnf4α; Rip-rtTA*) were isolated, and relative expression of *Hnf4α* was measured by qPCR on islet mRNA. *Human-Hnf4α8*, which is the transgene under the *Tre* promoter, is overexpressed 9 fold in islets of overexpressing group compared to islets of the control group (Fig 3.9a). Endogenous *mouse-Hnf4α* exhibited no change in expression. However, expressions of several HNF-4α targets are not up-regulated upon *Hnf4α* overexpression, including *Kir6.2* and *St5* (Fig 3.9a). Glucose tolerance tests were performed on *Hnf4α*


Overexpression model: TreHnf4a/R26-rtTA/MipCreER
TAM
Cre-ER
① + Tamoxifen →Cre translocates to the nucleus
Mip-CreER
Cytoplasm
Nucleus
Cre-ER
② STOP cassette excised by Cre
Rosa26-rtTA
Rosa26
STOP
rtTA
③ rtTA is expressed driven by Rosa26
Rosa26
rtTA
rtTA
Dox
④ +Doxycycline →rtTA binds to TRE element
Tre-Hnf4a
Tre
Hnf4a
⑤ Overexpression of Hnf4a under TRE promoter

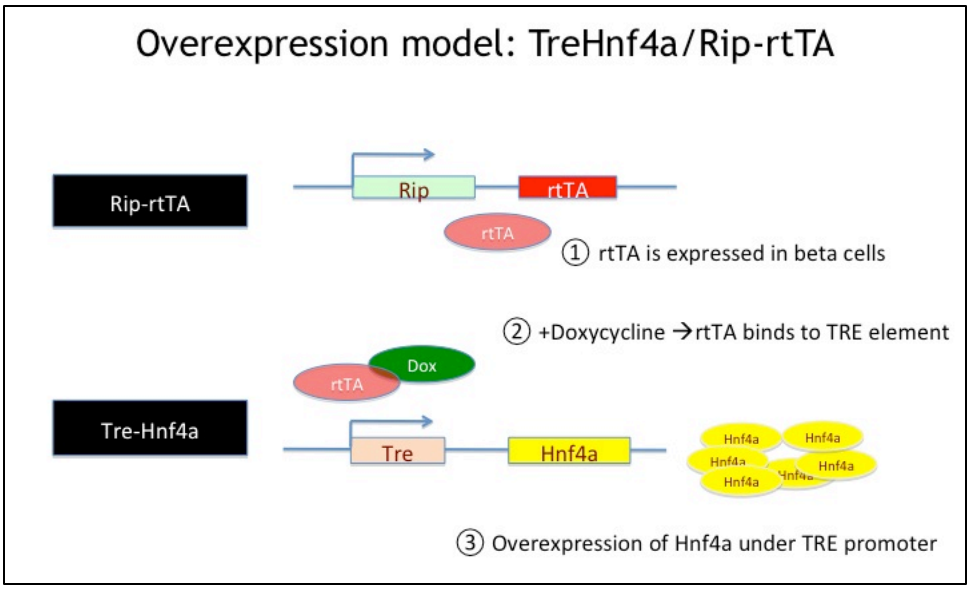

Overexpression model: TreHnf4a/Rip-rtTA
Rip-rtTA
Rip
rtTA
rtTA
① rtTA is expressed in beta cells
② +Doxycycline →rtTA binds to TRE element
Dox
rtTA
Tre-Hnf4a
Tre
Hnf4a
③ Overexpression of Hnf4a under TRE promoter

Fig 3.8 *Hnf4α* overexpression mouse models. **a** *Tre-Hnf4α /R26-rtTA/MipCre*^{ER} mouse model. The *Mip-Cre*^{ER} recombinase is expressed specifically in beta cells. After administration of tamoxifen, the Cre^{ER} recombinase translocates into the nucleus, and results in excision of the stop cassette from the *Rosa26-rtTA* locus. Once the stop cassette is excised, the *Rosa26* promoter induces the expression of *rtTA* (*reverse tetracyclin transcriptional activator*) solely in beta cells. Treatment with doxycycline activates rtTA, promotes its binding to the TRE element, and induces overexpression of the *Tre-Hnf4α* transgene specifically in beta cells. **b** *Tre-Hnf4α /Rip-rtTA* mouse model. *rtTA* is expressed specifically in beta cells under the *rat-insulin-promoter* (*Rip*). Upon administration of doxycycline, rtTA binds to TRE element, which in turn promotes *Hnf4α* overexpression solely in beta cells.

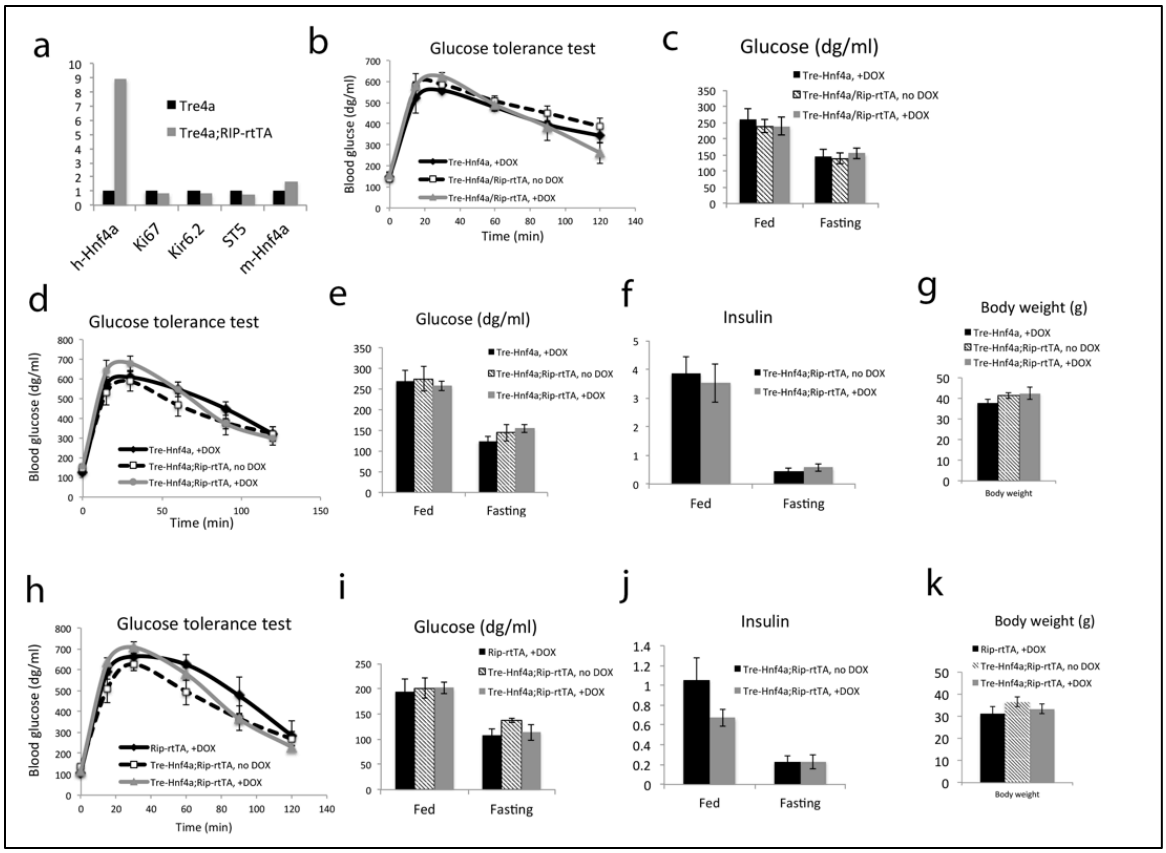

a
Tre4a
Tre4a;RIP-rtTA
h-Hnf4a
Ki67
Kir6.2
ST5
m-Hnf4a
b
Glucose tolerance test
Blood glucse (dg/ml)
Time (min)
Tre-Hnf4a, +DOX
Tre-Hnf4a/Rip-rtTA, no DOX
Tre-Hnf4a/Rip-rtTA, +DOX
c
Glucose (dg/ml)
Fed
Fasting
d
Glucose tolerance test
Blood glucose (dg/ml)
Time (min)
Tre-Hnf4a, +DOX
Tre-Hnf4a;Rip-rtTA, no DOX
Tre-Hnf4a;Rip-rtTA, +DOX
e
Glucose (dg/ml)
f
Insulin
g
Body weight (g)
Body weight
h
Glucose tolerance test
Rip-rtTA, +DOX
i
Glucose (dg/ml)
j
Insulin
k
Body weight (g)

Fig 3.9 *Hnf4α* overexpression has no protective role in HFD-induced hyperglycemia. **a** mRNA expression of *human-Hnf4α8*, *Ki67*, *Kir6.2*, *St5*, and *mouse-Hnf4α* in isolated islets from doxycycline treated control mice (*Tre-Hnf4α*) and overexpression mice (*Tre-Hnf4α; Rip-rtTA*). *Human-Hnf4α8* is upregulated for 9-fold in overexpression group. **b** Glucose tolerance tests of chow diet fed control mice and overexpression mice. n=4 of each group. All male mice were 4 months old. **c** Resting and fed glucose levels of chow diet fed control mice and overexpression mice. n=4 of each group. All male mice were 4 months old. Glucose tolerance test (**d**), resting and fasting glucose levels (**e**), resting and fasting insulin levels (**f**), body weight (**g**) of HFD-fed male mice. n=4-5 for each group. Glucose tolerance test (**h**), resting and fasting glucose levels (**i**), resting and fasting insulin levels (**j**), body weight (**k**) of HFD-fed female mice. n=4 for each group.

overexpression mice (*Tre-Hnf4α; Rip-rtTA* with doxycycline) and two control groups (*Tre-Hnf4α; Rip-rtTA* without doxycycline and *Tre-Hnf4α* mice with doxycycline). No differences in glucose tolerance were observed among the different groups (Fig 3.9b). Furthermore, fed and fasting glucose levels were measured and no differences were observed (Fig 3.9c). In summary, overexpressing *Hnf4α* is not sufficient to improve glucose tolerance under basal physiological conditions. This is not surprising since under basal conditions, there is no need for improvement of beta cell proliferation. Therefore, overexpressing a positive regulator of beta cell proliferation has no effect on glucose tolerance.

In order to challenge the mice with higher metabolic demand, mice were put on high fat diet for three months (one month old to four months old). Overexpression of *Hnf4α* was induced by doxycycline administration for a month (three to four months old). High fat diet successfully promoted obesity in both male and female mice, with male mice weighed around 40 grams and female mice weighed around 30 grams (Fig 3.9g,k). However, there were no differences in glucose tolerance (Fig 3.9d,h), fed and fasting glucose (Fig 3.9e,i), or fed and fasting insulin levels (Fig 3.9f,j) in *Hnf4α* overexpressing obese mice compared to control obese mice. In summary, *Hnf4α* overexpression does not provide protection against glucose intolerance in an obese mouse model.

Overexpression of *St5* has no effect on glucose tolerance or beta cell proliferation

HNF-4α regulates the Ras/ERK pathway to promote beta cell proliferation in an ST5-dependent manner during pregnancy (9). *St5* is a direct transcriptional target of HNF-4α and its expression decreases in *Hnf4α*-deficient islets. *In vitro* studies show that ST5 positively regulates Ras/ERK pathway. Therefore, I hypothesized that

overexpression of *St5* in beta cells might be exploited to increase beta cell proliferation via augmented Ras/ERK signaling.

The experimental strategy used to overexpress *St5* was similar to the *Hnf4α* overexpression model. Both control (*Tre-St5*) and overexpression (*Tre-St5/Rip-rtTA*) mice were given doxycycline in drinking water for 3 weeks (7 to 10 weeks old). Since mice have shown resistance to doxycycline drinking water and lagging of body weight growth compared to mice on normal water in a previous study, 2.5% sucrose was added into drinking water to improve taste. Islet mRNA from both control and *St5* overexpression mice were isolated. qPCR shows dramatic overexpression of *human-St5* in *Tre-St5/Rip-rtTA* mouse islets while endogenous *mouse-St5* expression remains the same, relative to controls (Fig 3.10a). Immunostaining of pancreas sections confirms the extensive upregulation of ST5 protein in beta cells of the overexpression group (Fig 3.10b). In control mice, ST5 protein is undetectable in islets. In the overexpression group, ST5 exhibited a mosaic overexpression pattern, with approximately 60% of beta cells producing high levels of ST5 protein (Fig 3.10b).

After validating the *St5* overexpression mouse model, I performed glucose tolerance tests to investigate the effects of beta cell specific *St5* overexpression on glucose homeostasis. 10 weeks old male mice from both control and overexpression group were tested and there is no difference between glucose tolerance or resting and fasting glucose (Fig 3.10c,d). Female mice also display no significant differences (Fig 3.10e,f). Next, I examined the effects of *St5* overexpression on beta cell proliferation. Ki67 and insulin immunostaining were used as markers for replicating cells and beta cells, respectively. Five control female mice and five *St5* overexpression female mice were

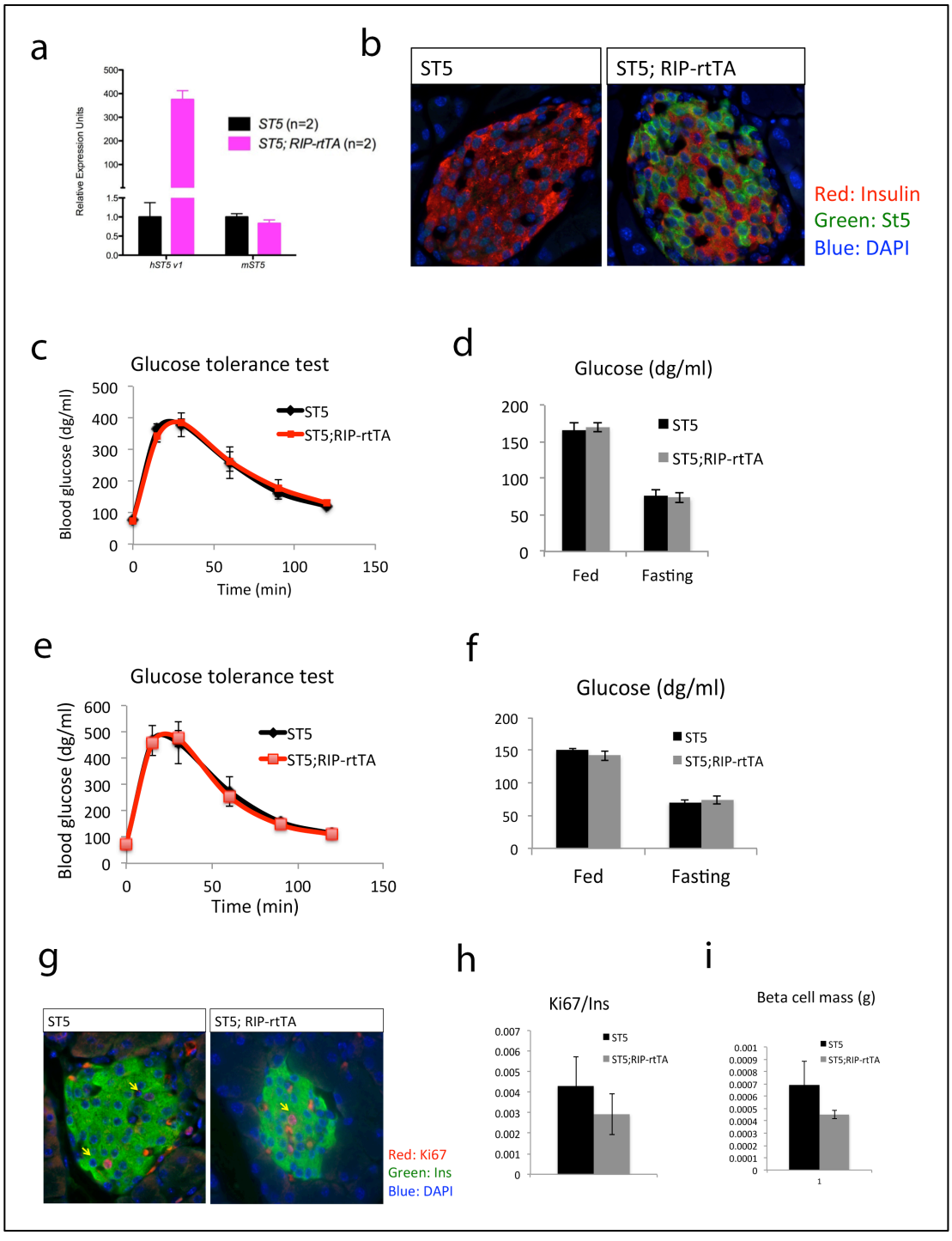

a
Relative Expression Units
ST5 (n=2)
ST5; RIP-rtTA (n=2)
hST5 v1
mST5
b
ST5
ST5; RIP-rtTA
Red: Insulin
Green: St5
Blue: DAPI
c
Glucose tolerance test
Blood glucose (dg/ml)
ST5
ST5;RIP-rtTA
Time (min)
d
Glucose (dg/ml)
ST5
ST5;RIP-rtTA
Fed
Fasting
e
Glucose tolerance test
Blood glucose (dg/ml)
ST5
ST5;RIP-rtTA
Time (min)
f
Glucose (dg/ml)
ST5
ST5;RIP-rtTA
Fed
Fasting
g
ST5
ST5; RIP-rtTA
Red: Ki67
Green: Ins
Blue: DAPI
h
Ki67/Ins
ST5
ST5;RIP-rtTA
i
Beta cell mass (g)
ST5
ST5;RIP-rtTA

Fig 3.10 Overexpression of *St5* has no effect on glucose tolerance or beta cell replication. **a** qPCR results demonstrated that *human-St5* is highly overexpressed in isolated mouse islets while endogenous *mouse-St5* expression is unaffected. **b** Immunostaining of pancreas sections reveals extensive upregulation of ST5 protein in islets of overexpression group. Glucose tolerance test (**c**) and resting and fasting glucose levels (**d**) of control and overexpression male mice at 10 weeks old. Glucose tolerance test (**e**) and resting and fasting glucose levels (**f**) of control and overexpression female mice at 10 weeks old. **g** Co-immunostaining of Ki67 and insulin in islets of both control and overexpression groups. **h** Quantification of proliferating beta cells. Proliferation rate was quantified as the ratio of the number of $Ki67^{+}$/$insulin^{+}$ cells divided by the total number of $insulin^{+}$ cells. n=4 for each group. All female mice were 10 weeks old. i Beta cell mass of control and overexpression mice. n=5 of each group. All mice were treated with doxycycline for 3 weeks (7 to 10 weeks of age). Data shown in **a** and **b** were generated by Jia Zhang.

analyzed, and no statistically differences in beta cell proliferation were observed between the two groups (Fig 3.10g,h). Beta cell mass was also measured, and there was no difference (Fig 3.10i). In stark contrast to my original hypothesis, there is actually a trend that *St5* overexpression might result in lower beta cell proliferation rate and lower beta cell mass.

Discussion

Previous studies in our lab have shown that HNF-4α is required for both beta cell function and beta cell proliferation (Gupta et al., 2007; Gupta et al., 2005). Mice lacking *Hnf4α* in beta cells are glucose-intolerant due to an impaired first-phase insulin secretory response (Gupta et al., 2005). Furthermore, HNF-4α also plays an essential role in the proliferative response of beta cells to the altered metabolic demands of pregnancy. Mice with beta cell specific *Hnf4a* ablation failed to replicate and expand beta cell mass during pregnancy, which led to lower insulin content and impaired glucose tolerance (Gupta et al., 2007). Mechanistic studies show that Ras/ERK signaling is downregulated in *Hnf4α* deficient beta cells (Gupta et al., 2007). ST5, a direct target of HNF-4α, is the molecular link between HNF-4α and Ras/ERK signaling. ST5 is homologous to known guanine exchange factors and its overexpression leads to increased ERK activation (Majidi, Hubbs, & Lichy, 1998). Min6 cells treated with *St5* shRNA display lower basal and EGF-stimulated p-ERK protein levels, suggesting that ST5 is a novel regulator of ERK signaling in beta cells.

Since *Hnf4α* deficiency is associated with both MODY1 in humans and gestational diabetes in mouse, it is important to investigate whether HNF-4α also plays a role in

obesity-induced beta cell compensation, which is much more relevant to human health. Interestingly, *Hnf4α* is upregualted in islets of B6$^{ob/ob}$ mice, which have compensatory beta cell proliferation during obesity, but not BTBR$^{ob/ob}$ mice, which fail to augment beta cell proliferation in obesity and become hyperglycemic. These results suggest that HNF-4α plays an important role in regulating beta cell replication during obesity-induced insulin resistance (Keller et al., 2008). Two obesity mouse models were investigated in my studies. The first was genetically induced obesity with a leptin gene mutation (*ob/ob* mouse model). The second was the high-fat-diet induced obesity mouse model.

HNF-4α is required for adaptive beta cell replication in *ob/ob* mice

In this study, I induced *Hnf4α* ablation in beta cells of obese mice to directly determine the requirement of HNF-4α in beta cell proliferation during obesity. B6$^{ob/ob}$ mice provide an ideal model for studying adaptive beta cell proliferation in response to obesity because they have only mild hyperglycemia achieved by upregulated beta cell proliferation between 6 to 14 weeks of age (31). Both control (*Hnf4a*$^{L/L}$*; ob/ob*) and mutant (*Hnf4α*$^{L/L}$; *Mip-Cre*^{ER}; *ob/ob*) mice were embedded with tamoxifen pellets from 7 to 10 weeks of age. Male mice were chosen for this study because they exhibit a higher capacity of beta cell compensation during obesity (31).

Immunostaining and western blot show >90% ablation of HNF-4α protein in islets from mutant mice. Staining for Ki67 in islets revealed a 50% proliferation rate in *Hnf4α* mutant beta cells compared to control beta cells, suggesting that HNF-4α is required for beta cell proliferation in obese mice. However, despite the deficiency in proliferation, beta cell mass remains the same in mutant mice compared to control mice. Additionally,

no differences are observed in fed or fasting glucose levels. Mutant mice also exhibit normal glucose tolerance. One possible explanation of the lack of physiological phenotype is that *ob/ob* mice gain more weight than lean mice from as early as two weeks of age (Q. Gao et al., 2007), indicating that their beta cells might start to increase proliferation at a very early age. Therefore, beta cell mass may have already been established when the mice are 7 weeks old. In this case, the reduced beta cell proliferation achieved by ablation of *Hnf4α* for three weeks might not be enough to lead to an observable decrease in beta cell mass. Two different approaches could be utilized to answer these remaining questions. First, prolonged *Hnf4α* ablation in *ob/ob* mice will provide more insight to the requirement of HNF-4α for maintenance of beta cell mass during obesity. Alternatively, it will also be informative to ablate *Hnf4α* from an earlier stage, such as two weeks of age, before beta cell mass starts to expand on the *ob/ob* background. In this way, the effect of *Hnf4α* ablation on beta cell proliferation might be more severe.

It will also be benificial to investigate the level of Ras/ERK signaling in *Hnf4α* mutant *ob/ob* mice, to determine whether HNF-4α regulates beta cell proliferation via the same pathway in obesity as during pregnancy. *St5* expression measurements in this model will help partially elucidate the mechanism of impaired beta cell proliferation in *Hnf4α*-deficient beta cells. Because the mechanisms of beta cell proliferation in obesity and pregnancy are not exactly the same (Rieck et al., 2009), it would be beneficial to investigate whether HNF-4α is involved in obesity-associated beta cell proliferation pathways. For example, high blood glucose levels and glycolytic pathways have been suggested to induce beta cell proliferation. Therefore, glycolytic pathway could be

investigated in *Hnf4α* mutant *ob/ob* beta cells by manipulating glucokinase by its activator. It would also be interesting to test the insulin secretion capacity of *Hnf4α* mutant beta cells in obese mice. This could be accomplished by isolating islets for glucose-stimulated insulin secretion assays, or by measuring insulin levels *in vivo* after injecting glucose. Since HNF-4α regulates insulin secretion by directly controlling *Kir6.2* expression, it would be valuable to compare the expression of *Kir6.2* in islets between control and mutant obese mice. A microarray on islet mRNA should be performed to compare the expression profiles between control and mutant mice. This will provide a comprehensive understanding of the role of the transcription factor HNF-4α in beta cell function during obesity.

Hnf4α overexpression is not sufficient to improve glucose tolerance in HFD induced obese mice

In this study, I investigated the role of HNF-4α on beta cell proliferation during obesity by overexpressing *Hnf4α* in beta cells of high-fat-diet induced obese mice. My hypothesis was that overexpressing *Hnf4α* would result in increased beta cell proliferation and improved glucose tolerance during obesity. Mice were given high-fat-diet from 1 to 4 months of age. Both control mice (*Tre-Hnf4α*) and *Hnf4α* overexpression mice (*Tre-Hnf4α; Rip-rtTA*) were administrated doxycycline in drinking water for the last 3 weeks of the high-fat-diet. qPCR results demonstrate that *human-Hnf4α8* is upregulated 9-fold in islets from the *Hnf4α* overexpression group compared to islets from the control group. Glucose tolerance and resting/fasting glucose levels are the same in control and *Hnf4α* mutant mice on chow-diet fed mice. Moreover, no differences are observed in

glucose tolerance, resting/fasting glucose, or resting/fasting insulin levels between the control and mutant groups on HFD (both males and females). Overall, these results suggest that *Hnf4α* overexpression is not sufficient to improve glucose homeostasis in obese mice.

It would be helpful to quantify beta cell proliferation in both control and mutant group. It is possible that overexpressing *Hnf4α* induces a small increase in beta cell proliferation, to an extent that no detectable physiological phenotype could be detected. A recent study in our lab demonstrates that *Hnf4α* overexpression in human islets results in increased beta cells entry into cell cycle; however, these cells fail to complete the cell cycle. Indeed, the rapidly proliferating beta cells harbored signs of DNA damage and apoptosis. It would be important to test if this is the case for *Hnf4α* overexpression in mouse beta cells.

St5 overexpression is not sufficient to increase beta cell proliferation

In this study, I investigated the effects of *St5* overexpression on beta cells proliferation and glucose homeostasis. Because ST5 is a positive regulator of ERK signaling, and ERK signaling is known to increase beta cell proliferation during mouse pregnancy, I hypothesized that overexpressing *St5* specifically in beta cells would cause increased beta cell proliferation. Both control mice (*Tre-St5*) and *St5* overexpression mice (*Tre-St5; Rip-rtTA*) were administrated doxycycline in drinking water from 7 to 10 weeks of age. Q-PCR results demonstrate that *human-St5* mRNA is upregulated more than 300-fold in islets from the overexpression group compared to islets from the control group.

Immunostaining confirmed the dramatic increase of ST5 protein in beta cells from the overexpression group.

Glucose tolerance and resting/fasting glucose levels are the same in the control and mutant groups, suggesting that *St5* overexpression is not sufficient to improve glucose homeostasis. Furthermore, no increase in beta cell proliferation or beta cell mass were observed between control and overexpression mice, suggesting that overexpressing *St5* in beta cells is not sufficient to induce beta cell proliferation.

It would be beneficial to repeat this study with an additional stress, such as pregnancy or obesity, to test whether ST5 plays a role in beta cell proliferation when there are higher metabolic demands. This is a possible outcome since ST5 is a knowb regulator of the ERK pathway, which has been shown to regulate beta cell proliferation during pregnancy.

Materials and methods

Mice: *Hnf4α* loxP mice and *Pdx1-Cre*^{Late} mice have been described previously(9). *Hnf4α*$^{L/+}$; *Pdx1-Cre*^{Late} mice were backcrossed with B6 mice for six generations to achieve congenic B6 background. Since *Hnf4α* is located at 94cM of chromosome 2, sets of genotyping primers containing polymorphisms between the donor 129 strain and the recipient B6 strain were generated at ~10cM intervals on chromosome 2, according to the list in Ref (Schalkwyk et al., 1999). After PCR of genomic DNA extracted from tails, non-denaturing 8% acrylamide gels were run as 45V for 3 hours at room temperature. Pups with the most acquisition of the recipient B6 gene background detected by the polymorphism markers were chosen for next generation backcrossing. This marker-

assisted breeding allowed us to generate congenic B6 mice in six generations instead of the conventionally used ten generations (Markel et al., 1997). After six generations, markers >8cM away from the *Hnf4α* locus were all from B6 background. In addition, these mice were all black. *Hnf4α*$^{L/+}$; *Pdx1-Cre*^{Late} mice were further crossed with B6$^{ob/+}$ mice to derive *Hnf4α*$^{L/+}$*; Pdx1-Cre*^{Late}*; ob/+* mice. These mice were crossed to produce mice homozygous for the *Hnf4α* loxP allele (*Hnf4a*$^{L/L}$*; Pdx1-Cre*^{Late}; *ob/+*). Since *ob/ob* mice are sterile, *ob/+* mice had to be bred to generate *ob/ob* mice. Specifically, *Hnf4a*$^{L/L}$*; Pdx1-Cre*^{Late}; *ob/+* mice were crossed with *Hnf4a*$^{L/L}$*; ob/+* mice to produce control *Hnf4a*$^{L/L}$*; ob/ob* mice and mutant *Hnf4a*$^{L/L}$*; Pdx1-Cre*^{Late}*; ob/ob* mice.

Hnf4α$^{L/L}$*; Mip-Cre*^{ER}*; ob/ob* mice were generated by crossing *Hnf4α*$^{L/L}$; *ob/+* mice with *Mip-Cre*^{ER} mice. To induce *Hnf4α* ablation, tamoxifen pellets (free base, 25 mg/pellet, 21 day release, E-361; Innovative Research of America) were implanted subcutaneously into the mice. Analysis was performed 3 weeks after implantation. Alternatively, 2mg/ml tamoxifen dissolved in sunflower oil was injected.

Pdx1-Cre^{Late} mice and *Mip-Cre*^{ER} mice were crossed with *Rosa-loxP-STOP-loxP-YFP* mice to determine Cre recombinase expression pattern and efficiency. *Tre-Hnf4α/R26-rtTA/MipCre*^{ER} mice were generated by crossing the three respective transgenic mice strains. *Tre-Hnf4α/Rip-rtTA* mice were generated by crossing the two respective transgenic mice strains. 1g/kg doxycycline was administrated in drinking water for both control (*Tre-Hnf4α*) and overexpressing (*Tre-Hnf4α; Rip-rtTA*) mice. Mice without doxycycline treatment (*Tre-Hnf4α; Rip-rtTA*) also served as controls. High-fat-diet was given to mice from 1 month to 4 months of age. Doxycyclin was given for the last 3 weeks of their lives. Mice were sacrificed at 4 months of age.

Tre-St5/Rip-rtTA mice were generated by crossing the two respective transgenic mice lines. 1g/kg doxycycline with 2.5% sucrose was administrated in drinking water for both control (*Tre-St5*) and overexpression (*Tre-St5; Rip-rtTA*) mice.

Islet isolation and real-time PCR: Islets were isolated using standard collagenase procedures followed by purification through a Ficoll gradient (Sigma-Aldrich Corp, Ficoll PM 400) as described previously (Kaestner et al., 2007). Islets were handpicked under a light microscope. Total RNA was isolated in Trizol (Life Technologies) and reversely transcribed using 1μg oligo (dT) primer, Superscript II Reverse Transcriptase, and accompanying reagents (Life Technologies). PCR reaction mixes were assembled using the Brilliant III SYBR Green QPCR Master Mix (Agilent Technologies, Inc., Santa Clara CA, USA). PCR reactions were performed on an Mx4000 Multiplex Quantitative PCR System (Agilent Technologies). All reactions were performed in triplicate with reference dye normalization. Median cycling threshold values were used for analysis.

Western Blotting: For western blots, islets or liver were isolated and lysed in lysis buffer containing 50mmol/l Tris (pH 8.0), 5mmol/l EDTA, 150mmol/l NaCl, 1% Triton, 1% SDS, 0.5% sodium deoxycholic acid and Complete Protease Inhibitor Cocktail Tablets (Genentech Roche, Newtown, PA, USA). Protein concentrations were measured by Bradford Assay using SpectraMax Plus 384 (Molecular Devices). Total lysate were heated at 95º C for 10 min and loaded on 4-12% Bis-Tris gel (Novex, Wadsworth, OH, USA). Proteins were transferred to PVDF membranes by iBlot Dry Blotting System (Life Technologies) and detected by antibodies against Hnf4α (R&D) and rat anti-beta-actin

(Cell Signaling). The ECL-Plus detection system (GE Healthcare Biosciences, Pittsburgh, PA, USA) was used to detect the signal.

Immunostaining: Pancreata were dissected, flattened by forceps, fixed in 4% para-formaldehyde for 24 hours, and paraffin embedded so that tissues with the maximum pancreatic footprint were sectioned. Tissues were sectioned to 5μm thickness. Deparaffinized and rehydrated slides were subjected to antigen retrieval by pressure cooker in 10mmol/l pH 6.0 citric acid buffer. For beta cell proliferation, simultaneous immunofluorescent staining was performed for insulin and BrdU, or insulin and Ki67. Beta cell proliferation rate was quantified as the number of BrdU(or Ki67)/ insulin double positive cells divided by insulin positive cells. For beta cell mass, three sections (40 μm apart) from each animal were immunostained for insulin using the standard DAB method without counterstaining. The entire tissue section was scanned with Meyer PathScan Enabler IV Histology Slide Scanner and Silverfast Pathscan 6.6 software. The percentage of beta cell area relative to the total pancreatic area was measured and calculated using ImageJ. Beta cell mass was derived from the total pancreas weight multiplied by the percentage of beta cell area. For Cre recombinase expression test, simultaneous immunofluorescent staining was performed for insulin and YFP.

Glucose tolerance test and insulin assay: Animals were fasted overnight and fasting and fed glucose levels were determined by glucometer. 2g/kg body weight of glucose (Sigma-Aldrich Corp., St. Louis, MO, USA) was injected intraperitoneally. For *ob/ob* mice, 1g/kg body weight of glucose was injected. Glucose levels were measured at 15, 30, 60,

90, and 120 minutes postinjection by Glucometer Breeze2 (Bayer AG, Leverkusen, Germany). To determine plasma insulin levels, blood was collected from the tail vein of mice before and after overnight fasting. Plasma insulin concentrations were measured by ELISA.

Chapter 4 Elevated hepatic betatrophin expression does not increase human beta-cell replication

Abstract

The recent discovery of the hormone betatrophin, a protein secreted by the liver and white adipose tissue in conditions of insulin resistance and shown to dramatically stimulate replication of mouse insulin-producing beta-cells, has raised high hopes for the rapid development of a novel therapeutic approach for the treatment of diabetes. However, at present the effects of betatrophin on human beta cells are not known. Here we employ administration of the insulin receptor antagonist S961, shown to increase betatrophin gene expression and stimulate beta-cell replication in mice, to test its effect on human beta cells. While mouse beta cells, both in their normal location in the pancreas and in islets transplanted under the kidney capsule, respond with a dramatic increase in beta cell DNA replication, engrafted human beta cells are completely unresponsive. These results put into question whether betatrophin can be developed as a therapeutic for human diabetes.

Results and Discussion

A common feature of both type 1 and type 2 diabetes is an insufficient number of insulin-producing beta cells in the endocrine pancreas. While promising in principle, targeted expansion of human beta-cells as a therapeutic strategy for diabetes has not yet been achieved (Bonner-Weir & Weir, 2005a). After beta cells have been specified from pancreas endocrine precursors in fetal life, postnatal beta cell mass expansion is the result of replication of preexisting beta cells, and is not dependent on tissue-resident progenitor cells, at least in rodents (Dor et al., 2004). The basic proliferation rate of beta cells in adult mammals is very low under normal physiological conditions, typically less than 1%

(Kaestner et al., 2007). However, when metabolically challenged, such as during pregnancy, diet-induced insulin resistance, and experimental beta cell ablation, beta cells have the capacity to expand by proliferation, at least in rodents (Heit, Karnik, & Kim, 2006a). In particular, hepatic insulin resistance has been shown decades ago to be a powerful promoter of beta-cell replication in mice (Bruning et al., 1997; Kido et al., 2000; Withers et al., 1998). Recently, increased glycolytic flux in beta cells, as occurs when blood glucose levels are elevated, was proposed as one mediator of increased beta-cell replication (Glaser et al., 2011). Whether additional signals contribute to beta-cell replication during the insulin resistant state remains unknown.

Using a pharmacological inhibitor of insulin action, Yi and colleagues screened for a potential secreted molecule that might represent the mitogenic signal to beta cells in conditions of insulin resistance (Yi et al., 2013). They identified betatrophin, also know as lipasin (R. Zhang, 2012) or angiopoetin-like 8 (Quagliarini et al., 2012), as a secreted protein synthesized in liver and to a lesser extent in white adipose tissue, as a potential beta cell mitogen (Yi et al., 2013). Betatrophin mRNA expression was induced four- to five-fold in the liver and white adipose tissue of mice treated with the insulin receptor antagonist S961 (L. Schaffer et al., 2008). This change in betatrophin expression following S961 administration was accompanied by a dose-dependent increase in beta-cell replication (~4-fold to 12-fold). Strikingly, overexpression of a myc-tagged betatrophin protein in the liver via hydrodynamic injection resulted in a similar increase in beta cell replication in mice, indicating that it is central to the proliferative response seen by inducing insulin resistance through treatment with S961 (Yi et al., 2013). While the mechanism of action of betatrophin is currently unknown, its description has lead to

wide-spread hopes that this hormone might represent a new therapeutic avenue for treating diabetes.

Importantly, it has not yet been determined whether betatrophin can also stimulate replication of human beta-cells. To address this question, I asked whether human islets transplanted under the kidney capsule of immunodeficient mice increase their proliferation rate in response to increased betatrophin levels. First, I established that immunodeficient NOD-Scid (NOD.CB17-Prkdcscid/J) mice respond to the insulin receptor inhibitor S961 in a fashion similar to that seen by Yi and colleagues in C57BL/6J mice (Yi et al., 2013). As shown in Fig 4.1a, delivery of S961 using osmotic minipumps indeed caused insulin resistance in NOD-Scid mice within 48 hours, and this effect persisted for the entire treatment period. Next, I evaluated whether expression of betatrophin is induced in the liver of NOD-Scid mice. Messenger RNA levels of betatrophin were induced about 5-fold as determined by quantitative reverse transcription PCR (Fig 4.1b), similarly to what was published previously for C57BL/6 mice (Yi et al., 2013). However, mRNA levels of betatrophin were not induced in white adipose tissues as shown in Yi's paper, probably due to the difference in mouse strains (Fig 4.1c). In addition, in the previous paper, betatrophin had a trend of being induced in fat tissue, but in a statistically non-significant manner. These results suggest that white adipose tissue is not the source of betatrophin that drives beta cell proliferation *in vivo*.

Next, I proceeded to assay replication of beta cells in the endocrine pancreas of S961-treated NOD-Scid mice by measuring incorporation of the thymidine analogue BrdU (bromodeoxyuridine) into beta cells. The number of replicating beta cells was dramatically increased in S961-treated mice compared to vehicle controls (Fig. 4.1d,g).

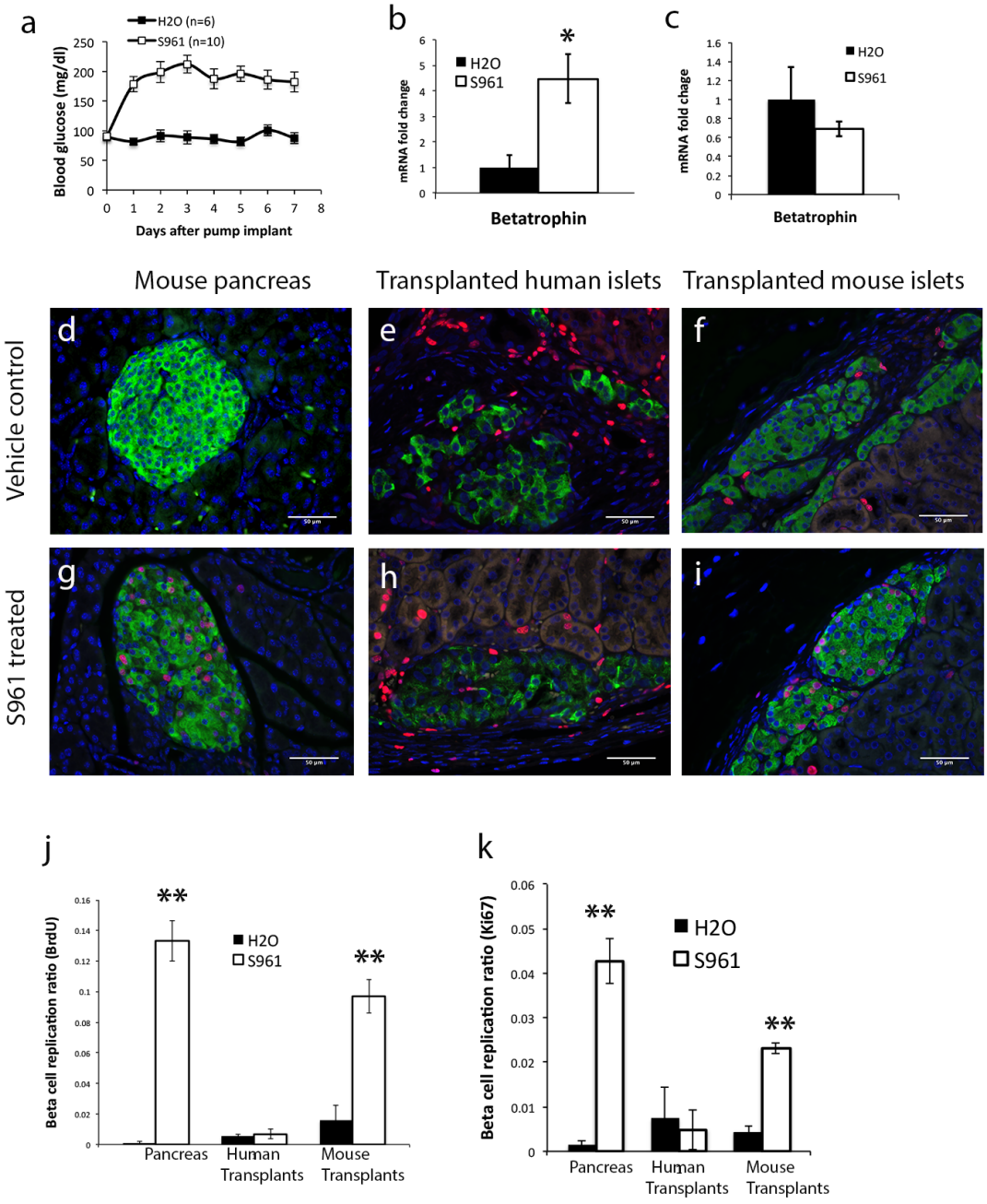

a
H2O (n=6)
S961 (n=10)
Blood glucose (mg/dl)
Days after pump implant
b
H2O
S961
mRNA fold change
Betatrophin
c
H2O
S961
mRNA fold chage
Betatrophin
Mouse pancreas
Transplanted human islets
Transplanted mouse islets
Vehicle control
S961 treated
d
e
f
g
h
i
j
Beta cell replication ratio (BrdU)
H2O
S961
Pancreas
Human Transplants
Mouse Transplants
k
Beta cell replication ratio (Ki67)
H2O
S961
Pancreas
Human Transplants
Mouse Transplants

Fig. 4.1. Elevated betatrophin expression does not stimulate replication of human beta-cells. **a** Random blood glucose levels of NOD/Scid mice treated with vehicle (n=6) or the insulin receptor antagonist S961 (n=10). Glucose levels in S961-treated mice were significantly elevated from day 1 to 7 ($p<0.001$). **b** Hepatic betatrophin mRNA levels in mice treated with vehicle (n=3) or S961 (n=4) as determined by qRT-PCR. Messenger RNA levels were normalized to those of TBP (TATA-box binding protein) as internal control, and are expressed as fold over vehicle. *, $p < 0.05$ **c** White adipose tissue betatrophin mRNA levels in mice treated with vehicle (n=6) or S961 (n=6) as determined by qRT-PCR. Messenger RNA levels were normalized to those of beta-actin as internal control. **d-i** Beta-cell replication in NOD/Scid mice treated with vehicle or S961was determined by BrdU incorporation (red signal). Beta cells were identified by insulin immunofluorescence staining (green). **d** Beta cells in the endocrine pancreas of vehicle-treated mice. **e** Transplanted human islets recovered from the kidney capsule of vehicle-treated mice. **f** Transplanted mouse islets recovered from the kidney capsule of vehicle-treated mice. **g** Beta-cells in the endocrine pancreas of S961-treated mice. Note the frequent BrdU-positive beta-cells (yellow arrow). **h** Transplanted human islets recovered from the kidney capsule of S961-treated mice. **i** Transplanted mouse islets recovered from the kidney capsule of S961-treated mice. Note the frequent BrdU-positive beta cells (yellow arrow). **j** Beta-cell replication was quantified as the percentage of $BrdU^+/insulin^+$ double-positive cells of the total number of insulin positive cells in beta-cells from the six conditions shown in **d-i**. n=5 for vehicle and for S961. **, $p < 0.01$. **k** Beta-cell replication was also quantified as the percentage of $Ki67^+/insulin^+$ double-positive cells of the total number of insulin positive cells in beta-cells from the six conditions.

Quantification of the data showed a mitogenic effect of about 15-fold, again in line with what had been observed in C57BL/6J mice (Fig 4.1j) (Yi et al., 2013). Co-immunostaining for insulin and Ki67, another marker of cell proliferation, revealed a 5-fold increase in beta cell replication in the S961 treated mice (Fig 4.1k). The discrepancy between the $BrdU^+$ cell ratio and the $Ki67^+$ cell ratio is not surprising since BrdU labeling captures all cells proliferating during the 72-hour time period of BrdU administration, while Ki67 staining only detects the cells that were actively replicating at the time of sacrifice.

Having validated the experimental model, I tested the effects of S961 treatment and elevated betatrophin levels on human beta cell replication. To this end, I transplanted islets obtained from deceased, non-diabetic organ donors (see Table 4.1 for donor information) under the kidney capsule of S961-treated NOD-Scid mice. In contrast to the dramatic increase in proliferation seen in the mouse endocrine pancreas documented above, human beta cells did not respond to S961 treatment and elevated betatrophin expression with increased BrdU incorporation (Fig 4.1e versus Fig 4.1h). Quantification of $BrdU^+$/$insulin^+$ double positive cells confirmed that the percentage of replicating human beta cells was negligible in both vehicle and S961-treated mice (Fig 4.1j). Co-immunostaining for Ki67 and insulin also confirmed that engrafted human beta cells do not respond to betatrophin elevation in the same manner as the beta cells of the mouse pancreas (Fig 4.1k).

An important caveat to this study is that we are not able to analyze human beta cells in their normal location, but only after transplantation under the kidney capsule. One could envision, for instance, that the betatrophin produced in the liver does not reach the

islets in the kidney capsule in the same concentration as is seen by beta cells in the endocrine pancreas. In addition, it is possible that betatrophin exerts its effects not directly by binding to a receptor on beta-cells, but indirectly, for instance by altering beta-cell innervation, or by activating another cell or signal not present under the kidney capsule. To address this issue, I simultaneously transplanted mouse islets under the right kidney capsule and human islets under the left kidney capsule of the same NOD-Scid mice. Strikingly, mouse islets were responsive to S961 treatment regardless of location, and showed a robust increase in beta-cell replication even when placed under the kidney capsule (Fig 4.1f,I,j). In fact, the replication rate of transplanted mouse beta cells was similar in magnitude to that seen in beta cells in the endocrine pancreas of the same mice. Co-immunostaining for Ki67 and insulin also confirmed that mouse islets transplanted under kidney capsules respond to S961 treatment and display an increased replication rate (Fig 4.1k).

Another potential explanation for why the human beta cells were unresponsive to S961 treatment is that mouse betatrophin may not act on its human receptor. To address this, I sought to overexpress the human version of betatrophin (h-betatrophin) in the mouse liver, then assess the impact on beta cell replication. cDNA encoding *h-betatrophin* was cloned into a plasmid with the sleeping beauty transposon backbone, then introduced into mouse hepatocytes via hydrodynamic tail vein injection. One week after the injection, mRNA expression of *h-betatrophin* in the mouse liver were measured by q-PCR (Fig 4.2a). Mice injected with *h-betatrophin* cDNA possess a 30-fold increase of *h-betatrophin* mRNA in their livers than mice injected with control plasmids (Fig 4.2b). Next, I co-stained BrdU and insulin in the mouse pancreas (Fig 4.2c,d).

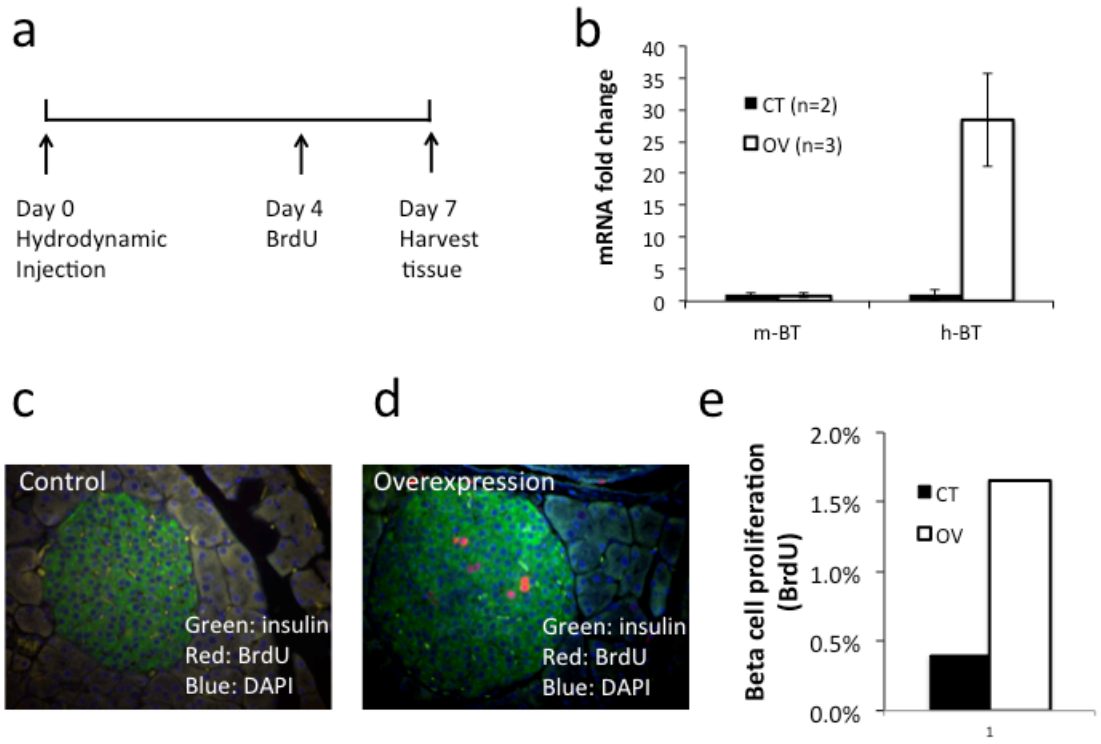


Fig 4.2 Overexpression of human betatrophin results in elevated beta cell proliferation **a** Experimental scheme of hydrodynamic injection. **b** Hepatic mRNA levels of both mouse betatrophin and human betatrophin in mice injected with either control plasmids (CT) or plasmids with h-betatrophin cDNA (OV), as determined by qRT-PCR. Messenger RNA levels were normalized to those of beta-actin as internal control, and are expressed as fold over control. **c,d** Beta-cell replication in mice injected with control or h-betatrophin plasmids was determined by BrdU incorporation (red signal). Beta cells were identified by insulin immunofluorescence staining (green). **e** Beta-cell replication was quantified as the percentage of $BrdU^{+}$/$insulin^{+}$ double-positive cells of the total number of insulin positive cells in mice injected with either control plasmid (CT) or plasmids with h-betatrophin cDNA (OV).

Overexpression of *h-betatrophin* mRNA resulted in a 4-fold increase of endogenous mouse beta cell proliferation (Fig 4.2e), indicating that human betatrophin protein can exert its effect on endogenous mouse beta cells.

To test the effect of h-betatrophin on engrafted human islets, I will repeat the transplantation experiments with NOD-Scid mice as mentioned previously. Mice harboring human and mouse islet grafts will be injected hydrodynamically with *h-betatrophin* or control plasmids, then the transplanted tissues, along with the liver and pancreas, will be collected for analysis. Furthermore, an adeno-associated virus (AAV) containing *h-betatrophin* cDNA is also being generated as a second approach to induce *h-betatrophin* overexpression in the mouse liver.

In summary, I have shown that the insulin-resistant state produced by treating mice with the insulin receptor antagonist S961 causes a dramatic increase in murine beta-cell replication, and that this effect occurs regardless of whether beta-cells are in their native environment in the endocrine pancreas, or in the ectopic location under the kidney capsule. This dramatic increase in replication rate is accompanied by the previously documented increase in betatrophin expression in the liver (Yi et al., 2013). Unfortunately, human islets transplanted under the kidney capsule were not responsive, and maintained negligible levels of beta-cell replication even when betatrophin production was elevated.

To test the effect of h-betatrophin protein on human islets, I first tested h-betatrophin protein on endogenous mouse islets. mRNA levels of h-betatrophin were increased 30-fold and endogenous mouse beta cell BrdU incorporation rate was increased

2.5-fold. Ongoing studies are being performed to test the effect of human betatrophin protein on transplanted human beta cells.

A second important issue is the age of the beta-cell responding to a mitogenic signal, as there is a well-documented decline in beta-cell replication rate with age in both mouse and human (Kushner, 2013). One of the three organ donors in our study was only four-years old (Table 4.1), and thus of an even younger relative age than the mice employed, indicating that even young human beta-cells are not responsive to betatrophin. In conclusion, these data put into question whether betatrophin is a mitogen for human beta-cells, and whether betatrophin will be a useful therapeutic approach for human diabetes.

Table 4.1 Summary information for the deceased organ donors used for the human islet transplantation studies.

Donor	ID	Age	Sex	BMI	Race	Cause of death
1	AAER055	53	M	29	Hispanic	Head Trauma
2	AAFC089	46	M	28.8	White	NA
3	ICRH-51	4	F	16.1	Hispanic	Head Trauma
4	AAGW388	43	M	30.6	White	Head Trauma
5	ICRH-52	18	M	23.5	White	Head Trauma

Materials and Methods

Mice and glucose measurements: Immunodeficient Nod-Scid (NOD.CB17-Prkdc^{scid}/J) female mice were purchased from Jackson Laboratory (Cat No. 001303) and used for experiments at 8 weeks of age. Random blood glucose levels were measured from tail vein blood using glucometer Breeze2 (Bayer AG, Leverkusen, Germany).

Mouse islet isolation: Adult C57BL6/J mice were used for islet isolation. Islets were released with standard collagenase treatment followed by purification through a Ficoll gradient (Sigma-Aldrich Corp, St. Louis, MO, USA, Ficoll PM 400) as described previously (Gupta et al., 2007). Islets were handpicked under a light microscope before transplantation.

Islet transplantation: During surgical procedures, mice were maintained on inhalation anesthesia (isofluorane 2-4%). 500 human islets were transplanted under the left kidney capsule and 100 mouse islets were transplanted under the right kidney capsule of each mouse. Mice were allowed to recover from the surgery for one week before implantation of osmotic minipumps.

S961 treatment: S961 was received as a generous gift from Dr. Lauge Schafffer (Novo Nordisk) (L. Schaffer et al., 2008). Vehicle (H_2O) or 10nmol S961 was loaded into Alzet 2001 osmotic pumps and implanted subcutaneously on the back of mice one week after

islet transplantation. Mice were sacrificed for tissue harvesting seven days after S961 or vehicle treatment.

Real-time PCR analysis: Total RNA from liver was isolated in Trizol (Life Technologies, Grand Island, NY, USA) and reverse transcribed using oligo (dT) primer, Superscript II Reverse Transcriptase, and accompanying reagents (Life Technologies). PCR reaction mixes were assembled using the Brilliant III SYBR Green QPCR Master Mix (Agilent Technologies, Inc., Santa Clara CA, USA). PCR reactions were performed on an Mx4000 Multiplex Quantitative PCR System (Agilent Technologies). All reactions were performed in triplicates with reference dye normalization. Median cycling threshold values were used for analysis.

Immunohistochemistry: 72 hours before euthanizing the mice, 1g/L BrdU (Sigma-Aldrich Corp., St. Louis, MO, USA) was administrated in the drinking water. Both kidneys were dissected and fixed with 10% formalin (Fisher PROTOCOL, Pittsburgh, PA, USA). The pancreas was dissected, flattened by forceps, fixed in 4% para-formaldehyde, and paraffin-embedded so that tissues with the maximum pancreatic footprint were sectioned. Tissues were sectioned to 5µm thickness. Deparaffinized and rehydrated slides were subjected to antigen retrieval by pressure cooker in 10mmol/l pH 6.0 citric acid buffer. Simultaneous immunofluorescent staining was performed for BrdU and insulin. The primary antibodies used were guinea pig anti-insulin (1:1000 dilution, Dako North America, Inc., Carpinteria, CA, USA) and rat anti-BrdU (1:500 dilution, AbD Serotec; Raleigh, NC, USA). Secondary antibodies were Cy2-anti-guinea pig (1:200) and Cy3-

anti-rat (1:200). The beta cell proliferation rate was quantified as the number of $BrdU^+$/$insulin^+$ double positive cells divided by the number of insulin positive cells. As least 1,000 beta cells were counted, respectively, for human islets under the kidney capsule, mouse islets under the kidney capsule, and endogenous mouse islets in the pancreas.

Construction of expression vectors: A plasmid containing human betatrophin cDNA was obtained from OriGene. The h-betatrophin cDNA sequence was amplified by PCR then cloned into the EagI site of a vector backbone containing the sleeping beauty (SB) transposon. In this context, h-betatrophin is under control of the SV40 Promoter. PCR-amplified h-betatrophin cDNA was also introduced into the NotI and HindIII sites of an AAV vector obtained from Penn Vector Core (PVC). The final AAV vector containing h-betatrophin under control of the liver-specific TBG promoter was then submitted to the PVC for packaging into a functional AAV. All clones were sequenced and subjected to diagnostic restriction digestion to ensure their fidelity.

Hydrodynamic tail vein injection: The injections were performed according to publish methods (Wilber et al., 2005). Briefly, 10μg of control or h-betatrophin-expressing plasmid DNA in the SB transposon backbone were first diluted in lactated ringer's solution. Mice were injected with 10% body weight volumes (ml/g) of diluted plasmid DNA within 7 s through the lateral tail veins and sacrificed eight days later. Livers were collected for expression analysis while pancreata were prepared for immunohistochemistry.

Chapter 5 Conclusions and Future directions

Mounting evidence in both human and mouse studies have emphasized beta cell deficiency as a key pathogenic determinant for both T1D and T2D [19, 52]. Therefore, cell replacement therapy is thought to be well suited for diabetic patients whose endogenous beta cell number is below the threshold to successfully alleviate hyperglycemia chronically. However, a major hurdle for cell replacement-based diabetes therapy is the difficulty to supply vast numbers of functioning insulin-producing cells. Beta cell proliferation has been shown to be the predominant mechanism of maintaining beta cell mass during adult life, at least in rodents [85]. Beta cells have exhibited adaptive capacity of expanding under metabolic stresses such as pregnancy, obesity, and pancreatic injury in mice [75, 76, 82]. Therefore, it is promising to utilize the regenerative capacity of beta cells to produce an unlimited supply of fully functional beta cells through the proliferation of pre-existing terminally differentiated adult beta cells.

My research attempted to investigate important molecular players associated with or responsible for beta cell proliferation during physiological expansion of beta cell mass. In the first part of my thesis, I utilized a pancreas-specific gene ablation mouse model to study the role of CISH in beta cell proliferation during pregnancy. In the second part of my thesis, I investigated the role of HNF-4α on beta cell proliferation in adaption to obesity-induced insulin resistance, which is a highly related model to human disease. I investigated *ob/ob* mice with *Hnf4α* ablation and HFD-fed mice with *Hnf4α* overexpression. Along this line, I also investigated the role of an HNF-4α target, ST5, which is a novel regulator of Ras/ERK pathway, on beta cell proliferation. In the third part of my thesis, I studied the role of betatrophin, a prominent mouse beta cell mitogen, on human beta cell proliferation. We transplanted human islets into kidney capsules of

immune-deficient mice and induced betatrophin overexpression by either administrating a drug inducing insulin-resistance (S961) or forcing ectopic human-betatrophin expression by hydrodynamic injection.

This dissertation makes several contributions to the knowledge of beta cell regeneration. First, this is the first time that a mouse model with pancreas-specific ablation of *Cish*, a proposed molecular suppressor of lactogen signaling, is reported. *Cish* ablation is not sufficient to promote elevated STAT5 signaling or induce increased beta cell proliferation during pregnancy. *Cish* mutant mice exhibited normal STAT5 signaling in concomitant with undisturbed beta cell proliferation rate and glucose homeostasis. This is probably due to a compensating effect from *Socs2* upregulation in *Cish*-deficient islets.

Second, I extend the role of HNF-4α as a regulator of beta cell proliferation from pregnancy model to obesity model. Obesity and pregnancy both induce insulin resistance and robust beta cell proliferation in rodents, but they utilize different molecular pathways for beta cell replication. Therefore, it is exciting to learn that HNF-4α regulates beta cell proliferation not only during pregnancy, but also during obesity. Beta cell-specific *Hnf4α* ablation results in decreased beta cell proliferation in *ob/ob* mice. Beta cell mass is not affected, probably due to a relatively short period of *Hnf4α* ablation in comparison to the length of overweight. Glucose homeostasis also remained normal in *Hnf4a* mutant mice, consistent with the unchanged beta cell mass. Furthermore, I also investigate whether overexpressing *Hnf4α* in beta cells affects glucose homeostasis in HFD-fed mice. Overexpression of *Hnf4α* is induced by administration of doxycycline in the drinking water of *Tre-Hnf4α; Rip-rtTA* mice. Nine-folds increase of *h-Hnf4α* mRNA in islets did not have any effect on glucose homeostasis in either chow-diet fed mice or HFD-fed

mice, indicating that overexpression of *Hnf4α* is not sufficient to promote a protective role in HFD-induced hyperglycemia. Along the same line, I also overexpressed *St5*, a target of HNF-4α, in beta cells. No increase of beta cell proliferation was observed in *St5* overexpressing mice.

Third, I investigated the role of betatrophin on human islets. Betatrophin has been shown to be a potent beta cell mitogen for mouse islets [162]. I first tested the role of endogenous mouse betatrophin on human beta cells by transplanting human islets into immune-deficient mice. Administration of S961 by osmotic pumps induces a 4-fold increase of betatrophin mRNA in the liver and more than 10-fold increase of BrdU incorporation in both endogenous mouse beta cells and transplanted mouse beta cells under kidney capsule. However, human islets in the grafts exhibited no response to mouse betatrophin, indicating that betatrophin is not a mitogen for human beta cells.

Future directions

Effect of *Cish* /*Socs2* double ablation on beta cell proliferation during pregnancy

Since both *Cish* and *Socs2* are up-regulated in the islets of pregnant mice and *Socs2* is further induced in *Cish*-deficient islets at pregnant day 9.5, I hypothesize that SOCS2 is compensating for loss of CISH as a suppressor for PRL/JAK2/STAT5 signaling. I am now breeding mice with *Cish* and *Socs2* double conditional alleles. The final $Cish^{L/L}$; $Socs2^{L/L}$; $Pdx1\text{-}Cre^{ER}$ mice will be compared with littermate $Cish^{L/L}$; $Socs2^{L/L}$ mice as controls.

It is very important to determine which gestational stage to investigate these mice. During normal pregnancy, *Cish* and *Socs2* expressions are elevated from P9, and keeps

elevated until the end of pregnancy [84]. Beta cell proliferation also starts to increase at around P9, peaks at around P14, and quickly decreases at P18. Interestingly, a cohort of cyclins expressions peak at P9.5, which is 5 days in advance of the peak of proliferation [209]. The jet-lag between the peak of cyclins and the peak of proliferations might be due to negative regulation of proliferation like CISH and SOCS2. Indeed, it seems CISH and SOCS2 regulation are finely-tuned at P9, when expression of *Socs2* is upregulated in *Cish* ablation mice, but not at P14.5. Therefore, it is worth investigating at P9 when CISH and SOCS2 might function to repress cyclin genes via JAK2/STAT5 signaling to prevent premature overshooting of beta cell proliferation.

Furthermore, it should be proceeded with caution when inducing *Cish* and *Socs2* ablation during pregnancy by tamoxifen injection. Tamoxifen has been shown to induce abortion at early stage of pregnancy in mice since it is an analog to estrogen. It should not be injected before pregnant day 5 and low dose should be used before pregnant day 8. Preliminary data have exhibited effective ablation of *Cish* at pregnant day 13 by tamoxifen injection without causing abortion. Further experiments are needed to determine whether *Socs2* could also be ablated in a similarly efficient way during pregnancy.

STAT5 signaling levels could be detected by western blot of p-STAT5 protein in islets and also qPCR of STAT5 target genes in pregnant mice. Levels of p-JAK2 should also be measured. Proliferation rate, insulin secretion, and glucose tolerance should be compared between control and mutant mice. Finally, it is worth proceeding with *Cish* and *Socs2* double ablation in human islets using lentiviral shRNA to further investigate whether ablation of these two genes has a mitogenic effect on human beta cells.

Effect of HNF-4α on beta cell proliferation and beta cell function in obesity

Ablation of *Hnf4α* for three weeks (from 7-10 weeks) in *ob/ob* mice results in decreased beta cell proliferation but unchanged beta cell mass and glucose homeostasis. It would be interesting to ablate *Hnf4α* from 3 weeks old *ob/ob* mice, when they start to gain more weight than lean mice and presumably trigger adaptive beta cell proliferation. *Hnf4α* deficiency from this earlier stage may lead to failure of beta cell mass expansion from the beginning and result in glucose intolerance.

It will be intriguing to study the mechanism of how HNF-4α regulates beta cell replication during obesity. One potential pathway is glucose metabolism. *ob/ob* mice have chronic mild hyperglycemia, and glucose metabolism in beta cells has been indicated to be a key regulator of beta cell proliferation. Expression of key regulators of glucose influx pathway should be measured, including *Glut-2* and *glucokinase*. Furthermoe, HNF-4α has been shown to regulate beta cell proliferation in pregnant mice by ST5-Ras-ERK signaling [197]. This is also a potential pathway of HNF-4α regulation in obesity.

In addition, HNF-4α might also affect insulin secretion function in beta cells. *Kir6.2*, a K_{ATP} channel subunit, has been shown to be a direct target of HNF-4α [59]. And *Hnf4α* mutant mice exhibited diminished first-phase insulin secretory response with decreased *Kir6.2* expression. Insulin secretion should be measured either with *in vivo* glucose injection or *in vitro* islet perifusion to study whether HNF-4α regulates insulin secretion pathway in obese islets.

Next, it would be helpful to perform microarray analysis on *Hnf4α* control and mutant islets from *ob/ob* mice to gain more knowledge of how HNF-4α regulates beta cell function during obesity. The mechanism of beta cell proliferation during pregnancy, obesity, and normal conditions are not the same. Therefore, comparing the microarray data from previous pregnancy model and the current obesity model will give us more information on the function of HNF-4α under different metabolic stresses.

Furthermore, ST5 is a direct target of HNF-4α. It is upregulated during pregnancy and it regulates Ras/ERK signaling. My preliminary data demonstrated that overexpression of *St5* has no effect on glucose homeostasis or beta cell proliferation under normal conditions. The function of ST5 should be further investigated under metabolic stress such as pregnancy and obesity since Ras/ERK pathway plays key roles in beta cell replication under these conditions.

Effect of betatrophin on human beta cell proliferation

It has been of great interest since the publication of Dr. Melton's paper reporting that betatrophin is a potent mouse beta cell mitogen, which brings a lot of hope to the development of the cell-replacement therapy of diabetes patients. The same insulin resistance-inducing drug, though, did not promote human islet proliferation under mouse kidney capsule although expression of betatrophin is upregulated in mouse liver.

Since mouse betatrophin protein is 74% homologous to human betatrophin protein, it is possible that human islet does not recognize mouse betatrophin protein. Therefore, we are now trying to overexpress human betatrophin in mouse liver to study whether transplanted human islets respond to human betatrophin. Two approaches are being

tested. First, AAV facilitated h-betatrophin cDNA injection. Second, hydrodynamic injection of h-betatrophin plasmid with a transposon backbone. Preliminary data demonstrated that h-betatrophin mRNA is overexpressed 30-fold in mouse liver by hydrodynamic injection. Endogenous mouse beta cells exhibited a 4-fold increase in proliferation, which is not as prominent as overexpressing mouse betatrophin. This is probably due to the relative lower levels of h-betatrophin mRNA comparing to endogenous mouse betatrophin even in the overexpressing mouse. We are now trying to increase the dose of h-betatrophin overexpression by injecting more plasmids. Ultimately, we will overexpress h-betatrophin in human-islets transplanted mice to analyze if h-betatrophin has an effect on human beta cell proliferation.

Appendix Transcriptional regulation of endocrine cell specification

Cell therapy for diabetes will be greatly aided by an unlimited and renewable supply of beta cells. Human pluripotent stem cells, including iPSCs and human ES cells, have the capacity to differentiate into insulin-producing cells (Hua et al., 2013). The differentiation and maturation of islet cells during embryogenesis involves a remarkably efficient and specific activation of a network of transcription factors, which either activate or silence their target genes, partially in combination with DNA methylation and/or histone methylation (Kroon et al., 2008). Although mouse genetics has provided mounting knowledge of the general functions of many key transcription factors, including PDX1, NKX6.1, NGN3, and NKX2.2, the precise molecular mechanisms by which they control their respective transcriptional targets during endocrine subtype specification have remained elusive. Therefore, approaches to generate beta cells from hES or iPSC have surpassed several milestones, but still needs perfection. One fundamental obstacle is to overcome the similarity of beta cells with other islet cell types, including alpha cells and delta cells, and induce uni-hormonal cells that express only insulin, instead of insulin and glucagon after in vitro differentiation. It would be intriguing to understand the mechanisms of how transcription factors and epigenetic modifications establish and maintain cell-type specific transcription.

During pancreatic development, the transcription factors NKX6.1, NKX2.2, and FOXA2 are each expressed in endocrine progenitors and later become restricted to specific endocrine subtypes (D'Amour et al., 2006). FOXA2 has been shown to play critical roles at multiple stages of pancreatic development, from the first induction of *Pdx1* in the pancreatic primordium, to the regulation of glucose sensing and insulin secretory machinery in the mature beta cells (N. Gao et al., 2007; Lantz et al., 2004; C. S.

Lee et al., 2002). Conditional ablation of *Foxa2* during pancreatogenesis results in loss of alpha cells, suggesting that FOXA2 also plays an important role in alpha cell specification (C. S. Lee et al., 2005).

Consistent with its restriction to beta cell, deletion of *Nkx6.1* results in selective beta cell loss (Sander et al., 2000). Moreover, NKX6.1 functions as a transcriptional repressor of alpha cell-specific genes and loss of *Nkx6.1* has been associated with ectopic activation of alpha cell-specific genes in mature beta cells *in vitro* (Schisler et al., 2005), suggesting that NKX6.1 specifies beta cell fate by repressing alpha cell specific genes. One recent study with endocrine precursor-specific ablation of *Nkx6.1* demonstrated that NKX6.1 is critical for establishing beta cell identity during development (A. E. Schaffer et al., 2013). Overexpression of *Nkx6.1* in *Ngn3* positive cells resulted in increased number of beta cells in concomitant with decreased other endocrine cell types, and ablation of *Nkx6.1* in *Ngn3* positive cells resulted in opposite phenotype (A. E. Schaffer et al., 2013). Moreover, conditional *Nkx6.1* ablation in adult beta cells resulted in rapid-onset diabetes and hypoinsulinemia resulted from partial loss of beta cell identity marked by decreased insulin production and secretion (Taylor, Liu, & Sander, 2013). And *Nkx6.1*-deficient beta cells gradually acquired molecular characteristics of delta cells, suggesting that NKX6.1 is critical for maintaining a functional beta cell state (Taylor et al., 2013).

Nkx2.2 is expressed in both alpha cells and beta cells and deletion of *Nkx2.2* resulted in the absence of all beta cells and most of alpha cells (Sussel et al., 1998), suggesting that NKX2.2 is necessary for the formation of both cell lineages. Further study showed that DNA methylation is one of the underlying mechanisms for the differential

programming of cell lineages. NKX2.2 formed a large repressor complex together with DNMT3a (a DNA methyltransferase), Grg3, and HDAC1 to maintain the methylation of promoter of *Arx*, an alpha cell factor (Papizan et al., 2011). Mutation of NKX2.2 tinman domain abolished the interaction between NKX2.2 and Grg3, resulted in ectopic expression of *Arx* in beta cells, and disrupted beta cell specification, suggesting that transcription factors and DNA methyltransferases interact with each other to regulate cell differentiation programs. Indeed, a beta cell-specific deletion of *Dnmt3a* resulted in de-methylation of promoter region of *Arx* and beta-to-alpha reprogramming (Papizan et al., 2011). Furthermore, epigenomic analysis suggested that differentiated alpha cells possess many more bivalently marked genes (genes that are marked by both activating H3K4me3 mark and repressing H3K27me3 mark) than exocrine and beta cells, indicating the potential of promoting alpha-to-beta reprogramming by manipulating the histone methylation signature of alpha cells. Intriguingly, treatment of cultured human islets with a histone methyltransferase inhibitor leads to bi-hormonal cells with both insulin and glucagon, proving the plasticity of islet cells (Bramswig et al., 2013).

Therefore, to successfully direct endocrine precursors toward the beta cell lineage or to reprogram alpha cells or other endocrine cells to adopt beta cell identity, it is necessary to identify the direct transcriptional targets of critical cell fate determining transcription factors. Furthermore, it will be beneficial to understand how these transcription factors mediate epigenetic modifications to establish and maintain endocrine subtype-specific gene expression programs. Elucidating how islet cell type-specific transcription factors co-regulate gene expression with chromatin modifying enzymes at a

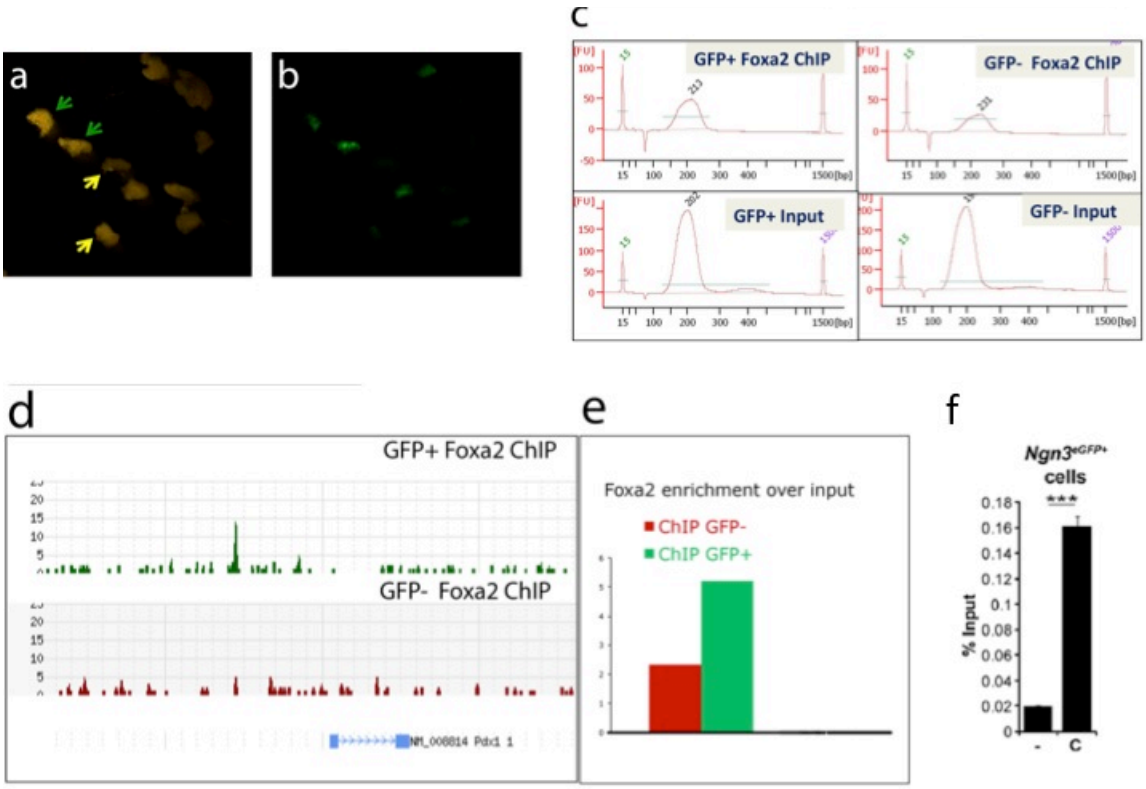
genome-wide level will be a major step forward in the understanding of how to promote beta cell fate.

Embryonic day 14.5 to 15.5 is the critical period of endocrine subtype specification. Ngn3 marks endocrine progenitors and their endocrine descendants at this stage. Therefore, purified endocrine progenitors were isolated from E15.5 pancreas by sorting GFP^+ cells from Ngn3-eGFP embryos. One Ngn3-eGFP male mouse was set up with multiple CD1 female mice to allow half embryos with Ngn3-eGFP transgene. Mice were checked everyday for plugs to determine start date of pregnancy. Usually, 5 to 7 cages of mating were set up together to ensure at least five pregnant mice on the same day. Fetuses were harvested on embryonic day 15.5 and embryonic pancreata were dissected under microscope. GFP^+ pancreata were separated from GFP^- pancreata under a fluorescence microscope (App. Fig 1a,b). GFP^+ pancreata were then dissociated into single cell suspension with trypsin and subjected to FACS sorting. Approximately 4% GFP^+ cells are expected, which is 5000-8000 GFP^+ cells per pancreas (P. White, May, Lamounier, Brestelli, & Kaestner, 2008).

First, 362,000 $Ngn3^+$ cells were sorted from 70 GFP^+ embryonic pancreata, lysed, sonicated, and subjected to Foxa2 chromatin immunoprecipitation (ChIP). Chromatin library were than prepared for ChIP-sequencing (App. Fig 1c). In $Ngn3^+$ cells, FOXA2 bound to 1,460 sites according to GLITR analysis. In $Ngn3^-$ cells, FOXA2 bound to 2,732 sites according to GLITR analysis. Some known targets of FOXA2 were checked by qPCR to ensure that ChIP-sequencing worked. For example, FOXA2 have higher occupancy at *Pdx1* promoter in $Ngn3^+$ cells than in $Ngn3^-$ cells (App. Fig 1d), which was also confirmed by qPCR (App. Fig 1e). Second, 1.5×10^6 GFP^+ cells were isolated from

about 300 E14.5 pancreata by FACS and subjected to NKX6.1 ChIP. ChIP analysis demonstrated that in $Ngn3^{+}$ cells at E14.5, NKX6.1 directly and specifically associates with the *Arx* enhancer, which is critical for *Arx* transcription repression (App. Fig 1f). Binding of NKX6.1 to *Arx* enhancer significantly reduces *Arx* expression, indicating that Nkx6.1 promotes beta cell lineage by repressing *Arx* expression in endocrine progenitor cells.

In conclusion, I established feasible protocol of ChIP-Seq analysis on a limited number of sorted embryonic cells (0.3 million cells). Genome wide DNA occupancy analysis were performed for FOXA2 and NKX6.1 in E14.5-E15.5 $Ngn3^{+}$ cells, and resulted in new knowledge of endocrine cell lineage specification during embryogenesis. Further profiling of histone modifications and DNA methyltransferases occupancy would enhance our understanding of how establishing and maintaining of DNA methylation by both transcription factors and DNA modifying enzymes are accomplished. This knowledge will facilitate the differentiation of beta cells from alternative cell sources. It will also provide insight into the mechanisms that ensure the beta cell fate is maintained in a transplant setting.



Appendix Fig 1 a,b E15.5 embryonic pancreas under microscope. GFP^+ pancreata are marked with green arrows and GFP^- pancreata are marked with yellow arrows. GFP^+ pancreata were chosen under fluorescence microscope for further FAC sorting. **c** Size analysis of ChIP-ed or input chromatin before library preparation for sequencing. **d** GLITR analysis of Foxa2 ChIP-Seq on GFP^+ cells and GFP^- cells. Shown here is the promoter region of *Pdx1*, where a peak is identified in GFP^+ cells but not GFP^- cells. **e** qPCR analysis confirmed enrichment at *Pdx1* promoter region in GFP^+ cells over GFP^- cells after Foxa2 ChIP, comparing to inputs. Primers amplifying *Pdx1* promoter region were used. **f** NKX6.1 binding is enriched at the *Arx* enhancer locus in sorted GFP^+ cells from E15.5 pancreata.

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