THE GENERATION OF FULLY FUNCTIONAL β-CELLS BY PROLIFERATION: LESSONS FROM PREGNANCY AND HNF4α

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Dedication

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

To my grandfather for being a great role model, and teaching me the value and the enthusiasm of „learning for fun"

To my wife for her unwavering devotion, love, inspiring me to reach towards my best, and for giving me reasons to be optimistic throughout my endeavors

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Abstract

THE GENERATION OF FULLY FUNCTIONAL β-CELLS BY PROLIFERATION: LESSONS FROM PREGNANCY AND HNF4α

Sebastian Rieck

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Diabetes mellitus is an increasingly prevalent metabolic disorder that is estimated to affect over 300 million people by 2025. Common to either type 1 or type 2 diabetes is a progressive inadequacy of functional β-cell mass. Recent studies have shown that during times of prolonged metabolic demand for insulin, the endocrine pancreas can respond by increasing β-cell mass, both by an increase in cell size and by changes in the balance of β-cell proliferation and apoptosis. Advances that further our knowledge of the molecular factors that control both β-cell proliferation and survival will be crucial for understanding the homeostasis of β-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 β-cells that are suitable for transplantation. However, no systematic study that investigates the expression profile of the islet‟s response to pregnancy *in vivo*, a physiological state of insulin resistance, has been reported thus far.

In the first part of my thesis, I characterized the gene expression signature of pancreatic islets during pregnancy by performing large-scale expression profiling of islets isolated from 4- to 5-month-old non-pregnant and pregnant female mice at day 14.5 of gestation, the peak of β-cell proliferation. I identified a total of 1,907 genes as

differentially expressed, and demonstrated the induction of both proliferative and survival pathways in the islet during pregnancy. A comparison of our pregnancy gene set with two additional models of islet expansion suggests that diverse mechanisms can be recruited to expand islet mass. One of the genes that is required for β-cell proliferation during pregnancy in mice is the transcription factor HNF4α.

In an attempt to translate knowledge gained using the pregnancy paradigm, I hypothesized that HNF4α is a human β-cell mitogen. To address this question, in the second part of my thesis, I employed adenoviral-mediated overexpression of a pancreas-specific isoform of HNF4α (HNF4α8) in primary human islets. HNF4α8 stimulated β-cells to enter the cell cycle, and led to a greater than 300-fold increase in the number of β-cells that entered S-phase, without detectable change in glucose stimulated insulin secretion. However, HNF4α8 overexpressing β-cells showed signs of cell cycle arrest, caused by activation of the DNA damage response associated with replication stress, ultimately resulting in a senescence-like phenotype independent of caspase-dependent apoptosis. Overexpression of HNF4α8 together with known β-cell mitogens, also further increased cell cycle entry of β-cells, strengthening the argument that HNF4α8 is a mitogenic signal in the human β-cell. Additionally, I observed a substantial proportion of β-cells stimulated to enter the cell cycle by CDK6 and CYCLIN D3 to also exhibit both markers of cell cycle arrest and double stranded DNA damage. In summary, the DNA damage response is a barrier to efficient human β-cell proliferation *in vitro*, and as such I suggest its evaluation in future attempts to stimulate β-cell replication.

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Introduction

Diabetes mellitus as a candidate for cell replacement therapy

Diabetes mellitus is a metabolic disorder characterized by the loss of a single cell type, the insulin producing β-cell, which then leads to failed glucose homeostasis. The phenotype of chronic elevation of glucose in the bloodstream, shared by all forms of diabetes, can lead to severe and life-threatening complications including cardiovascular disease, renal disease, and blindness (1). Diabetes mellitus is characterized by either an absolute insulin deficiency due to the autoimmune destruction of pancreatic insulin-producing β-cells (type 1), or relative insulin deficiency due to diminished insulin secretion and/or decreased insulin sensitivity as a result of insulin resistance in peripheral tissues (type 2). Both forms of diabetes are caused by complex interactions between genetics, environment and lifestyle choices (2), and accepted forms of treatment for diabetes reflect two basic mechanisms whereby one can increase insulin secretion; to mimic the ability of the β-cell to secrete insulin in response to glucose by injections of recombinant insulin and increase β-cell mass in diabetic patients by islet transplantation.

Since the discovery of insulin in the 1920s by Dr. Frederick Banting and Dr. J.J.R. Macleod, tight control of glucose levels by use of an intensive exogenous insulin therapy can be achieved. However, while this improves the life of a diabetic patient considerably, with side effects including an increasing frequency of hypoglycemic episodes and a lack of prevention of long- term complications (1, 3), insulin therapy cannot provide the fine-tuned control of glucose homeostasis ensured by an individual‟s endogenous β-cell. It has been shown that both individuals with type 1 and type 2 diabetes show decreases in β-cell mass and increases in β-cell apoptosis (4-6). Additionally, studies of obese patients (including non-diabetic, pre-diabetic and diabetic

groups) reported an inverse relationship between blood glucose levels and β-cell volume below a certain threshold (7), illustrating that another important target in the treatment of diabetes mellitus is the correction of β-cell mass deficit.

Indeed in 1999, the Edmonton protocol, or first successful transplantation of cadaveric islets into brittle type 1 diabetic patients using a glucocorticoid-free immunosuppressive regimen resulting in temporary insulin independence (8), has caused a paradigm shift towards the development of diabetes therapies centered upon improving β-cell mass in diabetic patients. While being able to show insulin independence and near normal control of blood glucose levels for up to a year post- transplant (8, 9), transplant success has proven to be short-lived and accompanied by significant side effects, such as impairment of islet function (10), nephrotoxicity (11), and possibly also impairment of β-cell proliferation (12), all caused from the use of immunosuppressive regimes themselves. Furthermore, the application of islet transplantation is limited by the current lack of sufficient organ availability and the availability of β-cells from deceased donors cannot meet the demand. The use of potential regeneration-compatible immunosuppressive drugs for islet transplantations, and alternative methods, such as tissue engineering (for example, growth of exogenous islets in an encapsulated coating designed to prevent rejection) (13, 14), are being investigated, and great interest into the mechanisms of β-cell proliferation has emerged.

Specifically, significant attention has been focused to the formation of new fully functional β-cells. Several methods of β-cell generation have been investigated including the replication of pre-existing β-cells (15), transdifferentiation of endoderm-derived cell populations (hepatocytes, enteroendocrine cells, α-cells, exocrine cells) (16, 17),

differentiation from progenitors (controversially thought to reside in ducts or originating during development and maintained into adulthood) (18, 19), and differentiation from human embryonic stem cells (hESC) *in vitro* (20) (Figure 1.1). For type 1 diabetes, being able to expand β-cell mass *ex vivo*, or *in vivo* after islet transplantation, could both increase the number of patients that can be treated with a limited supply of donor islets and improve the outcome after transplantation. For type 2 diabetes, the identification of targets and pathways that mediate proliferation and/or apoptosis might lead to the development of novel drugs that stimulate β-cell growth in the patient and thus allow for improved glycemic control.

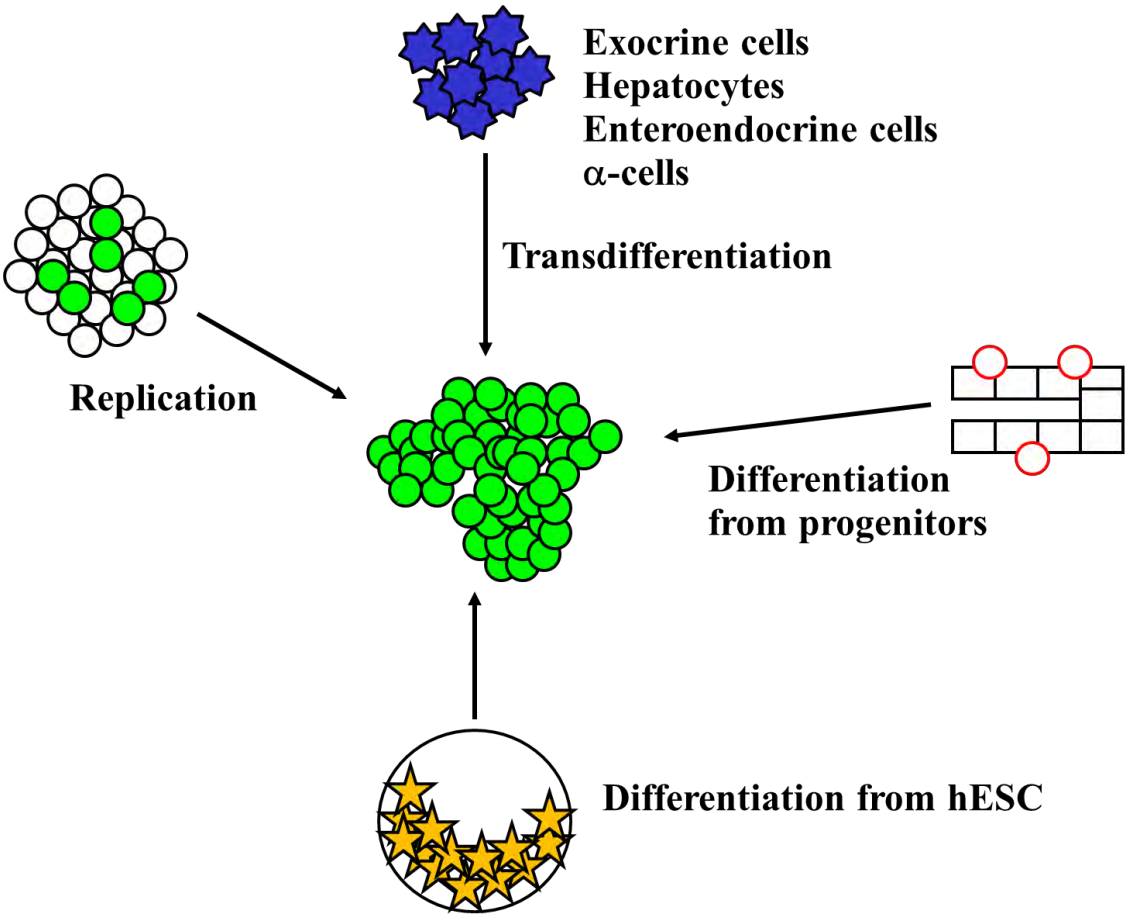

Figure 1.1. The mechanisms by which new fully functional β-cells can be generated.

Figure 1.1. The mechanisms by which new fully functional β-cells can be generated. Approaches toward generating new β-cells for the treatment of type 2 diabetes or for cell replacement therapy for type 1 diabetes include the use of instructive cues to induce the replication of terminally differentiated β-cells *in vitro* or *in vivo*, the direct reprogramming of endoderm-derived cell populations into β-cells by transdifferentiation *in vitro* or *in vivo*, the differentiation of purified pancreatic progenitor cells *in vitro*, and differentiation of human embryonic stem cells (hESC) *in vitro*. Green-filled circles represent fully functional adult β-cells, blue stars represent terminally differentiated cells of endoderm origin, red circles represent pancreatic progenitor cells (potentially residing in the pancreatic ducts), and yellow stars represent human embryonic stem cells.

Homeostatic control of β-cell mass

The β-cell population is restricted to the endocrine pancreas or islets of Langerhans, and is required for insulin production and secretion. The exocrine pancreas is composed predominantly of acinar cells which secrete digestive enzymes, and duct cells which transport these digestive enzymes out of the pancreas and into the duodenum. The endocrine pancreas is highly innervated by blood vessels and apart from endothelial cells, nerves and fibroblasts, is primarily composed of five endocrine cell types: the glucagon producing α-cell, insulin producing β-cell, somatostatin producing δ-cell, pancreatic polypeptide producing pp-cell, and ghrelin producing ε-cell. Together, the islets constitute about 1-2% of total pancreas volume, and range in size from just a few cells to several thousand cells (21). It should be noted that the three dimensional islet structure and relationship between endocrine cells differ between rodent and human islets, however, the significance of this concerning the maintenance of β-cell mass is unknown (Figure 1.2) (22, 23).

An organism‟s β-cell mass is determined by the product of the number and size of its pancreatic β-cells. In adult mammals, β-cell mass is maintained by the balance between cell renewal and growth (cell replication, hypertrophy, neogenesis), and cell loss (cell death, atrophy, autophagy) (Figure 1.3A). It is well established that adult pancreatic β-cells replicate very slowly after the establishment of β-cell mass during the neonatal period (a period of high β-cell replication rates both in rodents and in humans) (24-26). The absolute death rate of β-cells is extremely low, and counters the slow rate of β-cell replication (27). While the length of the replication refractory period (a period during which post-mitotic adult β-cells are prevented from immediately reentering the cell cycle)

is a subject of controversy (28, 29), a recent study demonstrates that all β-cells of an islet are similarly capable of numerous cell divisions (30), agreeing with gradual β-cell mass expansion as rodents age (31, 32). In addition, it is now known that adult β-cells can dynamically respond to systemic increases in insulin demand (here defined as an increase in metabolic load) by dramatically expanding their functional mass, at least in rodents and with limited certainty in humans, as seen during aging (32-34), pregnancy (35-37), obesity (38, 39), genetic insulin resistance (40), and even during hyperglycemic circumstances in models of β-cell loss (12, 41) and in both short- and long-term type 1 diabetic patients (an example of autoimmune β-cell loss in humans) (6, 42).

Current evidence suggests that dysregulation of the balance of β-cell gain (proliferation) and loss (cell death) mechanisms is an essential feature in the pathogenesis of diabetes mellitus. For example, a subset of obese individuals is unable to compensate for insulin resistance, and thus develop type 2 diabetes mellitus. In an attempt to explain why this occurs, gene expression studies identified a cell cycle regulatory module in islets that distinguishes between diabetes-resistant and diabetes-susceptible strains of leptin-deficient *Ob/Ob* mice, and successfully predicts their predisposition to diabetes onset (43). In line with this, a recent study comparing obese non-diabetic and type 2 diabetic human donors, correlates non-diabetic obesity with an increase in β-cell volume and proliferation, and obese individuals with type 2 diabetes with heightened rate of β-cell apoptosis without increases in β-cell volume and proliferation (44). Indeed, apoptotic β-cells are often organized in pairs in pancreatic tissue sections from type 2 diabetics, a finding that has been interpreted as β-cell apoptosis following mitosis as a mechanism of β-cell death (45). Thus, in this case, a β-cell renewal mechanism is attempted but is

overcome by β-cell loss, suggesting that diabetes onset is a result of a failure of β-cell expansion rather than a decrease in existing β-cell mass only.

While the ability of the pancreas to modulate β-cell mass is certain, the source of new endocrine cells in response to increased metabolic loads remains unclear and controversial. The use of lineage tracing in recent studies has clearly demonstrated the origin of newly derived β-cells in many physiological and pathological settings (Figure 1.3B). For example, evidence of neogenesis (the production of new β-cells arising from the differentiation of progenitors) was limited for a long time to the detection of insulin-positive cells within the ductal epithelium (46). However, a recent genetic lineage tracing experiment, showing that cells expressing Cre recombinase under the control of the carbonic anhydrase II promoter (a gene expressed at high levels in duct cells), strengthens the argument that a portion of β-cells can arise from the ductal compartment after birth and after pancreatic injury (18). Furthermore, the fetal differentiation program, as marked by Cre recombinase under the control of the bHLH factor *Ngn3*, can be reactivated in the adult mouse by the extreme injury stimulus of pancreatic duct ligation (47), though not in response to the milder insult of partial pancreatectomy (48). Similarly, the endoderm-derived α-cell, as marked by Yellow Fluorescent Protein under the control of glucagon expression, can spontaneously transdifferentiate into insulin-producing cells after extreme β-cell loss in mice (41). Nevertheless, genetic lineage tracing studies performed in the young adult mouse indicate that the great majority of new β-cells throughout adulthood and either after partial pancreatectomy or conditional ablation of β-cells are derived through the homogenous replication of pre-existing ß-cells and few, if any, newly formed β-cells stem from progenitor populations (12, 15, 28, 49). This highlights the

importance of proliferation in the normal maintenance of β-cell mass, at least in rodents. Of note, while the length of the post-replication quiescence period is prolonged with age, it is shortened during times β-cell regeneration, for example, after conditional ablation of β-cells (30).

There remains controversy regarding what β-cell gain mechanisms are dominant physiologically in humans, and the ultimate decision of which mechanism is best utilized to make new fully functional β-cells is still an unanswered question. In the next sections, I will concentrate on the generation of new β-cells through the replication of pre-existing β-cells using the pregnancy paradigm as an example, with the ultimate goal of deriving a well-defined, step-by-step protocol to drive efficient non-oncogenic expansion of human β-cells purified from donor pancreata *in vitro*.

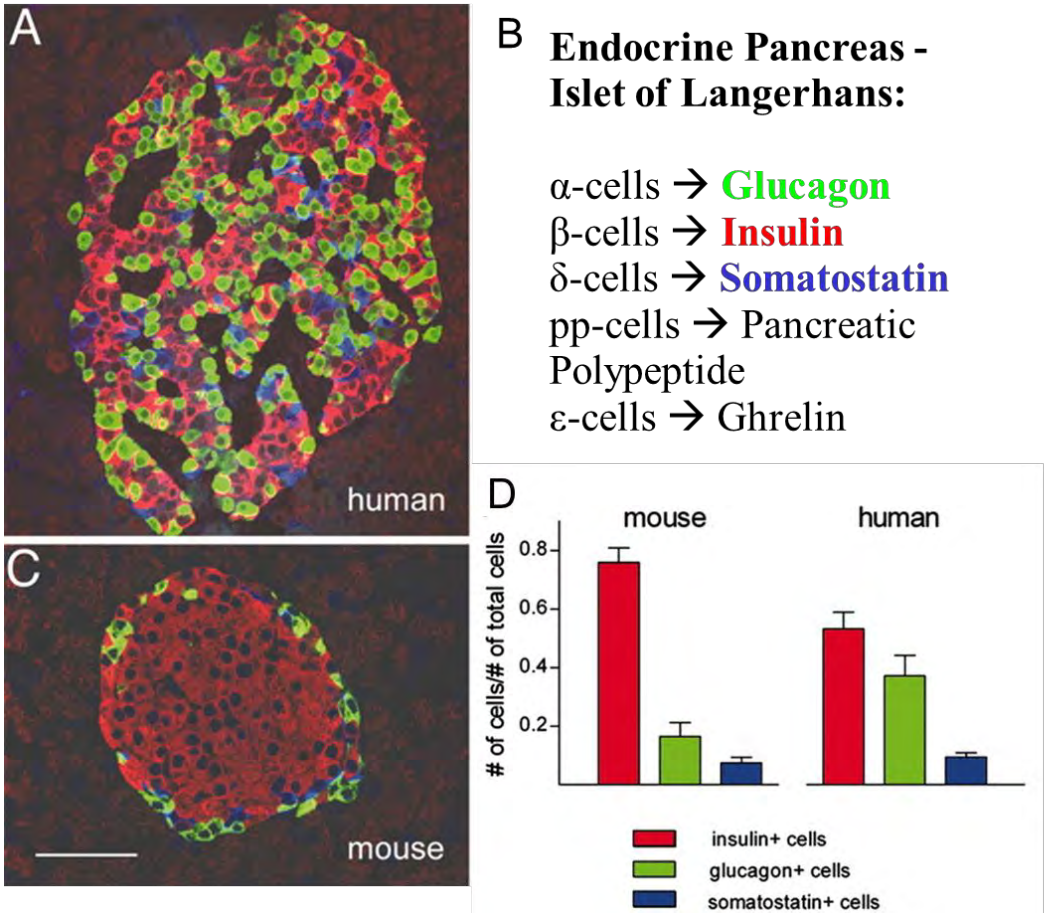

Figure 1.2: Anatomy of the mouse and human islet.

Figure 1.2: Anatomy of the mouse and human islet. Mouse islets exhibit a high degree of cell segregation, favoring β- and α-cell homologous contacts (C). In striking contrast to the „œcore-mantle‟ organization of mouse islets, human islets display β-cells intermingled with α- and δ-cells, with frequent heterologous interactions between β- and α-cells (A). Additionally, (D) adult human islets have fewer β-cells, but more α-cells, than mouse islets (23). α-cells are colored in green, β-cells in red and δ-cells in blue.

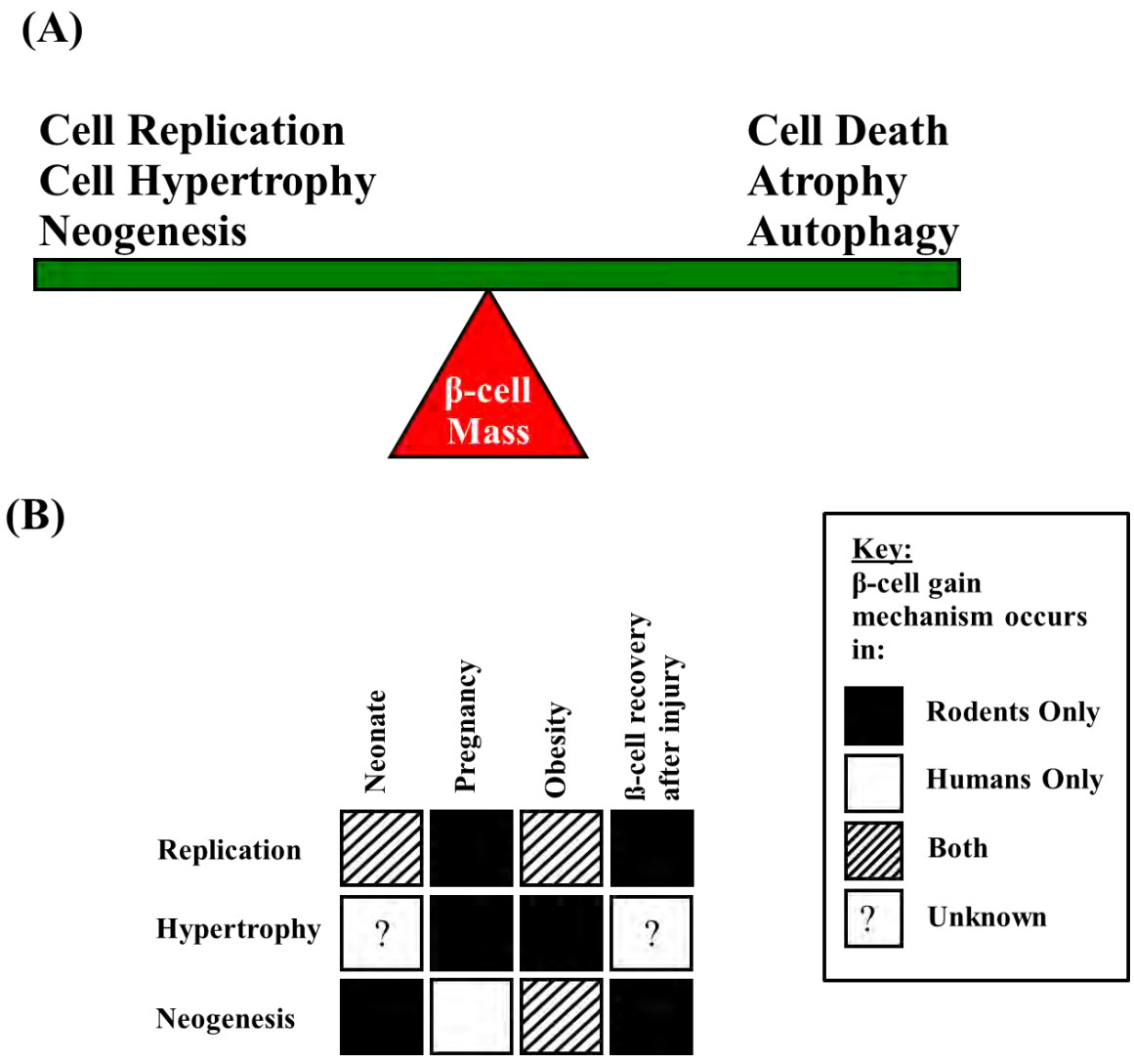

Figure 1.3: Homeostatic control of β-cell mass in rodents and humans.

Figure 1.3: Homeostatic control of β-cell mass in rodents and humans. (A) Control of β-cell mass (the fulcrum of the balance) is based on the relative contribution of processes that result in β-cell gain (replication, hypertrophy, neogenesis) and β-cell loss (death, atrophy, autophagy). A net increase in β-cell mass occurs when mechanisms involved in β-cell gain exceed those of β-cell loss. (B) This section depicts the experimental evidence in rodents and humans of β-cell gain mechanisms during adaptive increases in β-cell mass (neonatal period, pregnancy, obesity, and β-cell recovery after injury). This highlights the plasticity of the β-cell‟s ability to increase its mass during different physiological and pathophysiological (hyperglycemic) states and the relatively large amount of knowledge that remains to be uncovered, especially with respect to human β-cell biology. While the rodent evidence for neogenesis during the neonatal period and after injury is from work by Dr. Susan Bonner-Weir and colleagues (31, 50), this data is under much scrutiny based on recent lineage tracing showing that no β-cells originate from the pancreatic ductal epithelium during both instances (51). Dark squares represent evidence for β-cell gain mechanism only in rodent models; white squares represent evidence found only in human autopsy pancreatic samples; striped squares represent evidence both in rodents and humans; and question mark squares denote that there is no current evidence.

Intracellular glucose metabolism controls β-cell proliferation

β-cells compensate for increased systemic demands for insulin by both an increase in insulin secretory capacity, and an increase in β-cell mass. Recent studies suggest that these two processes are inherently linked. While the exact mechanisms underlying these processes are controversial, there is evidence that an unknown circulating factor in insulin resistant animals induces β-cell proliferation in transplanted islet grafts (52). Not excluding the importance of insulin signaling with respect to β-cell proliferation, glucose is a possible candidate for this factor since it is well established that glucose infusion, i.e. food intake, increases β-cell proliferation in mice (53). When glucose enters the bloodstream, the workload imposed on a β-cell (the net insulin secretion per β-cell as regulated by intracellular glucose metabolism) increases in order to maintain euglycemia. Briefly, the catabolism of glucose principally through glucokinase (Gck) and glycolysis raises the intracellular ATP concentration, which leads to the closing of ATP-dependent potassium channels (K_{ATP}), depolarizing the plasma membrane and opening voltage-gated calcium channels, allowing calcium to enter the β-cell, and triggering insulin release by exocytosis. A negative feedback loop indirectly restricts insulin release on the same β-cell, as insulin lowers blood glucose by stimulating glucose uptake by peripheral tissues. The reduced blood glucose is detected by the β-cell as a diminished glycolytic flux, slowing the release of insulin, and holding blood glucose nearly constant despite large fluctuations in dietary intake (21, 54).

Recent studies have demonstrated that β-cell proliferation rates are also controlled by the intracellular glucose metabolism (the rate of glycolysis of the β-cell itself) (55). Based upon the β-cell workload model, several predictions about β-cell proliferation can

be made in response to metabolic load. First, increasing glycolytic flux in the β-cell increases β-cell replication. For example, if systemic insulin demand is constant, decreasing β-cell mass would increase the workload of the remaining β-cells, increasing their compensatory proliferation rates. Conversely, an excess of functional β-cells would lead to a reduced glucose metabolism per β-cell and a reduced β-cell proliferation rate. In line with this, wildtype mice into which additional wildtype islets have been transplanted show decreased endogenous β-cell proliferation (55). Second, if glucose metabolism is blocked, as in diabetic patients exhibiting mutations in glucokinase and the K_{ATP} channel (56, 57), extracellular glucose levels would uncouple from the intracellular metabolic flux of the β-cell. Indeed, β-cells lacking glucokinase do not respond to a hyperglycemic environment and instead behave as if exposed to hypoglycemia, exhibiting blunted glycolytic flux and decreased β-cell proliferation (55). Conversely, a patient with an activating mutation in glucokinase exhibits increased β-cell proliferation (57). These data show that metabolic demand during adult life is a key determinant of cell cycle re-entry of the β-cell. This suggests that adjusting β-cell number is equally as important as increasing insulin secretion in successfully maintaining euglycemia during states of physiological insulin resistance, as occurs during pregnancy.

Reversible β-cell mass expansion during pregnancy

It has been recognized for decades that increased β-cell mass is an adaptation to the progressive insulin resistance related to increased fetal burden that develops during pregnancy in women (35, 36, 58). The precise mechanism of β-cell mass expansion, i.e. proliferation, neogenesis or increase in size, has been elucidated only in part (Figure 1.4) (36, 59, 60). However, as with obese individuals, based on rodent studies, when compensatory β-cell mass expansion fails during gestation, diabetes results (61). Interestingly, long-term follow-up studies show that a significant percentage of women who are diabetic during pregnancy develop type 2 diabetes later in life, emphasizing that the ability of β-cells to successfully adapt to increases in metabolic load is a common theme for preventing both gestational diabetes and type 2 diabetes (62).

In addition to the increased sensitivity of the β-cell to secrete insulin in response to glucose during gestation, studies in rodents found a 2-fold increase in β-cell mass and demonstrated that β-cell proliferation also increases dramatically during pregnancy (Figure 1.4A-B) (35, 63). The peak of bromodeoxyuridine (BrdU) incorporation, an indicator of DNA synthesis during S-phase, occurs about two-thirds of the way through the gestational period, with labeling returning to pre-pregnancy levels shortly before parturition. Notably, this peak in DNA synthesis coincides with increased placental lactogen (PL) levels, suggesting that lactogenic activity is vital for the ability of β-cells to enhance proliferation and function in response to pregnancy (Figure 1.4D) (35, 58). Furthermore, studies in primary human islets demonstrate that lactogen treatment increases insulin secretion and islet cell proliferation (64). Of note, both morphometric

and DNA-to-protein ratio methods indicate β-cell hypertrophy in addition to β-cell hyperplasia as a mechanism towards β-cell expansion during pregnancy in rodents (65, 66). Intriguingly, β-cell mass returns to normal levels within ten days after birth through increased β-cell apoptosis, decreased proliferation and reduced β-cell size (Figure 1.4C) (59, 60). In spite of the small case size considered, β-cell mass has also been shown in a recent study to increase by 40% in pregnant women (37). Although the molecular mechanisms underlying these processes are not yet known, the pregnancy paradigm is a unique example of rapid and reversible β-cell mass expansion, with distinct bursts of both β-cell proliferation and β-cell apoptosis occurring in a physiological setting.

As a proof of principle for direct regulation of β-cell proliferation by placental lactogens, overexpression of PL in the β-cell caused a dramatic increase in β-cell proliferation and β-cell mass, even resulting in hypoglycemia (67). Similarly, global deletion of the prolactin receptor (*Prlr*), through which placental lactogens signal, reduces β-cell mass and mildly impairs insulin secretion in non-pregnant mice (68). The requirement of the prolactin receptor for β-cell adaptation during pregnancy was demonstrated using pregnant mice heterozygous for the prolactin receptor null mutation. These mice exhibited reduced β-cell proliferation, decreased β-cell size and mass, and impaired glucose tolerance (66). Interestingly, the maternal genotype had a significant effect on the phenotype of female offspring that became pregnant, as assessed by heightened serum glucose levels (66), suggesting that *in utero* exposure to impaired glucose homeostasis alters the epigenetic memory of β-cells (69).

These findings provide a link to the well-known phenomenon that the intrauterine milieu affects the glucose homeostasis and the capacity of β-cell mass to expand when

facing insulin resistance in the adult (69, 70). Indeed, individuals born to mothers with gestational diabetes mellitus have a higher risk of obesity and type 2 diabetes (71). Epidemiological studies in humans show very clearly how caloric intake by the mother affects the future glycemic health of the child. Intrauterine growth retardation in rodents is an experimental approach that has been used to investigate this phenomenon on the molecular level. In this model, epigenetic marks at the promoter of the β-cell transcription factor *Pdx1* were found to be altered in the offspring of dams in which the uterine arteries had been ligated, causing intrauterine growth retardation (72). Again, it is clear that the metabolic state of the fetus determines the epigenetic fate of the β-cell, and highlights the importance of understanding the physiological mechanisms underlying maternal β-cell expansion during pregnancy.

Although activation of multiple signaling pathways (such as Stat5, Mapk and classic insulin signaling mediators, such as phosphatidylinositol 3-kinase, Insulin response substrate 1/2 and Akt) enhances β-cell compensation downstream of the prolactin receptor *in vitro* (73-75), it is not clear whether placental lactogens stimulate these pathways *in vivo*. In addition, Stat5- dependent downregulation of the tumor suppressor gene menin (Men1) and subsequent inhibition of p18 and p27 are crucial events in β-cell expansion during pregnancy (76). While substantial progress has been made in elucidating the contribution of selected genes to β-cell compensation during pregnancy, no systematic study investigating the global expression profile of islets in response to pregnancy exists to consider which mechanisms specifically drive β-cell replication during pregnancy *in vivo*.

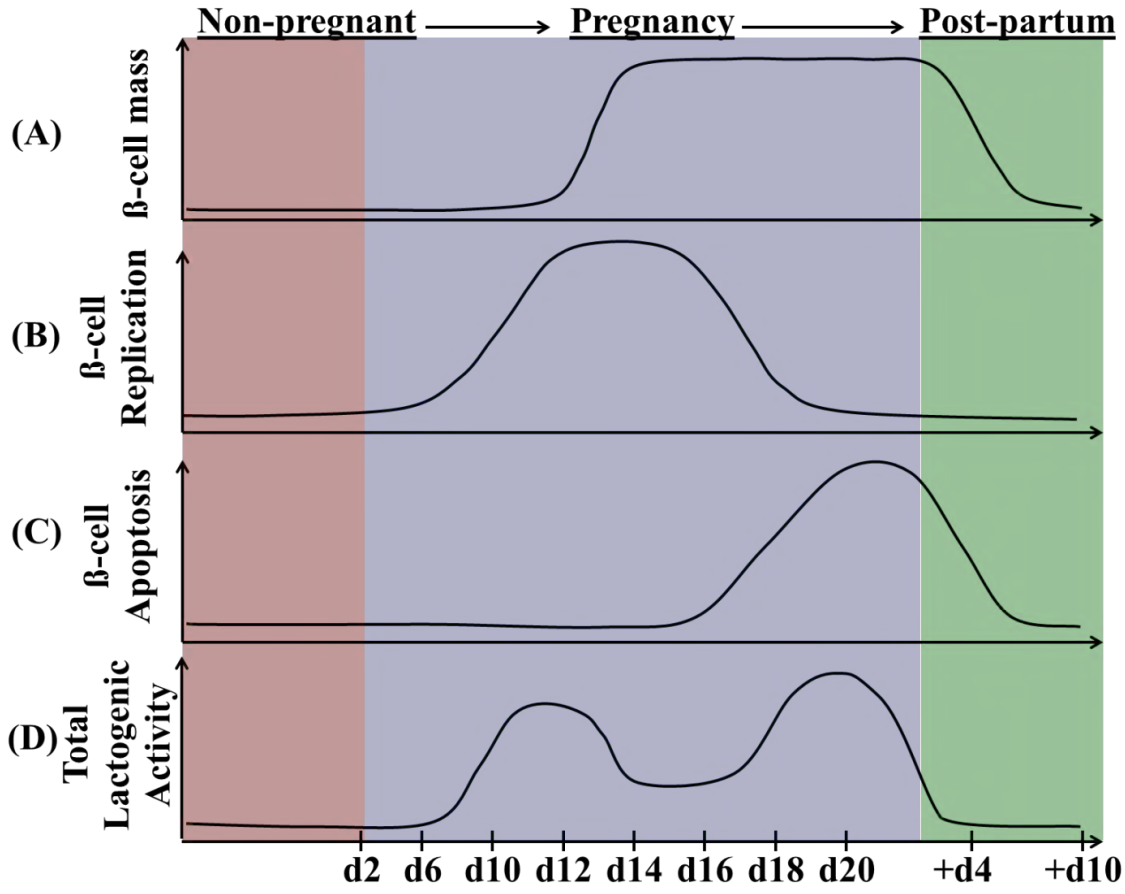

Figure 1.4: β-cell dynamics during pregnancy in the mouse.

Figure 1.4: β-cell dynamics during pregnancy in the mouse. (A) β-cell mass is increased by (B) β-cell replication during the first two-thirds of gestation. After parturition, maternal β-cell mass returns to non-pregnant levels by (C) β-cell apoptosis, which increases through the end of pregnancy and is still detected 4-6 days after birth. The graphs represent approximate changes in these processes before pregnancy (red, non-pregnant), over the course of pregnancy (light purple) and post-partum (green), and show what is believed to occur during rodent pregnancy based on previous studies. (D) Total serum lactogenic hormone levels (such as placental lactogens) during pregnancy in the mother are increased from gestational day 10 to 20, pointing to their key role in the adaptation of the islet to pregnancy. Specifically, of the two identified rodent placental lactogens, levels of PL-I peak at mid-gestation. As such, PL-I is considered to be the first trigger to enhance β-cell proliferation and function, while PL-II is initially detectable on day 12 of gestation and does not reach peak levels until closer to delivery (35, 77). Additionally, it is thought that steroid hormones present at relative high levels at day 19 of pregnancy, such as progesterone, counteract the stimulatory effects of elevated lactogenic activity on β-cell proliferation during the last third of pregnancy (58, 78).

Regulation of β-cell proliferation by cell cycle genes

The cell cycle of the adult β-cell is comprised of four phases: (i) The first gap (G_1) growth phase, (ii) the DNA synthesis (S) phase, (iii) the second gap (G_2) phase, and (iv) the mitotic (M) phase (Figure 1.5). The expression of regulatory subunits called cyclins fluctuates periodically throughout the cell cycle. The association of distinct cyclins with specific cyclin-dependent kinases (cdks) activates the catalytic activity of their cdk partners, and powers the cell through different phases of the cell cycle. Mitogenic stimulation initiates exit from quiescence cells (G_0), entry into G_1-phase of the cell cycle and up-regulation of D-type cyclins. Cyclin D complexes with its catalytic partner Cdk4 and/or Cdk6 to execute critical regulatory events in G_1- phase. E-type cyclin expression is up-regulated next, enabling the binding and activation of Cdk2. Together, these active Cyclin/Cdk complexes inactivate the retinoblastoma protein (Rb) by hyperphosphorylating it and/or both of its functional homologs (p107 and p130). This facilitates progression through S-phase by the subsequent release of the E2F family of transcription factors and the transcription of their target genes, including A-type cyclins, allowing for the formation of the Cyclin A/Cdk2 complex. During G_2- and M-phase, both A-type and B-type cyclins associate with and activate the kinase activity of Cdk1 that is required for progression through mitosis. Inactivation of Cdk1 in late mitosis, by the decreasing availability of appropriate cyclins contributes to reset the cell to G_1-phase (79, 80).

Surveillance mechanisms called checkpoints impose quality control in the cell cycle to ensure that a cell has properly completed all the requisite steps of one phase

before it is allowed to proceed into the next phase (Figure 1.5). For instance, a cell will not be permitted to enter into S-phase until all G_1 processes have been properly completed, for example the licensing of replication origins to form a pre-replicative complex (81). Entry into G_2-phase is blocked until a cell‟s entire chromosomal DNA has been properly replicated. Furthermore, an activated checkpoint will not allow a cell to enter into anaphase, when the paired chromatids are pulled apart, until all of its chromosomes are properly assembled on the mitotic spindle during metaphase. In addition, a cell is not allowed to advance into S- or M-phase if its DNA has been damaged and not yet repaired. Cell cycle arrest is achieved during checkpoint activation by two families of inhibitory kinases, the inhibitors of Cdk4 (INK4s) and the cyclin inhibitory proteins (CIPs) or kinase inhibitory proteins (KIPs), which physically associate with Cyclin/Cdk complexes to block their kinase activities. The INK4s include $p15^{ink4b}$, $p16^{ink4a}$, $p18^{ink4c}$ and $p19^{ink4d}$, and specifically inhibit the activity of the Cdk4/6-Cyclin D complex present during G_1/S-phase. The CIP/ KIP family which includes $p21^{cip1}$, $p27^{kip1}$ and $p57^{kip2}$ inhibit the activity of Cyclin E/Cdk2, Cyclin A/Cdk2, Cyclin A/Cdk1 and Cyclin B/Cdk1 activity present during later stages of the cell cycle. On the other hand, sequestration of $p21^{cip1}$ into Cyclin D/Cdk4 complexes alleviates $p21^{cip1}$- mediated inhibition of Cyclin E/Cdk2 activity and promotes G_1/S phase transit. Of note, it is thought that once cells have passed the restriction point in late G_1-phase, they no longer require extracellular mitogenic cues to complete the cell cycle (80).

There is overwhelming evidence that cell cycle genes controlling the G_1/S-phase transition in the cell cycle are likely central to the control of β-cell proliferation. While the importance of other cell cycle genes regulating other aspects of the cell cycle such as

the G_2/M-phase transition cannot be understated, unfortunately there is a lack of investigation into their predicted role in the β-cell. Indeed, the vast majority of cell cycle genes governing G_1- and S-phase are expressed in mouse and human islets (82, 83). However, it is worth noting that there are important differences in the activity profile of cell cycle genes between human and mouse islets. Fiaschi-Taesch and colleagues catalogued the G_1/S proteome of the human islet and found that Cdk4 and Cdk6 are expressed at comparable levels, whereas only Cdk4 was found in the mouse (84).

Perturbation of G_1/S-phase cell cycle regulators in the β-cell by the use of genetic mouse models yields either deficiency or unconstrained proliferation (Figure 1.5). While many tissues are unaffected by the global deletion of Cdk4 in mice, islets displayed β-cell hypoplasia, leading to diabetes (85). This phenotype can be rescued by replacing the endogenous *Cdk4* locus with a constitutively active form of Cdk4 still under the control of its endogenous promoter. Again, this mouse yielded a restricted phenotype, showing marked β-cell hyperplasia leading to islet neoplasms (86). Additionally, global ablation of both *Cyclin D2* alleles in combination with a single allele of *Cyclin D1* result in greatly reduced β-cell mass and β-cell proliferation after birth, and severe diabetes by 3 months of age (87). Conversely, overexpression of Cyclin D1 is frequently seen in human pancreatic endocrine tumors (88). Mice with β-cell specific ablation of Rb and global deletion of p130 leads to unrestrained cell cycle reentry as well as activation of apoptosis (89). Together the aforementioned studies demonstrate that the D-type Cyclin/Cdk4/ phospho-Rb pathway is required for β-cell replication during adult growth in the mouse.

Interestingly, the decreased capacity with age of the β-cell to expand by proliferation in response to injury or a high-fat diet correlates with epigenetic changes at

the *Cdkn2a* locus, which encodes the INK4 members $p16^{ink4a}$ and $p19^{ink4d}$ (34, 90, 91). In fact, manipulation of $p16^{ink4a}$ expression in transgenic mice dramatically alters the proliferative capacity of β-cells, precisely as would be expected if $p16^{ink4a}$ limits proliferation in aging β-cells (92). Furthermore, recent genome-wide association studies link both CDKN2A ($p16^{ink4a}$) and CDKN2B ($p15^{ink4b}$) to the risk of type 2 diabetes (93), illustrating that G_1/S-phase cell cycle regulators are not only important in rodent β-cell proliferation, but also during the ontogeny of type 2 diabetes.

Finally, a complete picture encompassing all the signaling pathways that control many of the mentioned cell cycle regulators in the β-cell is still not attained (Figure 1.5). For example, while it is acknowledged that prolactin (PRL) signaling via the Jak2/Stat5 pathway increases expression of Cyclin D2, it is not known whether this is the only or most important mechanism for cell cycle activation in the β-cell during pregnancy *in vivo* (94). A known upstream regulator of cell cycle genes is the multiple endocrine neoplasia (MEN) type 1 syndrome protein, menin. Mice deficient in menin specifically in β-cells lead to the development of dramatic β-cell hyperplasia, and islet tumors (95). Increased rates of β-cell proliferation are observed, and associated with reduced expression of $p18^{ink4c}$ and $p27^{kip1}$, both downstream targets of menin- dependent histone methylation (96). Additionally, the transcription factor p53 is thought, among other functions, to be a tumor suppressor, exemplified by its target gene $p21^{Cip1}$. Unlike parental mice containing either *Rb*-null or *p53*-null alleles, mice both heterozygous for *Rb* and null for *p53*, develop frequent insulinomas similar to menin-deficiency (97, 98). These studies demonstrate that p53 in addition to Rb is a critical upstream checkpoint of the cell cycle of the β-cell. As we continue to learn about the complexity of cell cycle control in the β-

cell whether extrinsic or intrinsic, the next challenge will be to identify the most feasible and appropriate β-cell mitogens for driving cell cycle entry and progression in human β-cells *in vitro*.

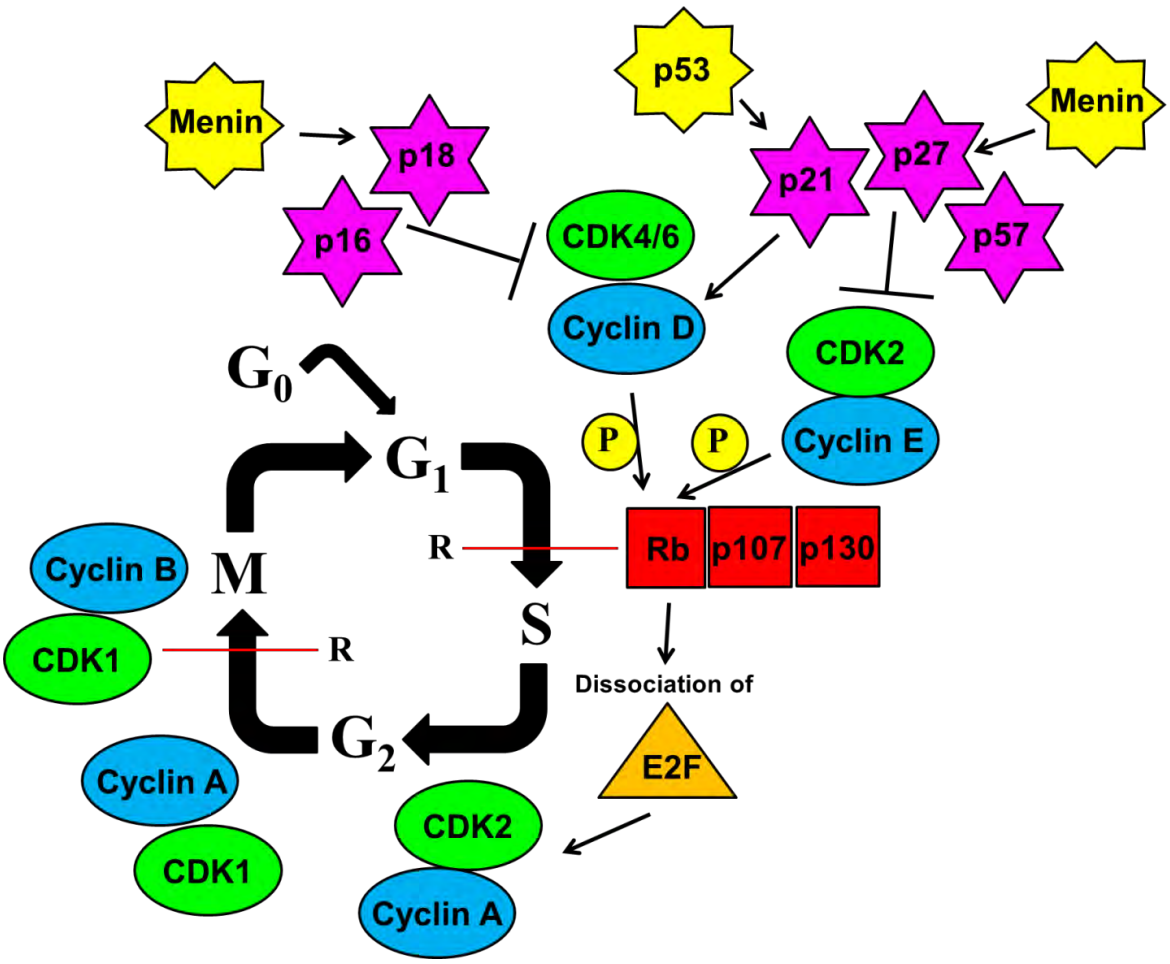

Figure 1.5: Regulation of β-cell proliferation by cell cycle genes.

Figure 1.5: Regulation of β-cell proliferation by cell cycle genes. An overview of the proteins that control β-cell cycle with emphasis on the G_1/S-phase transition. The "P" in the yellow circle indicates phosphorylation. The "R" next to the red line indicates the G_1/ S- and G_2/ M-phase checkpoints, respectively. All of the genes are discussed in the body of the text. It should be noted, as indicated by the yet unexplained observation that embryonic and adult cell division seem to be regulated independently (99, 100), that the study of cell cycle control in the β-cell is still incomplete.

Attempts to expand functional β-cell mass by proliferation *in vitro*

Initial efforts to expand functional β-cells by proliferation have not been successful. Lineage tracing in purified mouse islets demonstrates that β-cells divide very slowly, undergo gradual dedifferentiation to an insulin-, Pdx1-, and Glut2- negative state, and are eventually replaced by cells of non-β-cell origin *in vitro* (101). In spite of this, lineage tracing of primary adult human β-cells with a Cre-loxP system demonstrates that dedifferentiated human β-cells, but not mouse, can be grown in culture for up to 16 passages (102). It is clear that spontaneous replication in primary human islets is ultimately halted over time by cellular senescence, which is characterized by loss of differentiated function (103). However, the redifferentiation of cells derived from adult human β-cells expanded in culture could provide a sufficient number of β-cells needed for islet transplantation at the current availability of human islet donors (104). Understanding the molecular mechanisms involved in β-cell fate *in vitro* is critical for attempts to redifferentiate these cells back into functional glucose-sensing, insulin releasing β-cells after expansion (105).

Because primary β-cells do not proliferate in culture, attempts to bypass the senescence program have been made by immortalizing pancreatic β-cells through the forced expression of oncogenes such as the human homologue of the retrotranscriptase subunit (hTERT), simian virus large T antigen (SV40T), and constitutively activated Ras (106). While some attempts to do this still lead to dedifferentiation of human β-cells (107), the use of reversible immortalization strategy for expansion of human islets has produced a human pancreatic β-cell line that appears functionally equivalent to primary

pancreatic β-cells. Although many safeguards were used to eliminate the possibility of developing tumorigenesis in this human β-cell line, the large scale cell growth needed to meet the amount of cells required for islet transplantation therapy might result in somatic mutations leading to increased cancer risk in the patient (108). Indeed, the cell cycle regulators one might hope will be sufficient to drive cell cycle progression in the β-cell, such as the G_1/S-phase mediators described previously, are themselves members of oncogenic pathways. Whether forced cell cycle entry by these molecules allows non-oncogenic and safe progression of the cell cycle in the β-cell remains an important unanswered question.

With the increased understanding of cell cycle regulation in the past five years, it has become clear what single or a combination of potential human β-cell mitogens need to accomplish to successfully expand functional human β-cell mass by proliferation *in vitro* (Figure 1.6A). First, a β-cell cycle regulator must stimulate entrance into the cell cycle (exit from G_0- and entry into G_1-phase), and ensure that precise duplication of the genome is achieved to maintain genomic stability (progression from G_1- into and through S-phase). Without cell cycle entry, the β-cell would stay in G_0-phase. Also, in addition to the required high fidelity of DNA polymerases, the cell must be able to distinguish between replicated and unreplicated DNA during G_1-phase to ensure the precise duplication of chromosomal DNA during S-phase of a single cell cycle. If the chromosomal DNA is under-replicated as a result of too few active replication origins, the chromosome is likely to be broken near the unreplicated region upon sister chromatid separation during anaphase. The firing of a replication origin more than once would lead to re-replication of the DNA in the vicinity of the over-firing origin, and if initiated

sufficiently close together can undergo head-to-tail collision and lead to extruded double stranded DNA fragments (109, 110). Of note, an additional level of yet unclear regulation exists during S-phase called the „replication timing program" in which sequential patterns of domains containing discrete foci or factories of DNA synthesis occur during the course of S-phase (111).

The licensing of replication origins by the stable binding of the „pre-replicative complex" (Pre-RC) only during G_1-phase affords the cell the ability to distinguish between active and inactive replication origins, defined here as a site on chromosomal DNA where a bidirectional pair of replication forks initiate (81, 109). The major component of the Pre-RC are the mini-chromosome maintenance 2-7 proteins (Mcm2-7), whose binding onto origin DNA is essential for the replication origin to be able to initiate a pair of replication forks. Mcm2-7 is thought to function as a DNA helicase traveling ahead of the replication fork, as it is subsequently displaced from origin DNA as forks are initiated. Therefore the Mcm2-7 complex is never associated with replicated DNA, precluding the origin from being fired again during later phases of the cell cycle (81). Furthermore, the down-regulation of other components of the Pre-RC complex at the G_1/S-phase transition such as chromatin licensing and DNA replication factor 1 (Cdt1), thought to be the rate-limiting factor responsible for loading of the replicative Mcm-helicase onto DNA, is critical to preventing re-licensing of replicated origins (110). While there is no formal way of distinguishing early G_1-phase from G_0, the detection of Mcm2-7 expression is a potentially powerful way of assessing cell cycle entry of β-cells (109).

To date, experiments in primary human islets provide the proof of principle that human β-cells can be stimulated to enter the cell cycle *in vitro*, confirming recent human cadaver studies which determined that β-cell proliferation occurs neonatally (26) and in increased frequency with greater proximity to pancreatic tumors (112). For example, overexpression of a D-type Cyclin with its partner Cdk clearly stimulates β-cells to enter the cell cycle, as assessed by BrdU incorporation, and Ki67 expression, a thymidine analog marker of S-phase and a marker of late G_1- to M-phase, respectively (83, 113). In addition, overexpression of other regulators of the G_1/S-phase transition, such as E2F1 in primary rat islets also leads to activation of multiple phases of the cell cycle, as assessed by BrdU incorporation, Ki67 expression and phosphorylation of histone H3 (pHH3), a phosphorylation event that occurs in M-phase (114). Other examples of factors sufficient for induction of multiple phases of the cell cycle in primary human β-cells include overexpression of CYCLIN E in combination with CDK2, FOXM1, Nkx6.1, and parathyroid hormone- related protein (PTHrP) (115-117). While it is clear that human β-cells can be forced to enter the cell cycle *in vitro*, no assessment of genomic stability has yet been demonstrated.

As implied in the previous section, progression through the remaining cell cycle phases and subsequent cell cycle exit depend primarily on the successful completion of activities during G_1- and S-phase, namely the precise duplication of chromosomal DNA during S-phase (Figure 1.6A). Conversely, genomic instability resulting from DNA breaks generated during DNA replication (defined here as replication stress) can activate a G_1/S-phase checkpoint, also referred to as the DNA damage response (Figure 1.6B). Of the first responders to replication stress, two proteins are activated by phosphorylation;

ATM (ataxia telangiectasia mutated) and ATR (ataxia telangiectasia and Rad3-related protein). These PI3K-like kinases in turn phosphorylate many adaptors and sensors, including gamma-H2AX (γH2AX) on serine 139 which recognizes double stranded DNA breaks and marks megabase lengths of DNA adjacent to break sites, and checkpoint-transducer serine/threonine kinases, Chk1 and 2 (118). Originally thought to obey strict phosphorylation dependence, Chk1 on ATR and Chk2 on ATM, crosstalk among these kinases have now been documented (119). Activated ATM and ATR, directly phosphorylate the p53 transcription factor within its amino-terminal transactivation domain, specifically on serine 15. Also, Chk 1 and 2 converge on p53 phosphorylation, particularly threonine 18 and serine 20. Serine 15 phosphorylation of p53 inhibits Mdm2 binding, an ubiquitin ligase that ensures rapid p53 turnover (118). Together these modifications lead to the stabilization and subsequent accumulation of p53 protein in response to DNA damage.

Once expressed, p53 functions as an integrator of diverse stress signals into different cellular outcomes, including cell cycle arrest, senescence, DNA repair, and apoptosis (120) (Figure 1.6B). For example, the transcriptional upregulation of its target gene $p21^{Cip1}$ silences the activation of the Cyclin E/Cdk2 kinase, blocking the inactivation of Rb, leading to G_1/S-phase cell cycle arrest in an attempt to give time for DNA repair mechanisms to repair DNA damage (118). However, the exact response of the cell to sustained p53 expression depends on the combination of, among others; cell-type, tissue-type, type of stimulus, protein localization, and regulation of target gene selection (120). Indeed, in support of the DNA damage response being utilized in the β-cell, a mouse model deficient in nonhomologous end-joining (NHEJ) and expressing a

hypomorphic mutant of p53, defective in apoptosis but not in cell cycle arrest, develops diabetes. In these mice, β-cell mass is progressively depleted due to accumulated DNA damage (sustained γH2AX expression), promoting a decrease in β-cell proliferation through p53/p21-dependent cell cycle arrest (121). In addition, interventions that activate the cell cycle in β-cells can also activate cell death pathways. For example, β-cell specific overexpression of the oncogene c-Myc in transgenic mice not only increases β-cell proliferation, but also increases apoptosis leading to the development of diabetes (122). Furthermore, while sufficient to induce progression through one cell cycle, the ultimate fate of primary rat β-cells overexpressing E2F1 is apoptosis (114). Multiple demonstrated (dedifferentiation, apoptosis, senescence) and potential barriers (DNA damage response) to attempted β-cell proliferation exist, and unfortunately, an *in vitro* protocol for efficient, step-wise non-oncogenic stimulation of human β-cell proliferation to completion remains elusive.

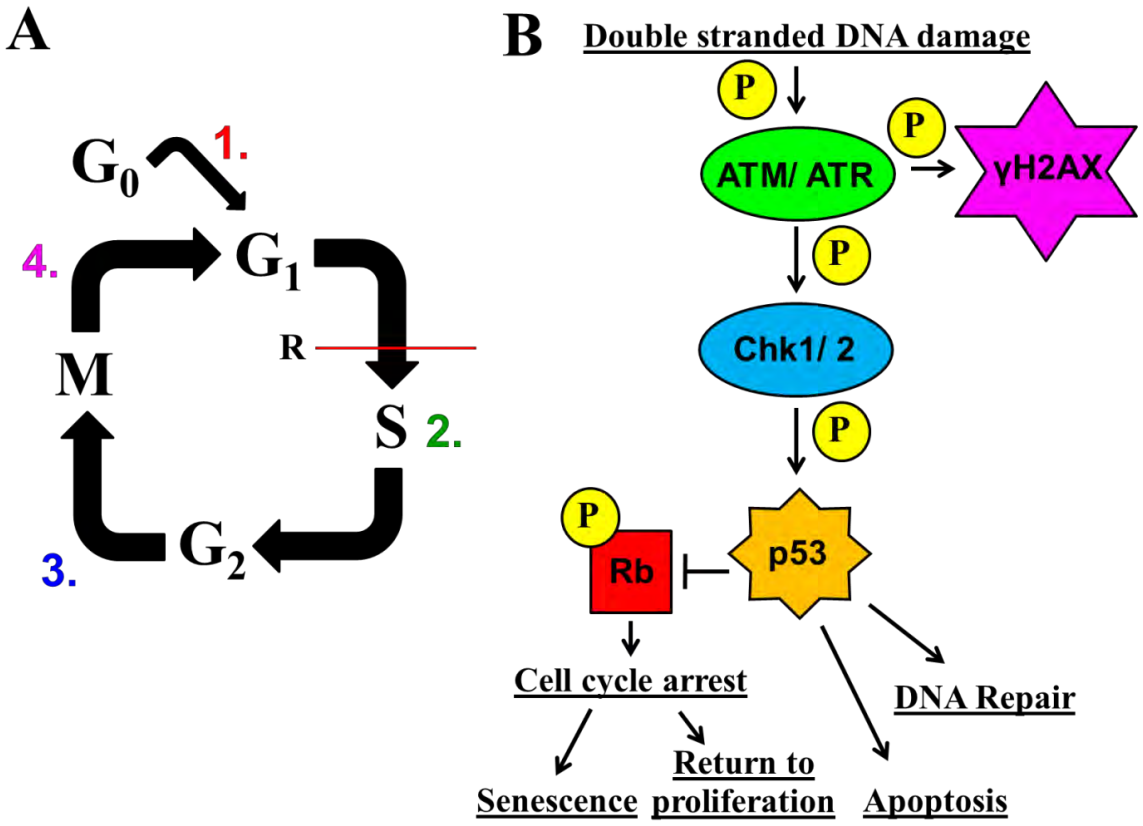

Figure 1.6: Challenges that need to be overcome to successfully expand functional β-cells by proliferation *in vitro*.

Figure 1.6: Challenges that need to be overcome to successfully expand functional β-cells by proliferation *in vitro*. (A) To successfully replicate, a sufficiency factor would have to (1) enter the cell cycle, and license DNA at each replication origin, (2) properly duplicate chromosomal DNA, (3) progress through G_2/M- phases, and (4) exit the cell cycle. We use this as a model and basis for characterization of potential β-cell mitogens. (B) An oversimplified model of the DNA damage response pathway, showing the main mediators of the double stranded DNA stimulus to cellular outcome. Briefly, ATM/ATR are activated by double stranded DNA damage, and phosphorylate their targets, γH2AX, and Chk1/2. Chk1/2 in turn with ATM/ATR stabilizes p53 expression. Depending on many variables, p53 can activate diverse processes such as cell cycle arrest, senescence, DNA repair and apoptosis.

Maturity Onset Diabetes of The Young (MODY) transcription factors regulate proliferation and survival in the adult β-cell

Maturity onset diabetes of the young (MODY) is a monogenic form of type 2 diabetes. The clinical criteria used to characterize MODY include (a) diagnosis before 25 years of age in at least one family member, (b) autosomal dominant inheritance pattern and (c) defects in insulin secretion (123). Mutations in at least six identified genes define the molecular genetic etiology of MODY. All encode various transcription factors including hepatocyte nuclear factor 4α (HNF4α; MODY1), hepatocyte nuclear factor 1α (HNF1α; MODY3), pancreatic and duodenal homeobox 1 (Pdx1; MODY4), hepatocyte nuclear factor 1β (HNF1β; MODY5) and neurogenic differentiation factor 1 (NeuroD1; MODY6), except for glucokinase (Gck; MODY2), a glycolytic enzyme important for glucose sensing (124). The identification of glucokinase as a type 2 diabetes susceptibility gene highlights MODY as an attractive model for studying the multifactorial polygenic disorder of type 2 diabetes (125). It is now clear that mutations in the different MODY genes result in clinical heterogeneity. While the frequency of specific MODY gene mutations varies in MODY families from different countries, the high percentage of the diabetic phenotype in carriers heterozygous for a MODY mutation indicates the high penetrance of all known mutations (126, 127). Since the diabetic phenotype of not all MODY families can be explained by the existing MODY genes, additional MODY genes have been described such as kruppel-like factor 11 (Klf11; MODY 7), carboxyl ester lipase (Cel; MODY8), and paired box 4 (Pax4; MODY9) (126, 128-130).

MODY genes, well known for their role in glucose stimulated insulin secretion, have been shown recently to be involved with β-cell mass homeostasis through the regulation of proliferation and survival pathways. A recent study indicates that the rate of β-cell proliferation is controlled by glycolysis or workload placed on an individual β-cell, rather than blood glucose per se, linking together two previously assumed separate cellular functions of the β-cell (55). Furthermore, it seems that the modulation of glucokinase (MODY2) activity through genetic deletion or pharmacological activation, is key to determining the rates of β-cell proliferation during adulthood, high fat diet and following β-cell injury (55, 131). However, in line with the known toxicity of high concentrations of glucose on β-cells (132), a human patient exhibiting an activating mutation in glucokinase exhibits not only enhanced replication but also increased apoptosis, although the reason for β-cell death is unknown (57). Indeed, mice haploinsufficient for Pdx1 (MODY4) show blunted β-cell mass expansion in response to high fat diet through increased endoplasmic reticulum (ER)-stress induced apoptosis (133). Also, Pax4 (MODY9) protects adult β-cells from stress-induced apoptosis (134). Together these studies of MODY genes demonstrate the importance of further identifying molecular players leading to β-cell proliferation and/or β-cell survival during times of increased metabolic demand.

Hepatocyte Nuclear Factor- 4α: The MODY1 Gene, β-cell function and β-cell proliferation

Hepatocyte nuclear factor 4α (HNF4α) is a highly conserved family member of the nuclear receptor superfamily of transcription factors. A typical nuclear receptor is characterized by a variable amino-terminal transactivation domain (AF-1; A/B region), a conserved zinc-finger DNA binding domain (DBD) (C region), a hinge domain (D region), a conserved ligand binding domain (LBD) containing a second (AF-2) transactivation domain (E region), and an inhibitory carboxy-terminal domain (F region) (135). The members of the nuclear hormone superfamily can be categorized based on the following criteria: protein dimerization, structure of the cognate DNA binding site, and intracellular localization. HNF4α is thought to define a distinctive subclass of nuclear receptors, defined by stable homodimerization, predominantly nuclear localization, and a binding preference for direct repeats of a hexamer half-site (DR+1 element) (136). While the classical model of nuclear receptor transcriptional activation requires ligand binding to induce a conformational change in the LBD that recruits co-activator complexes (137), questions remain whether HNF4α transcriptional activity is always ligand- dependent.

The mechanism by which HNF4α is transcriptionally activated is unclear. The crystal structure of the hydrophobic ligand binding domain of bacterially expressed HNF4α has been solved, and demonstrates the ability of the binding pocket to irreversibly hold various fatty acids (138). However, further structural studies on bacterially expressed HNF4α show that co-activator binding and not the binding of a ligand determines the active confirmation of the α-helix containing the AF-2 domain (139). Indeed, HNF4α functionally interacts with the co-activator PGC-1α independent of

the addition of exogenous ligand to transcriptionally activate the promoter of the gluconeogenic gene PEPCK *in vitro* (140). Also, tyrosine phosphorylation can affect the potential of HNF4α to bind to DNA and its localization within the nucleus (141). The recent use of mammalian cell lines and tissues identified linoleic acid (LA), a single polyunsaturated fatty acid obtained from the diet, as an endogenous ligand for HNF4α. While significant ligand-independent transcriptional activity of HNF4α was observed, linoleic acid binding was found to be bound to HNF4α in the livers of fed and not fasted mice (142). This demonstrates that its linoleic acid binding is reversible *in vivo*, suggesting that in addition to being a nuclear receptor, HNF4α is a potential drug target. While physiological function of linoleic acid is not known, the mechanism of HNF4α activation might be dependent on physiological context as described for other pancreatic transcription factors, for example FoxA2 (143).

Although the mechanism underlying HNF4α transcriptional activity is still controversial, its physiological role is clear. HNF4α is expressed primarily in the liver, gut, kidney and pancreas (144). At least six isoforms of HNF4α exist and their tissue specific expression is accomplished by a proximal promoter (P1) and an alternative promoter (P2) located 46kb upstream of P1 (145). Specifically, HNF4α isoforms 7-9 are expressed in adult pancreas, and not adult liver via utilization of the P2 promoter (Figure 1.7) (146). The clinical phenotypes of patients with MODY1 reflect either a pancreas defect characterized by progressive loss of insulin secretion in response to a glucose and arginine challenge (147) or a liver defect described as impaired lipid homeostasis prior to hyperglycemia (148). To investigate whether the loss of insulin secretion might reflect a primary defect in MODY1 patients and not a long term consequence of loss of HNF4α in

other tissues, a mouse model that specifically ablates HNF4α in the β-cell ($HNF4\alpha^{LoxP/LoxP}$; Insulin.Cre) was utilized. These adult mice were glucose intolerant, and exhibited diminished insulin release in response to glucose and sulfonylureas. Furthermore, HNF4α was required for the expression for potassium channel subunit Kir6.2, clearly demonstrating that HNF4α is required in the pancreatic β-cell for the maintenance of glucose homeostasis *in vivo*. In addition, the lack of overt diabetes in this mouse model makes it likely that contributions from other HNF4α-deficient metabolic organs are required for the progression to type 2 diabetes in MODY1 patients (149). Interestingly, the mild hypoglycemia and elevated tonic insulin secretion seen in these mice reflect the clinical phenotypes of children born haploinsufficient for HNF4α, later evolving into diabetes due to reduced insulin secretion (150).

Indeed, in addition to loss-of-function mutations found near the P2 promoter of the *HNF4α* locus in MODY1 families (151), there is evidence that variants in the regulatory regions of *HNF4α* contribute to type 2 diabetes risk in specific human populations. For example, single nucleotide polymorphism (SNP) markers were genotyped in case and control DNA pools from sibling-pair families in Finland. To eliminate cases of type 1 diabetes and MODY, diabetes age-of-onset was constrained to greater than 35 years of age. 291 SNPs were discovered at 20q13, the chromosomal location of the *HNF4α* locus, of which ten showed association with diabetes disease status (Figure 1.8). The two SNPs that showed the strongest association with diabetes status, rs1884613 and rs2144908, flank the β-cell specific P2-promoter. Furthermore, analysis of unaffected offspring carrying at least one copy of the rs2144908 risk allele exhibited lower acute insulin response to glucose as detected by an oral glucose tolerance

test, consistent with known HNF4α function in the β-cell (152). Strikingly, similar findings were found in independent and candidate gene studies of Ashkenazi Jewish (153), Mexican American (154), and Danish populations (155), suggesting that genetic variation near the promoters of HNF4α might predict susceptibility to late onset diabetes.

A role in β-cell proliferation can also be extended to HNF4α (MODY1). While β-cell mass is unchanged during normal conditions in the β-cell specific ablation of HNF4α (149), there is a defect in β-cell expansion that normally occurs in response to pregnancy leading to further glucose intolerance. Specifically, HNF4α is required in β-cells for the proliferative response of pregnancy through activation of the Ras/ERK signaling cascade. The down regulation of suppression of tumorigenicity 5 (St5), a novel positive regulator of ERK signaling in β-cells and a direct transcriptional target of HNF4α, contributes to the reduction of ERK activation caused by HNF4α deficiency (156). Its membership in the nuclear receptor family, expression of islet-specific isoforms, and its required role in β-cell proliferation during pregnancy, makes HNF4α an attractive potential β-cell mitogen warranting future investigation.

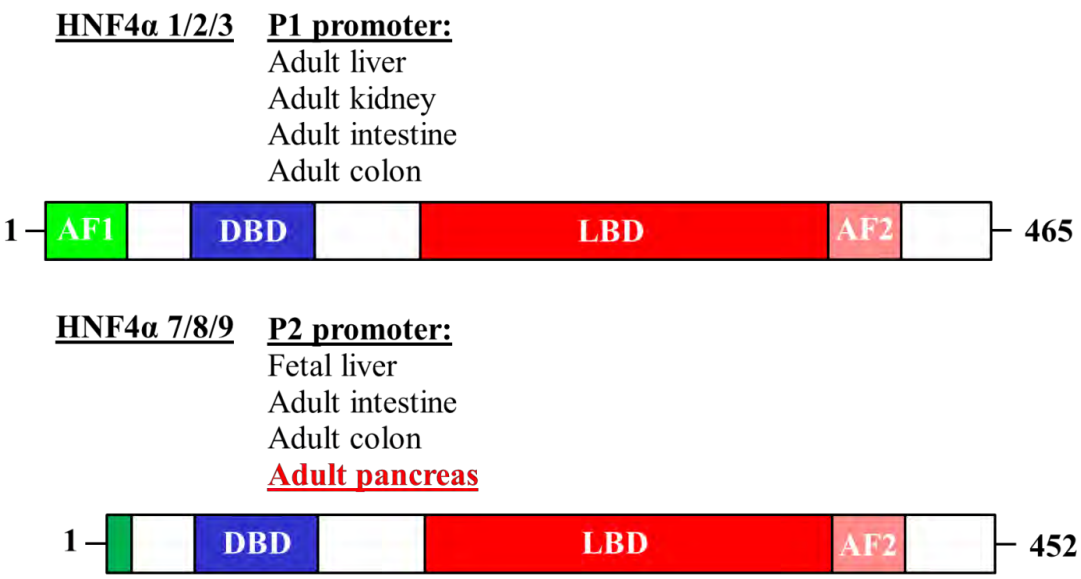

Figure 1.7: HNF4α isoforms and their functional domains.

Figure 1.7: HNF4α isoforms and their functional domains. The nuclear receptor HNF4α is located on human chromosome 20q13.1-13.2, and has two promoters (P1 and P2). At least 6 isoforms of HNF4α are generated by differential use of the promoters and varying splicing events specific to the C-terminus. Representative protein structures of P1- and P2- derived HNF4α isoforms are shown. 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. HNF4α contains different protein domains: an amino-terminal transactivation domain (AF1; green), a conserved zinc-finger DNA binding domain (DBD; blue), and a ligand binding domain (LBD; red) containing a second (AF-2; light red) transactivation domain. P2 derived transcripts do not contain the AF1 domain in the amino-terminal domain, indicated as dark green.

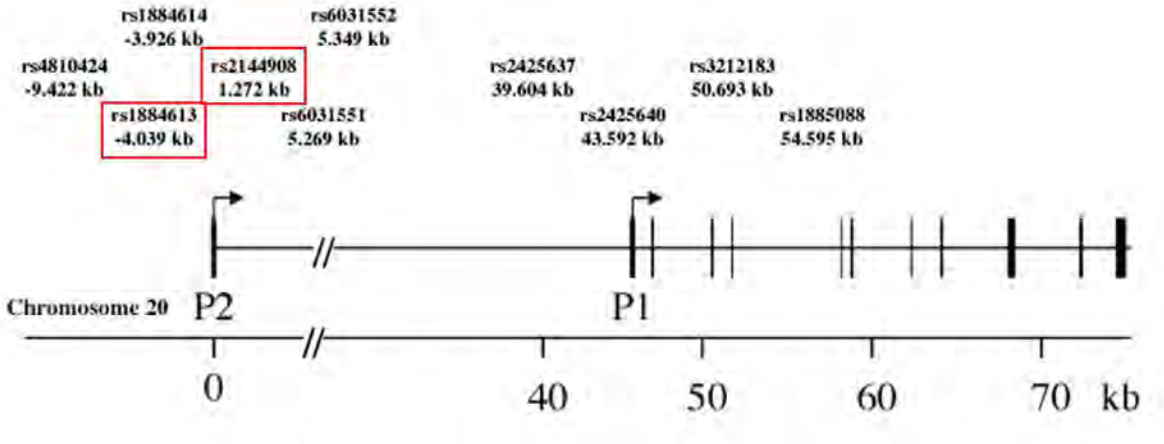

Figure 1.8: Location of SNPs associated with late-onset type 2 diabetes in the HNF4α locus.

Figure 1.8: Location of SNPs associated with late-onset type 2 diabetes in the HNF4α locus. This illustration was taken from (157). Shown here are ten SNPs associated with late-onset type 2 diabetes in the Finnish and Ashkenazi Jewish populations along with their locations relative to the P2 promoter (152, 153). Highlighted in the red boxes are two SNPs that are most commonly associated with diabetes disease status across various populations, rs2144908 and rs1884613.

Note: This illustration has been published in TRENDS in Molecular Medicine in a Review article entitled,

HNF-4alpha: from MODY to late-onset type 2 diabetes.

Gupta, R.K., and Kaestner K.H. 2004. HNF-4alpha: from MODY to late-onset type 2 diabetes. *Trends Mol Med* 10:521-524.

Summary and Specific Aims

Generating new β-cells *de novo* as a cellular therapy is a realistic long term goal for treatment for diabetes mellitus. One approach is to use instructive cues to induce proliferation of terminally differentiated β-cells. However, a well-defined protocol to drive efficient non-oncogenic proliferation of human β-cells *in vitro* remains elusive. Presently, the molecular mechanisms that control the expansion of β-cell mass during physiological states of insulin resistance such as occurs during pregnancy are poorly understood. I propose that a more complete knowledge of the mechanisms and factors that govern physiological β-cell expansion can help translate into an effective and defined human protocol for the generation of fully functional β-cells through the replication of pre-existing β-cells.

The following chapters will describe the results of a microarray experiment designed to examine the gene expression signature of the islet during pregnancy in mice, and an attempt in evaluating the ability of HNF4α as a human β-cell mitogen. **The goal of these experiments is to (1) determine what mechanisms are involved in controlling islet replication during pregnancy *in vivo* in mice and (2) assess the consequence of the overexpression of a pancreas specific isoform of HNF4α on human β-cell proliferation *in vitro*.** These experiments will identify many novel genes involved in islet expansion during pregnancy, clarify the mechanisms associated with general islet expansion, validate HNF4α‟s role in human β-cell proliferation and uncover the DNA damage response as a cell cycle progression inhibitor in human β-cells *in vitro*.

Note: Portions of this chapter have been published in TRENDS in Endocrinology and Metabolism as a Review article entitled,

Expansion of β-cell mass in response to pregnancy.

Rieck S, Kaestner KH. *Trends Endocrinol Metab*. 2010 Mar; 21(3):151-8.

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Chapter II:

The transcriptional response of the islet to pregnancy in mice

Abstract

The inability of the β-cell to meet the demand for insulin brought about by insulin resistance leads to type 2 diabetes. In adults, β-cell replication is one of the mechanisms thought to cause the expansion of β-cell mass. Efforts to treat diabetes require knowledge of the pathways that drive facultative β-cell proliferation *in vivo*. A robust physiological stimulus of β-cell expansion is pregnancy, and identifying the mechanisms underlying this stimulus may provide therapeutic leads for the treatment of type 2 diabetes. The peak in β-cell proliferation during pregnancy occurs on day 14.5 of gestation in mice. Using advanced genomic approaches, we globally characterize the gene expression signature of pancreatic islets on day 14.5 of gestation during pregnancy. We identify a total of 1,907 genes as differentially expressed in the islet during pregnancy. The islet‟s ability to compensate for relative insulin deficiency during metabolic stress is associated with the induction of both proliferative and survival pathways. A comparison of the genes induced in three different models of islet expansion suggests that diverse mechanisms can be recruited to expand islet mass. The identification of many novel genes involved in islet expansion during pregnancy provides an important resource for diabetes researchers to further investigate how these factors contribute to the maintenance of not only islet mass, but ultimately β-cell mass.

Introduction

The onset of type 2 diabetes in both human and rodent models is accompanied by a progressive decrease in β-cell mass, resulting from increased β-cell apoptosis (1). Many physiological and pathophysiological states such as pregnancy, nondiabetic obesity, aging, genetic insulin resistance, and acute illness increase systemic insulin demand (2). In order to compensate for relative insulin deficiency, pancreatic β-cells dynamically expand their mass. Although insulin resistance arises during metabolic stress, it is the inability of the β-cell to meet the demand for insulin that ultimately leads to type 2 diabetes.

Whereas the ability of the adult pancreas to modulate β-cell mass has been clearly demonstrated, at least in rodents, there is controversy regarding the mechanisms underlying the expansion of β-cell mass. These mechanisms include replication of pre-existing β-cells, differentiation of progenitors within the ductal epithelium, transdifferentiation of acinar cells, and differentiation of pancreatic stem cells and/or progenitors that are not of β-cell, ductal or acinar origin (3). Recent studies indicate that during adulthood the great majority of new β-cells in mice are derived through replication or pre-existing β-cells and few, if any, newly formed β-cells stem from progenitor cell populations (4). Only under extreme conditions is the fetal differentiation program, involving the basic helix loop helix factor Ngn3, reactivated in the adult mouse (5), indicating that terminally differentiated rodent β-cells retain a significant capacity to proliferate *in vivo*.

Adult β-cells undergo very little turnover and are estimated to have an average lifespan of approximately 60 days in the mouse (6), with turnover being even rarer in

islets of adult humans (7). Therefore, it is often necessary to introduce a metabolic stress that increases the demand for insulin in order to expose the effects of mutations affecting β-cell expansion. Such an approach has been used to study the role of Pdx1 (8) and Hnf4α (9) in the maintenance of β-cell mass.

One of the most robust physiological stimuli of β-cell expansion is pregnancy. Studies in rats demonstrated that β-cell proliferation increases dramatically during pregnancy, with a peak occurring about two-thirds of the way through gestation and returning to prepartum levels after day 18.5, as assessed through BrdU incorporation into DNA (10). This peak in proliferation coincides with the peak of placental lactogen levels. However, it remains unclear which mechanisms specifically drive β-cell replication during pregnancy *in vivo*. In this study we systematically uncover pathways and factors underlying the islet‟s proliferative response to pregnancy *in vivo*, because these targets might be exploited in the treatment of diabetes.

Results

β-cell proliferation and β-cell hypertrophy dramatically increase β-cell mass during pregnancy

To rigorously examine the modulation of β-cell mass in the adult mouse during pregnancy, we assessed β-cell mass in both non-pregnant control and pregnant female mice specifically at day 14.5 of gestation, corresponding to the peak bromodeoxyuridine (BrdU) labeling in the rodent (10). Using morphometric analysis, we found that β-cell mass was increased 3.8 times on day 14.5 of pregnancy compared to non-pregnant controls (Figure 2.1A). The increase in β-cell mass was strongly correlated with increased BrdU incorporation into DNA. On average, non-pregnant controls exhibited 1.14 BrdU-positive cells per islet crosssection and 1.04% of all islet nuclei were BrdU-positive. Conversely, BrdU labeling was increased to 4.02 BrdU-positive cells per islet crosssection and 3.58% of cells on day 14.5 of gestation (Figure 2.1B-C). In pregnant animals almost all islets contained at least one BrdU-positive cell, but many contained five to ten or even more BrdU-positive cells (Figure 2.1D). Insulin and BrdU immunostaining confirmed that it was indeed β-cells that were proliferating (Figure 2.2A-F). Figure 2.2 also illustrates the appearance of doublet BrdU stained nuclei, showing an increase of newly formed daughter cells, specifically as a result of β-cell mitosis. Ki67 antigen detection also confirmed the dramatic increase in β-cell proliferation on day 14.5 of gestation (Figure 2.2G-H), and closely mirrored a 4.8-fold increase in mKi67 mRNA expression (data not shown). In addition, the expression levels of *cyclin A2*, *B1*, *B2*, *D3*, *E1*, *F* and *Cdk4* were significantly increased, while expression of *Cdk6* and *Cdk7* was significantly decreased as compared to islets from control mice (Figure 2.1G).

Concurrently with β-cell proliferation, β-cell hypertrophy occurred (Figure 2.2I-J), as β-cell size, as measured by volume was increased approximately three times on day 14.5 of pregnancy (Figure 2.1E) with an overall shift towards larger β-cells (Figure 2.1F).

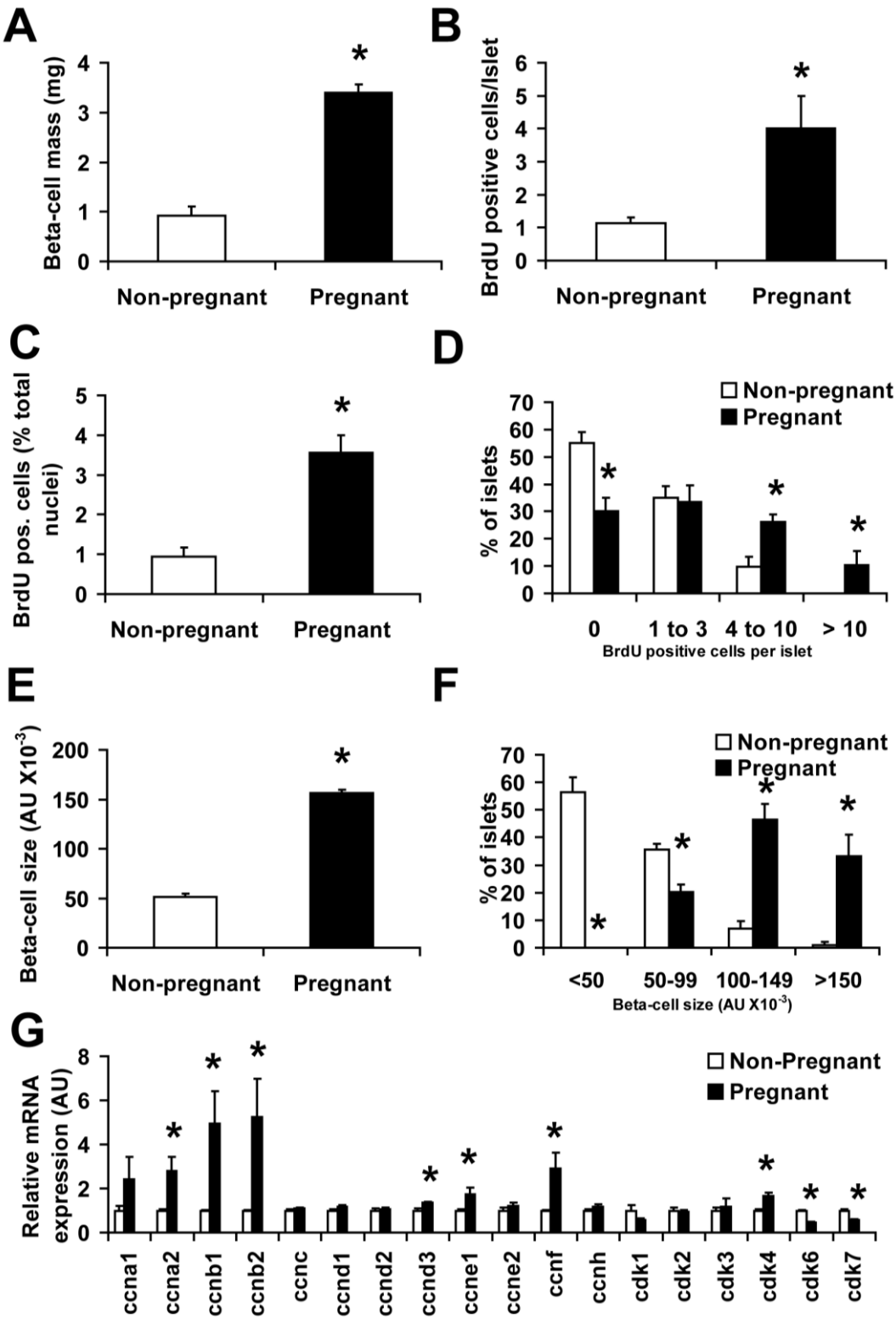

Figure 2.1: β-Cell proliferation with β-cell hypertrophy dramatically increase β-cell mass at day 14.5 of pregnancy in mice.

Figure 2.1: β-Cell proliferation with β-cell hypertrophy dramatically increase β-cell mass at day 14.5 of pregnancy in mice. (A) β-Cell mass of nonpregnant and pregnant (d 14.5) mice (n = 3–4/group; *, $P < 0.05$ *vs.* nonpregnant control). Quantification of (B) BrdU-positive cells per islet (n = 4–5; *, $P < 0.03$ *vs.* control), (C) BrdU-positive cells as % of total nuclei (n = 4–5; *$P < 0.01$ *vs.* control), and (D) percentage of islets with certain number of BrdU-positive cells. Determination of (E) β-cell size and (F) percentage of islets with certain β-cell size (n = 4–5; *, $P < 0.05$ *vs.* control). Gene expression changes (G) of cell cycle regulators (n = 4–5; *, $P < 0.05$ *vs.* control). AU, Arbitrary units; pos., positive.

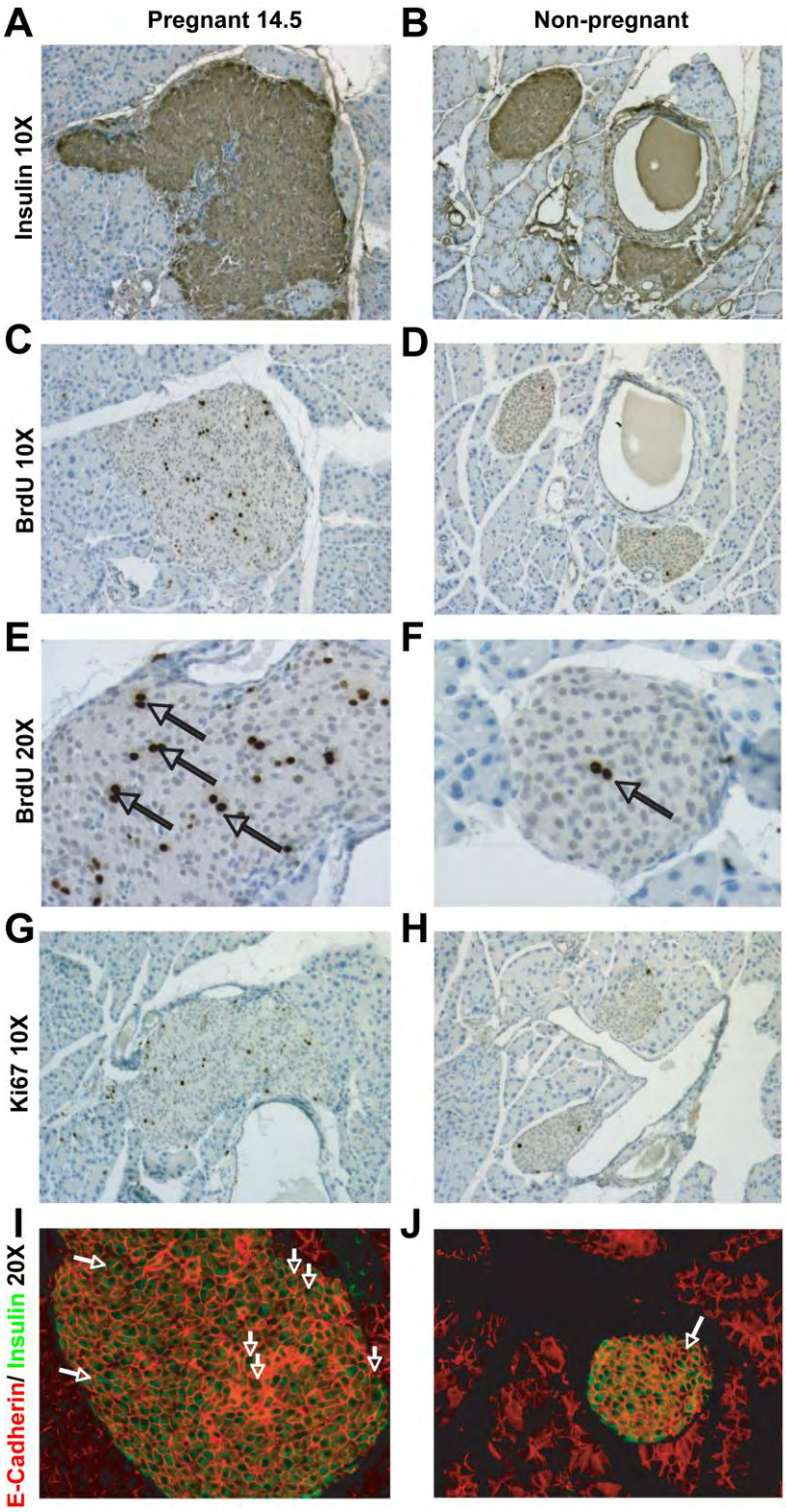

Figure 2.2: Histological analysis of β-cell proliferation, hypertrophy, and mass at day 14.5 during pregnancy.

Figure 2.2: Histological analysis of β-cell proliferation, hypertrophy, and mass at day 14.5 during pregnancy. Insulin staining (A) and BrdU incorporation into β-cells (C) of pregnant mice, insulin (B) and BrdU (D) staining of nonpregnant female mice, and BrdU staining of pregnant (E) and nonpregnant (F) mice (×20). Ki67 staining of pregnant (G), and nonpregnant (H) mice, dual fluorescent staining of cell surface marker E-cadherin (*red*) and insulin (*green*) of pregnant (I) and nonpregnant (J) mice.

Identification of differentially expressed genes throughout pregnancy in the islet

Whereas the dramatic proliferative response of the pancreatic β-cell that occurs during pregnancy has been known for some time (10), no systematic study that investigates the expression profile of this response has been reported thus far. To elucidate additional physiologic factors important for driving β-cell specific expansion during pregnancy *in vivo*, we isolated total RNA samples from islets of 4-5 month-old non-pregnant controls and pregnant (at day 14.5 of gestation) females for large-scale expression profiling. Quantitative RT-PCR (qPCR) was used to determine the relative levels of endocrine (prohormone convertase-2) and exocrine (amylase and chymotrypsinogen) gene expression levels in each sample, minimizing differential gene expression originating from the islet preparation technique. Using SAM (Significance Analysis of Microarrays), we identified nearly 2,000 genes that were differentially expressed in islets on day 14.5 of gestation when compared to non-pregnant controls. qPCR confirmation of selected significantly differentially expressed genes agreed with the great majority of the microarray data (Table 2.3). Many of the genes identified in our analysis have not been previously reported to play a role in β-cell expansion, and include key enzymes involved in serotonin biosynthesis *(Tph1/2), Itk, Gdf3, Tnfrsf11b, Ngfr, Bmp1, Cish, Socs2, Ilrn, Pax8, Hopx, Birc5, Nupr1* and the potential cell cycle regulators *Fbwx15, Fbxl17, Fbxl21, Fbxo27*. Factors linked to β-cell neogenesis and islet regeneration (*Reg3a*, *Pap*) were also significantly upregulated during pregnancy day 14.5 (11). In addition, not only are these genes differentially expressed concurrently with the peak of β-cell proliferation, but, for the 18 genes tested, also at day 10.5 when β-cell

replication is thought to initiate (Figure 2.4A-R). The majority of genes are also differentially expressed during 18.5 possibly reflecting potential further roles in the islet during pregnancy independent from replication.

Gene Symbol	Fold Change	qPCR (p<0.05)	Functional Category	Description
C85627	53.1	425.2	F-box domain containing protein	Expressed sequence C85627
Cldn8	51.1	74.3	Cell adhesion molecule/ Tight junction	Claudin 8
Tph1	19.1	218.3	Serotonin biosynthesis/ Regeneration	Tryptophan hydroxylase 1
Itk	18.2	46.3	Protein tyrosine kinase activity/ Proliferation	IL2-inducible T-cell kinase
Gdf3	17.9	16.6	Growth factor activity	Growth differentiation factor
Tph2	12.1	9.4	Serotonin biosynthesis/ oxidoreductase activity	Tryptophan hydroxylase 2
Cish	10.9	13.3	Regulation of growth	Cytokine inducible SH2 containing protein
Fmo1	7.20	10.72	Oxidoreductase activity	Flavin containing monooxygenase 1
Il1rn	5.1	27.3	Insulin secretion	Interleukin 1 receptor antagonist
Pax8	4.9	6.3	Cell differentiation/ Transcription	Paired box gene 8
Rasgrp1	4.8	4.6	Proliferation	Ras guanyl releasing protein 1
Igfbp5	4.4	9.3	Regulation of cell growth	Insulin-like growth factor binding protein 5
Fbxw15	4.2	34.8	Cell cycle regulation	F-box and WD-40 domain protein 15
Chgb	4.2	3.4	Secretory granule biosynthesis	Chromogranin B
Txnrd2	3.9	3.5	Oxidoreductase activity	Thioredoxin reductase 2
Reg3a*	3.6	5.5	Neogenesis	Regenerating islet-derived 3 alpha
Ngfr	3.4	3.2	Proliferation/ apoptosis	Nerve growth factor receptor
Pap (Reg3b)*	3.4	3.2	Neogenesis	Pancreatitis-associated protein
Tnfrsf11b	3.3	2.6	Receptor activity/ apoptosis	Osteoprotegerin
Angptl6	3.1	3.1	Angiogenesis	Angiopoietin-like 6
Socs-2	3.1	5.2	Regulation of growth	Suppressor of cytokine signaling-2
Hopx	3.0	3.2	Transcription/ Growth	Homeobox only protein
Mbc2	2.8	2.8	Insulin Secretion	Membrane bound C2 domain containing protein
Bmp1	2.7	3.1	Proteolysis/ Growth factor activity	Bone morphogenetic protein 1
Birc5	2.7	4.9	Anti-apoptosis/ cell cycle	Baculoviral IAP repeat-containing 5 (Survivin)
Prlr	2.6	3.0	Proliferation	Prolactin receptor
Acvr1c	2.5	3.4	Insulin secretion/ apoptosis	Activin A receptor, type 1C
Nupr1	2.5	2.4	Proliferation/ apoptosis	Nuclear protein 1
Id4	2.2	2.0	Proliferation	Inhibitor of DNA binding 4
Rab3d	2.0	1.4	Insulin secretion	Member Ras oncogene family
Cdca3	1.5	3.1	Proliferation	Cell division cycle associated 3
Cdc20	1.5	2.6	Proliferation	Cell division cycle homolog 20

*PancChip 6.2

Table 2.3: Most differentially expressed genes during pregnancy day 14.5.

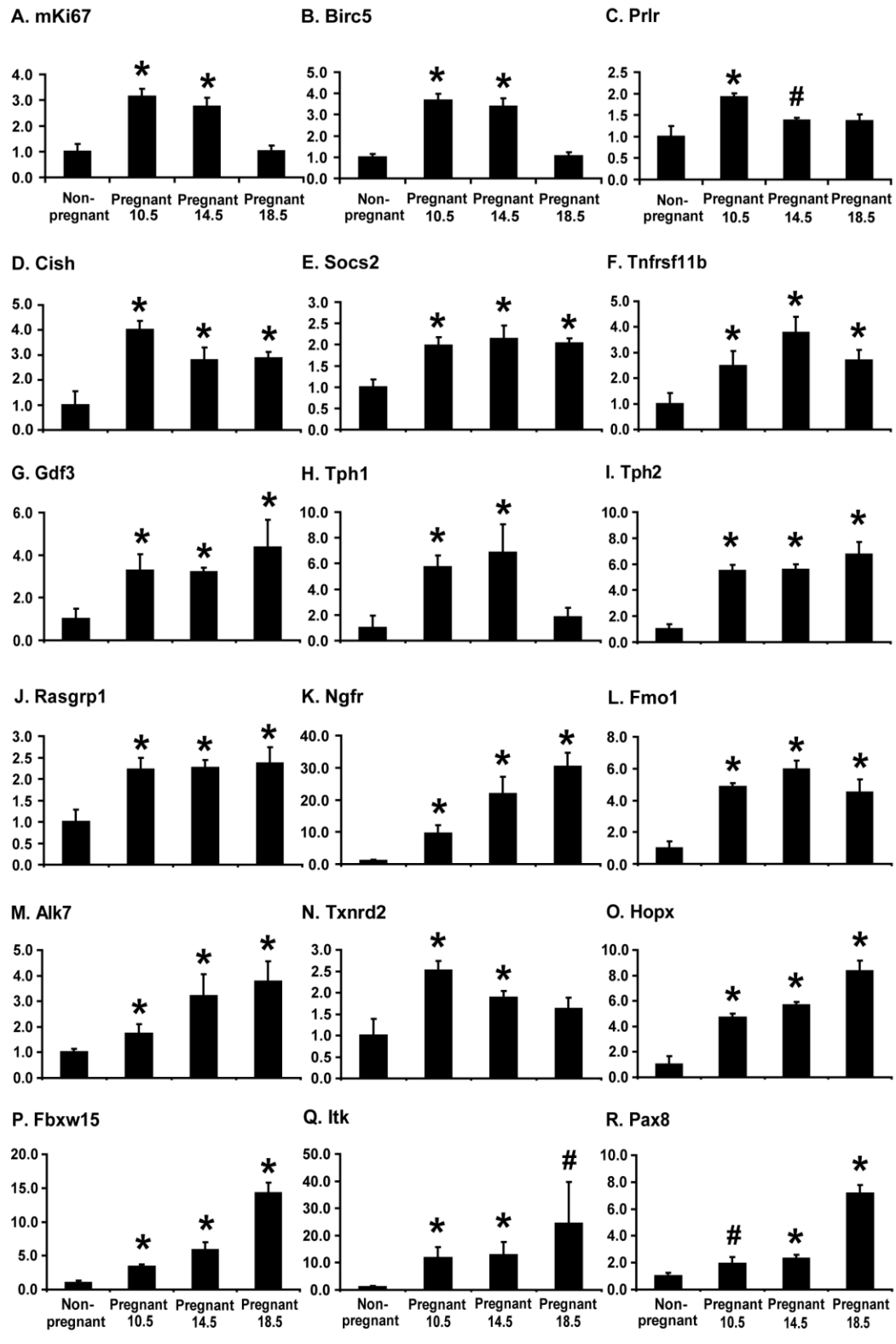

Figure 2.4: Temporal gene expression analysis of selected differentially expressed genes throughout pregnancy.

Figure 2.4: Temporal gene expression analysis of selected differentially expressed genes throughout pregnancy. (A–R) Gene expression changes for each respective gene during d 10.5, d 14.5, and d 18.5 during pregnancy (n = 4–5 per time point; *, $P < 0.05$; #, $P < 0.09$).

Differentially-expressed genes are present in β-cells

To investigate whether the expression of differentially expressed genes during pregnancy we found in islets are expressed in β-cells, we used Mip-GFP (mouse insulin promoter- green fluorescent protein) transgenic mice to enrich for β-cells (12). We prepared single-cell suspensions from isolated islets and separated GFP+ β-cells from GFP- non-β-cells, by fluorescence-activated cell sorting. Although sorting of GFP+ and GFP- fractions was successful (Figure 2.5), significant levels of *Insulin* and *Pdx1* mRNA were present in the GFP- fractions, at levels many orders of magnitude higher than RNA isolated from wildtype livers (Figure 2.6A-B). This indicates that not all β-cells in Mip-GFP mice express high enough GFP to allow for sorting. Despite this limitation, we observed significantly elevated expression of a panel of genes in GFP+ compared to GFP- cells derived from pregnant 14.5 islets for all genes except *Gdf3* (Figure 2.6E-O). This confirms that the majority of the islet-specific differentially expressed genes described above are indeed expressed in β-cells during pregnancy, and is consistent with their proposed role in regulating β-cell replication during gestation.

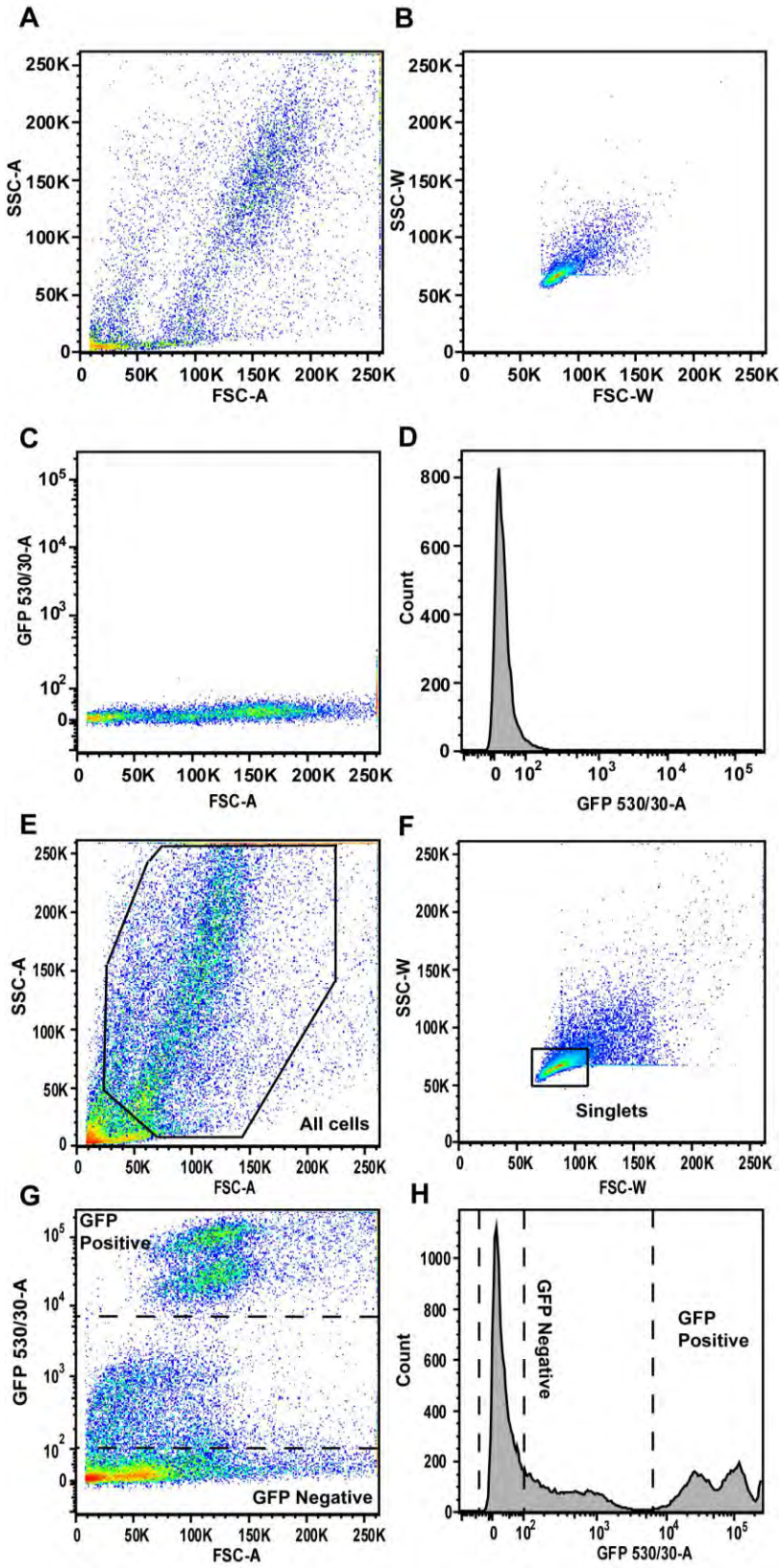

Figure 2.5: Separation of GFP+ and GFP- populations from Mip-GFP mice.

Figure 2.5: Separation of GFP+ and GFP- populations from Mip-GFP mice.

(A) SSC (side scatter) is proportional to cell complexity and depends on the number of organelles inside the cell. FSC (forward scatter) correlates with cell size. GFP 530/30-A measures strength of an individual cell‟s GFP intensity. (A-D) Freshly isolated islets from CD1+ female mouse (no GFP expression) were used to calibrate the cell sorter before GFP sorting began. (E) Plotting both SSC and FSC shows all cells collected as shown by the highlighted gate. (F) Only singlet or non-aggregated cells were used for efficient cell sorting. (G-H) Two distinct populations of cells were collected separately for further analysis.

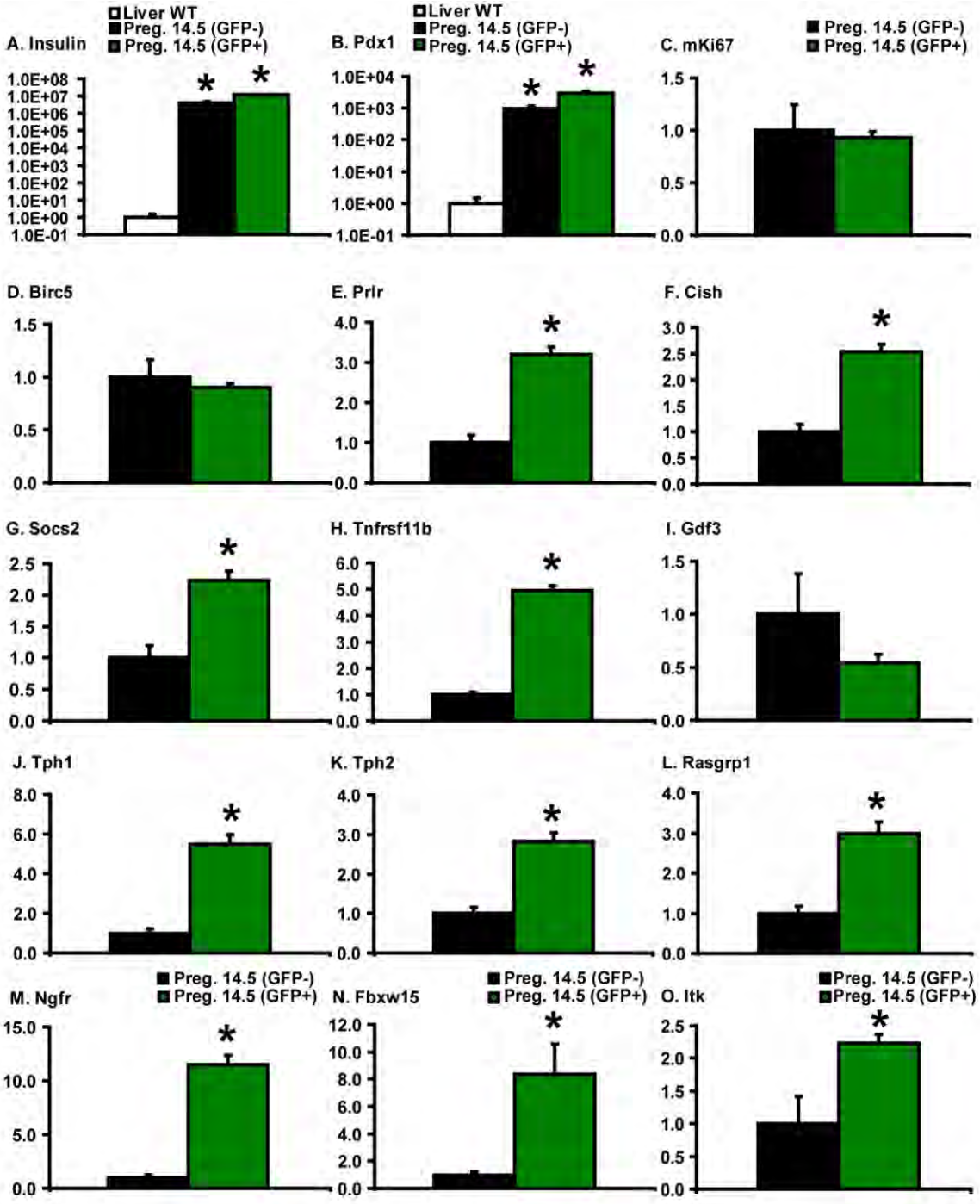

Figure 2.6: Differentially expressed genes identified in the islet are expressed in β-cells during pregnancy day 14.5.

Figure 2.6: Differentially expressed genes identified in the islet are expressed in β-cells during pregnancy day 14.5. Gene expression changes for insulin (A) and *Pdx1* (B) in wild-type liver, pregnant d 14.5 GFP− and pregnant day 14.5 GFP+ single-cell fractions. (C–O) Gene expression levels of selected genes in pregnant day 14.5 GFP− compared with pregnant day 14.5 GFP+ single-cell fractions (n = 3 per fraction; *, $P < 0.05$).

Although both proliferative and survival signals are required for islet expansion, the mechanisms to attain increased islet mass differ between pregnancy, obesity, and β-cell injury models

To identify specific pathways and mechanisms that potentially contribute to β-cell proliferation observed during day 14.5 of pregnancy, we analyzed our expression data with the Database for Annotation, Visualization and Integrated Discovery (DAVID), which is specifically designed to systematically extract biological meaning from large gene lists (13). Among the biological functions demonstrating significant enrichment between the genes differentially expressed in islets during pregnancy, 228 genes are involved in processes relating to cellular proliferation, and 60 with apoptosis. Interestingly, other functions enriched during pregnancy day 14.5 include antioxidation and free radical removal, vesicle mediated transport, ubiquitin cycle, proteolysis, and chromatin packaging and remodeling (Table 2.7). Gene Set Enrichment Analysis (GSEA) identified gene sets up-regulated during pregnancy involved in both tryptophan metabolism and ERK pathway (data not shown) (14).

Functional characterization of genes differentially expressed during pregnancy day 14.5 suggests that the islet‟s ability to compensate during metabolic stress requires the simultaneous induction of both proliferative and survival pathways. During pregnancy the increase in proliferation specifically in the β-cell is accompanied by a 5-fold increase in *Birc5* gene expression in the islet (Table 2.3). Conditional deletion of Birc5, an inhibitor of apoptosis, specifically in the endocrine pancreas showed a severe inability to maintain normal postnatal β-cell mass throughout adulthood *in vivo* (15). We

hypothesized that simultaneous induction of *Birc5* expression in the islet with β-cell proliferation during other models of β-cell expansion is essential for the ability of the islet to expand its mass in response to diverse metabolic stressors. To address this issue, we assessed *mKi67* and *Birc5* gene expression in two additional β-cell expansion models: the diabetes-resistant B6 *leptin*$^{ob/ob}$ and diabetes-susceptible BTBR *leptin*$^{ob/ob}$ mice (16), and the PANIC-ATTAC transgenic mouse model of inducible and reversible β-cell ablation (17). Leptin-deficient B6 mice that remain non-diabetic, irrespective of obesity, were able to induce the expression of both *mKi67* and *Birc5* throughout obesity both at 4 and 10 weeks of age (Figure 2.8A). In contrast, while initially able to significantly induce *Birc5* expression at 4 weeks, the 10 week old BTBR *leptin*$^{ob/ob}$ mice were unable to induce the expression of either gene, in part accounting for the onset of severe diabetes that this age (16). The expression of *Birc5* was significantly greater in the 10 week B6 *leptin*$^{ob/ob}$ compared to the 10 week BTBR *leptin*$^{ob/ob}$ mouse (Figure 2.8A).

β-cell mass is reversibly ablated by activation of caspase-8 mediated apoptosis in the PANIC-ATTAC model, and recovers in 30 days to pre-treatment levels even in the face of severe hyperglycemia (17). Islet proliferation was assessed by *mKi67* gene expression, showing a significant increase on day 8, with expression returning back to normal levels by day 30. *Birc5* expression closely mirrored the proliferative profile during the recovery of β-cell mass (Figure 2.8C), suggesting the ability to successfully expand islet mass during pregnancy, obesity, or in the setting of injury-induced diabetes requires both the induction of both islet proliferation and survival pathways.

Given the divergent physiologic contexts of pregnancy, obesity and experimental β-cell ablation, the molecular mechanisms responsible for both the compensatory

increase in islet mass response are probably distinct. To address this issue, using qPCR, we compared the expression of selected genes most differentially expressed during pregnancy day 14.5 to two other models of β-cell expansion: the 10 week old diabetes-resistant B6 *leptin*$^{ob/ob}$ mouse and the PANIC-ATTAC transgenic mouse. Whereas the expression of some genes such as *Tnfrsf11b* and *Hopx* significantly increased, the majority of gene expression did not change, declined, or was not detectable during obesity (Figure 2.8B). Genes whose temporal gene expression significantly changed during β-cell recovery after injury included *Itk*, *Tnfrsf11b*, *Il1rn*, *Rasgrp1*, and *Ngfr* with the majority of gene changes occurring on day 8, the peak of proliferation (Figure 2.8D-E). Again, the majority of gene expression profiles did not change or declined in the PANIC-ATTAC model (Figure 2.8E). Interestingly, *Birc5*, and *Tnfrsf11b* induction is connected to all three *in vivo* models of β-cell mass expansion analyzed. However, the dramatic differences in gene induction occurring between pregnancy, obesity-induced β-cell compensation, and recovery from β-cell ablation suggest that diverse mechanisms can be utilized by the β-cell to expand its mass.

		Number of genes	Benjamini p-value
Cellular Proliferation	Cell cycle control	25	3.80E-02
	Meiosis	42	2.30E-02
	Mitosis	66	4.60E-03
	Cytokinesis	47	2.70E-02
	Chromosome segregation	48	5.50E-03
Death	Apoptosis	60	3.40E-02
Response to Stress	Antioxidation and free radical removal	24	1.90E-02
Cell Communication	Receptor protein tyrosine kinase signaling pathway	48	3.60E-03
	Cell surface receptor mediated signal transduction	172	7.60E-04
	Ligand mediated signaling	45	1.30E-02
	G-protein mediated signaling	274	2.75E-05
	Cell adhesion-mediated signaling	43	1.20E-03
Cellular physiological process	Ubiquitin cycle	57	3.90E-02
	Proteolysis	391	1.60E-06
	Vesicle mediated transport	53	3.60E-02
	Secretion by cell	38	3.50E-02
	Chromatin packaging and remodeling	74	8.20E-03
Cellular metabolic process	Amino acid metabolic process	44	5.90E-05
	Lipid, fatty acid and steroid metabolism	82	5.90E-04
	Amine metabolic process	54	7.00E-04
	Electron transport	68	2.50E-02

Table 2.7: Gene Ontology (GO) functions significantly enriched during pregnancy day 14.5.

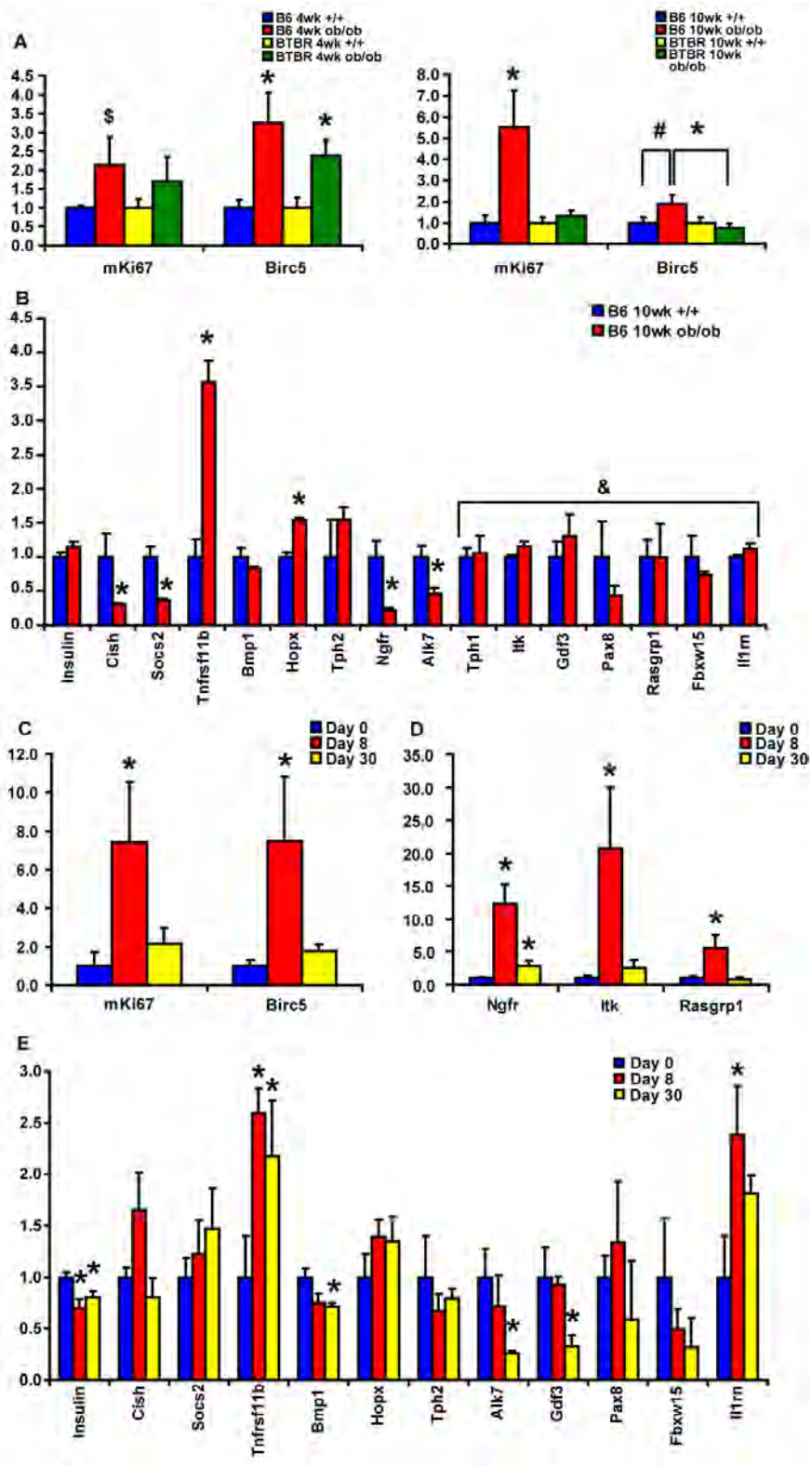

Figure 2.8: Gene expression profiles differ between the pregnancy, obesity, and β-cell injury models of β-cell regeneration.

Figure 2.8: Gene expression profiles differ between the pregnancy, obesity, and β-cell injury models of β-cell regeneration. (A) Strain-dependent comparison of mRNA expression of both *mKi67* and *Birc5* between both 4- and 10-wk-old $B6^{ob/ob}$ and $BTBR^{ob/ob}$ leptin-deficient mice and lean controls (n = 5–7 per strain; *, $P < 0.05$ *vs.* respective lean control; #, $P = 0.055$; $, $P = 0.08$). (B) Expression of selected differentially expressed genes identified in the pregnancy paradigm during obesity at 10 wk in the B6 strain (n = 5–7 per strain; *, $P < 0.05$ *vs.* lean control; &, not detectable). (C) Temporal gene expression of *mKi67* and *Birc5* before (d 0), after (d 30), and during (d 8) recovery from chemically induced β-cell ablation. (D and E) Temporal gene expression of selected pregnancy-induced genes during recovery from chemically induced β-cell ablation in the PANIC-ATTAC model (n = 4–5 per time point; *, $P < 0.05$ *vs.* day 0).

Discussion

The ability for the β-cell to compensate for an increased insulin demand includes not only an increase in insulin secretion, but also an expansion of β-cell mass by modulating the balance between both β-cell proliferation and apoptosis. Here we investigated the global gene expression profile of the expanding islet during pregnancy at the peak of β-cell proliferation. We identified nearly 2,000 genes that are differentially expressed at this time when compared to non-pregnant controls. Several of the genes observed to be differentially expressed during pregnancy are known to function in the maintenance of β-cell mass, including not only *c-Myc*, *Cdk4*, but both *Prl2c5*, and *Prlr*, consistent with the known role of placental lactogens during pregnancy-induced β-cell mass expansion (18-21). Strikingly, many of the genes identified in our analysis have not been previously reported to play a role in islet expansion.

One of the most significantly regulated genes was the F-box and WD-40 domain protein 15 (*Fbxw15*). F-box proteins have been identified as substrate recognition components of the multi-subunit ubiquitin ligase SCF (SKP1-CUL1-F-box protein) and contribute to the regulation of cell cycle progression and cell fate via the ubiquitylation and consequent degradation of many cell cycle inhibitors such as p27 (22) and Cyclin D1 (23). Differential expression of other F-box family members (*C85627*, *Fbxw14*, *Fbxl17*, *Fbxl27*, and *Fbxo27*) also occurs during pregnancy, signifying that tight regulation of specific cell cycle proteins by way of proteosomal protein degradation is a crucial process in the islet‟s adaptive ability response to metabolic stress.

Surprisingly, expression of key serotonin synthesizing enzymes, tryptophan hydroxylase 1 and 2 (*Tph1/2*), were strongly induced during pregnancy. In *Drosophila*, serotonergic neurons in the brain were shown to control body size by regulating the Insulin/IGF pathway in peripheral tissues (24). In mice gene ablation studies deletion of only the 5-HT_{2C} receptor produces insulin resistance and type 2 diabetes (25), resulting to the extensive development of 5-HT2C receptor agonists which improve glucose homeostasis in leptin-deficient obese mice (26). Serotonin itself has been shown to enhance insulin synthesis and secretion on primary rat islets *in vitro* (27). However, though the relatively elevated presence of serotonin in pancreatic islets has been known for years (28), a specific growth related role in β-cells remains to be established.

We demonstrate by utilizing three different models of β-cell expansion that both the islet‟s and β-cell‟s ability to respond to different metabolic stresses is associated with both an increase in proliferation and survival mechanisms, supported by increases *mKi67* and *Birc5* gene expression. β-cell specific overexpression of c-Myc in mice causes increased β-cell proliferation, but also apoptosis leading to the development of diabetes (29). Conversely, deletion of Caspase 3 protects the β-cell from c-Myc induced apoptosis and diabetes, supporting the notion that the β-cell‟s ability to compensate during metabolic stress requires the simultaneous induction of both proliferative and survival pathways (30). For example, dysregulation of this homeostatic control of β-cell mass, based on the relative contribution of cell proliferation and cell death, is not only a major contributor to gestational diabetes, but also the onset of type 2 diabetes, because 70% of women who are diabetic during pregnancy develop type 2 diabetes later in life (31). Our

pregnancy gene list shows potential signaling factors involved in coordinating both of these processes not only in the islet, but potentially in the β-cell itself.

Both Cish and Socs2, members of the suppressor of cytokine signaling (SOCS) family of proteins, have been shown to act as negative regulators of a variety of tyrosine kinases and receptors impacting β-cell proliferation *in vitro*, including Prlr (32) and Igf1r (33), via a negative feedback loop and proteosomal degradation mechanisms, inhibiting their downstream Janus family of tyrosine kinase 2/signal transducer and activator of transcription signaling cascades. Cish and Socs2 are known to decrease activation of Stat5 (32, 34), whose gene products induce proliferative (Cyclin D2), anti-apoptotic (Bcl-xL, Bcl6) (35-37) and functional mechanisms (Gck, Glut2, Insulin) (38, 39). This suggests that the high levels of *Cish* and *Socs2* expression observed during pregnancy play a critical role in limiting the rate of cell proliferation and promoting survival of β-cells. Conversely, during obesity the gene expression levels of *Cish* and *Socs2* significantly decrease in the B6 *leptin*$^{ob/ob}$ mouse at 10 weeks, suggesting an enhancement of these properties during a more chronic metabolic stress (Figure 2.8A). Additionally, Socs7-deficient mice display enlarged islets and increased insulin function, consistent with the notion that this family has a role in regulating β-cell mass in the adult mouse (40).

Additionally, our comparison of the three paradigms of β-cell expansion suggests that diverse mechanisms can be utilized by the islet to expand its mass. The majority of genes investigated are not similarly induced between models, strongly suggesting that not only does the β-cell have the ability to modulate its mass to cope with metabolic demand, but can also activate diverse signaling mechanisms depending on the physiological

condition. In support of this notion, several surprising mechanisms controlling β-cell mass homeostasis through bone-derived signals (41), and transmission of pro-proliferative signals from the liver via the peripheral and central nervous system to the β-cell (42) have been described recently. Conversely, *Birc5* and *Tnfrsf11b* are commonly induced in all three models of β-cell expansion, suggesting they act as universal mediators of β-cell expansion. Recently, Birc5 has been shown to exhibit a β-cell specific and bifunctional role important in both attenuation of β-cell proliferation and inhibition of apoptosis *in vivo* (15). We show that *Birc5* closely mirrors the proliferative profile (*mKi67*) throughout the β-cell injury time course (Figure 2.8C), providing additional evidence to suggest that *Birc5* is a critical component of the mechanism for maintaining β-cell homeostasis, not only physiologically, but also in the setting of diabetes.

Tnfrsf11b (Osteoprotegerin) is a secreted and soluble member of the tumor necrosis factor (TNF) receptor superfamily (43). Tnfrsf11b has anti-apoptotic properties in β-cells (44), and overexpression of this osteoblast-derived factor in islets suggests regulation of β-cell expansion by peripheral tissues, a mechanism supported by transplantation studies in mice (45). In support of this notion, osteocalcin, a peptide secreted by osteoblasts in the bone, affects energy metabolism, by increasing β-cell proliferation, insulin secretion, and insulin sensitivity (41). We hypothesize that Tnfrsf11b is an additional bone-derived hormone that exhibits a pro-proliferative effect on β-cells during pregnancy. In addition, Keller et al. (16) identified genes in insulin target tissues, specifically muscle, with expression profiles highly correlated to the islet cell cycle regulatory module activated during obesity, including several genes induced in islets during pregnancy, *Ngf*, *Bmp1*, and *Gdf10* (*Gdf3* during pregnancy), suggesting

these as further candidates for potentially mediating β-cell proliferation. The upregulation of TGF-β family members, *Bmp1*, *Alk7* and *Gdf3* is consistent with their already known role in establishment of β-cell mass (46, 47).

In conclusion, we identified a large number of genes correlating with both the initiation and peak of β-cell proliferation during pregnancy in the mouse. Additionally, we confirmed that many of the genes are expressed in the β-cell, and provide a platform for diabetes researchers to further explore their role in the maintenance of β-cell mass.

Note: The data presented in this chapter has been published in Molecular Endocrinology as an Original Research Article entitled,

The Transcriptional Response of the Islet to Pregnancy in Mice.

Rieck S, White P, Schug J, Fox AJ, Smirnova O, Gao N, Gupta RK, Wang ZV, Scherer PE, Keller MP, Attie AD, Kaestner KH. Mol Endocrinol. 2009 Oct;23(10):1702-12.

Materials and Methods

β-cell mass, β-cell proliferation, β-cell hypertrophy. For histological assessment, pancreata were dissected, fixed in 4% PFA for 24 hr, and then laid flat for paraffin sectioning. Sections with the greatest surface area were used in all experiments. β-cell mass was determined on 4 month-old non-pregnant and pregnant (at day 14.5 of gestation) female mice by point-counting morphometry as previously described (9). Sections from each animal were stained with guinea pig anti-insulin antibody (1:1000; Linco Research, Inc, St. Charles, MO) diluted in phosphate-buffered saline plus 0.1% Tween and incubated overnight at 4°C. The secondary antiserum was a biotinylated anti-guinea pig antibody (1:200; Vector Laboratories). For color development, horseradish peroxidase-conjugated avidin-biotin complex reagent was used with 3,3‟-diaminobenzidine as a substrate (Vector Laboratories, Burlingame, CA). Images covering the entire section were used from which the percentage of β-cell area was measured and calculated using MetaMorph Imaging Software (Universal Imaging Corp., West Chester, PA). β-cell mass was derived from total pancreas weight multiplied by percentage of β-cell area (9). For β-cell proliferation measurements, mice were injected with 10μL of Zymed BrdU Solution (3mg/mL; Zymed Laboratories, Inc., South San Francisco, CA) per gram body weight 24 hr before they were euthanized. BrdU staining was assessed using a BrdU-specific sheep monoclonal antibody (1:1000; United States Biochemical Corp., Cleveland, OH) as described (9). In addition, Ki67 antigen detection using a Ki67-specific rabbit monoclonal (1:2000; Vector Laboratories) antibody was employed to confirm β-cell proliferation assessment. For β-cell size determination, the same slides used for BrdU incorporation were used to determine the volume and number of nuclei for

each islet via ImageJ Software (Image Processing and Analysis in Java; National Institutes of Health). At least 20 islets per pancreas from at least four mice per experimental group were analyzed for determining β-cell size and proliferation.

RNA isolation and qPCR. Adult islets for all experiments were prepared using the standard collagenase procedure, as previously described (9). Total RNA was isolated using the RNeasy Mini Kit (QIAGEN, Chatsworth, CA) and eluted in water. 200ng of total RNA of each sample were amplified using the MessageAmp aRNA Kit (Ambion, Inc., Austin, TX) (9). The RNA 6000 Nano Lab Chip Kit with a 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA) was used to ensure samples of high RNA quality (48). cDNA was synthesized from approximately 500ng amplified islet RNA using 1µg of Oligo(dT) primer, SuperScript II Reverse Transcriptase, and accompanying reagents (Invitrogen). PCR reaction mixes were assembled using SYBR Green Quantitative PCR Master Mix (Stratagene, La Jolla, CA). Reactions were performed using the SYBR Green program on the Mx4000 Multiplex Quantitative PCR System (Stratagene). All reactions were performed in duplicate on at least four biological replicates with Rox reference dye normalization. *Hprt*, *Gapdh*, and *Ubc* were tested for suitability as housekeeping genes using the GeNorm analysis package. Median C_T values were used for analysis and normalized to the expression of two housekeeping genes; *Hprt*, and *Gapdh*. Primer sequences are available at http://www.med.upenn.edu/kaestnerlab/.

Microarray expression profiling and data analysis.

Non-pregnant controls vs. pregnant at day 14.5: To allow hybridization of samples that were well-matched for purity in terms of the amount of exocrine contamination, qPCR was used to determine the relative levels of endocrine (Prohormone convertase-2) and

exocrine (Amylase and chymotrypsinogen B1) gene expression in each sample. As a result, differential gene expression originating from varying islet purity was minimized. Total RNA (50 ng) from each sample was used for labeling and hybridization. Amplified cDNA was prepared using the WT-Ovation Pico Amplification System (NuGEN Technologies, San Carlos, CA). Amplified cDNA (2µg) was directly labeled using the BioPrime Array CGH Genomic Labeling System (Invitrogen) with Cy3- and Cy5-labeled nucleotides (GE Amersham Biosciences, Piscataway, NJ). Of the four biological replicates, two were labeled with Cy3 (Test) and the other with Cy5 (Control) and the other two with Cy5 (Test) and Cy3 (Control), eliminating variations introduced by dye bias. Labeled samples were hybridized overnight to the Agilent 4X44 Whole Mouse Genome Array. Arrays were washed and then scanned with the Agilent DNA microarray Scanner, Model G2565B (Agilent Technologies). Median intensities of each element on the array were captured with Agilent Feature Extraction version 9.53 (Agilent Technologies). Quality control diagnostic plots were prepared for each array, and those failing to exhibit high-quality hybridizations were excluded from further analysis, resulting in the final dataset containing four biological replicates for each condition.

The data were normalized by the print tip loess method using the LIMMA (Linear models for microarray data) package in R as described (49). For statistical analysis, genes were called differentially expressed using the Significance Analysis of Microarrays (SAM) one class response package with a false discovery rate (FDR) of 20% (50). Genes marked as absent, i.e. with expression levels near background, were omitted. Differentially expressed genes were confirmed using real-time qRT-PCR as described

above. All expression data were deposited into Arrayexpress (Accession no. E-MTAB-120).

Mip-GFP sorting. Freshly isolated islets from Mip-GFP mice (12) were placed in 1.0 mL of prewarmed Trypsin at 37C in an eppendorf tube for 12 minutes mixing every three minutes until no clumps were visible. Dissociation into a single-cell suspension was stopped with the addition of 1mL of heat inactivated FBS. The cells were then transferred to a 15mL Falcon tube through a 40-μm nylon mesh cell strainer cap (BD Biosciences, Palo Alto, CA), washed 3 times in PBS containing 2% FBS, and placed immediately on ice ready for sorting. GFP+ and GFP- cells were sorted by the University of Pennsylvania Flow Cytometry and Cell Sorting Resource Laboratory using the FACSVantage SE (BD Biosciences). Cells were gated for non-aggregates to achieve a high-purity sort. Both GFP+ and GFP- cells were separated and collected directly into a 1.5 mL eppendorf tubes containing 0.5 mL of Trizol and the samples were snap frozen in liquid nitrogen and stored at -80C. Depending on yield some samples were pooled to obtain 20,000 cells per sample.

Obesity, and reversible β-cell injury model. Islet RNA isolated from both 10 week old $B6^{ob/ob}$ and $BTBR^{ob/ob}$ leptin-deficient mice was used as an animal model representing obesity. The PANIC-ATTAC mouse was used as an animal model of inducible and reversible β-cell ablation (17). Islet RNA was isolated on day 0, 8, and 30 after administration of dimerizer AP20187 and cDNA synthesized using the Ovation RNA Amplification System V2 (P/N 3100; NuGEN Technologies). At least four biological replicates per time point were used for real-time qPCR analysis.

Statistical analysis. Statistical analysis between two groups was done were analyzed using one-tailed Student *t* test. Values are considered significant when $P < 0.05$.

Addendum

Following publication of the data presented in Chapter 2, several studies were published confirming a role in β-cell proliferation of a number of genes I identified as highly differentially expressed in the islet during pregnancy. Of transcriptional mediators, FoxM1 has been shown to directly activate the transcription of Birc5, the anti-apoptotic gene during pregnancy discussed previously, Cyclin B1, and prevent the nuclear expression of $p27^{Kip1}$ (51). Both *Birc5* and *Cyclin B1* is shown by our gene expression analysis to increase in expression (Figure 2.9), and protein levels of $p27^{Kip1}$ have been proven to decrease in the islet during pregnancy (52). Other known FoxM1 targets such as Skp2 have already been implicated in β-cell compensation in response to diet-induced obesity (22), and indeed, the proliferative response of the β-cell to pregnancy in mice is dependent on FoxM1 itself (52).

Two genes with the most dramatic fold change in steady state mRNA levels in islets during pregnancy were tryptophan hydroxylase 1 and 2 (*Tph1* and *Tph2*) (Figure 2.9). Although it has been known for a long time that serotonin (5-hydroxytryptamine; 5-HT) is synthesized within β-cells and stored together with insulin in secretory granules (53, 54), the physiological role of this synthesis and storage was unknown until very recently. Just this year, mice deficient for Tph1 exhibit impaired insulin secretion. They further showed that 5-HT is coupled via the action of transglutaminases to two small GTPases, rendering the latter constitutively active and promoting insulin secretion (55). It is tempting to speculate that dramatic increase of *Tph1/2* expression in the islet during pregnancy increases the insulin secretory response of the β-cell.

Furthermore, a recent study demonstrated that Tph1 expression is downstream of lactogenic signaling, and found that local serotonin signaling drives β-cell proliferation leading to β-cell mass expansion in pregnant mice (Figure 2.9). Specifically, expression of the $G\alpha_q$- linked serotonin receptor 5-hydroxytryptamine receptor-2b (Htr2b) is closely correlated with the increase of β-cell proliferation during rodent pregnancy, blocking of which leads to decreased β-cell expansion and glucose intolerance. Interestingly, Tph1 protein expression in islets is also increased in response to pregnancy in humans (56). Thus, serotonin signaling can be major determinant in regulating both β-cell expansion and heightened insulin secretion occurring during physiological insulin resistant states.

Of note, investigation into the role of the suppressor of cytokine signaling protein Cish in β-cell proliferation by genetic ablation specifically in β-cells is a future aim of this thesis (Chapter 4).

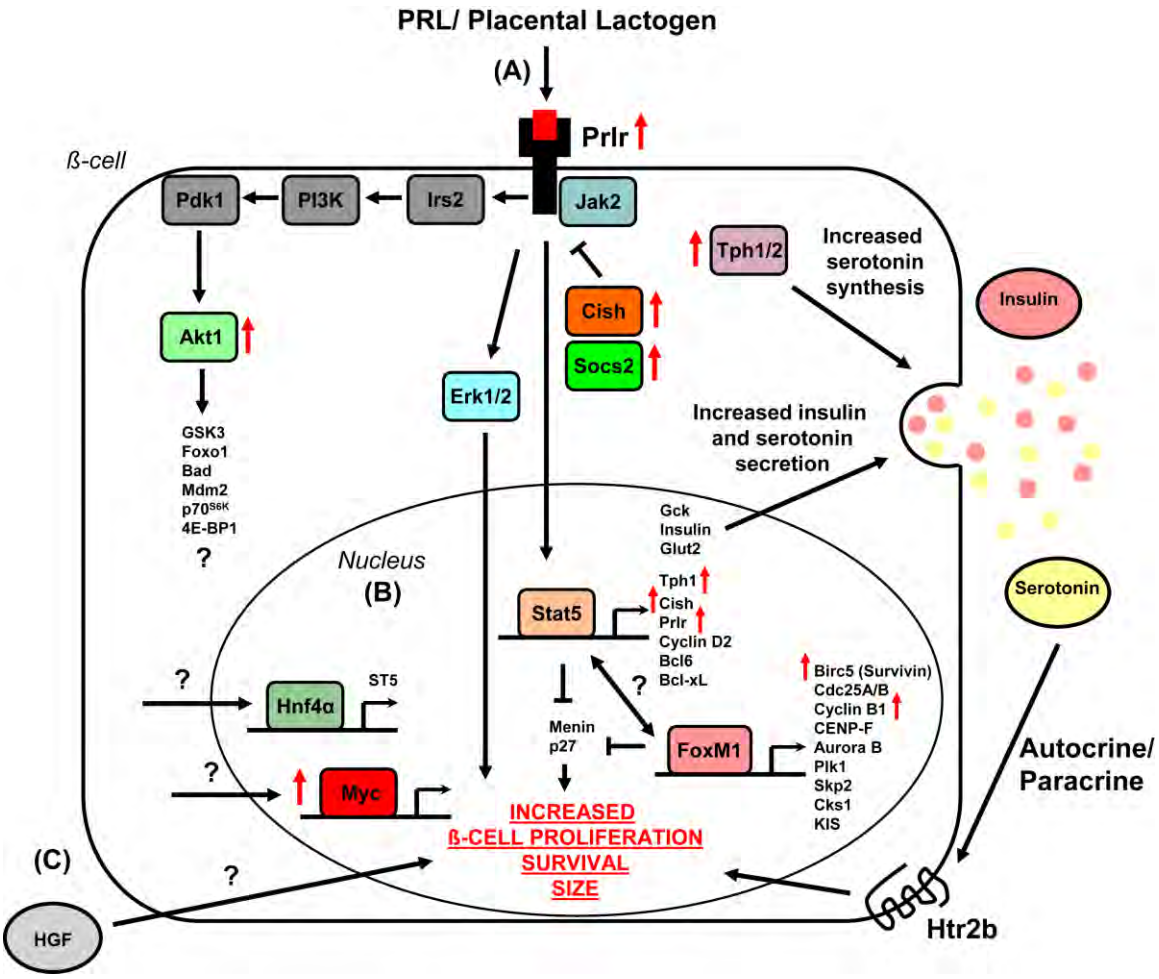

Figure 2.9: Known mechanisms responsible for β-cell gain during pregnancy.

Figure 2.9: Known mechanisms responsible for β-cell gain during pregnancy. (A) Activation of PRL receptors upon binding of lactogens (Prolactin or placental lactogen) plays a pivotal role in the adaptation of the β-cell to pregnancy. Downstream signaling pathways of the prolactin receptor include Stat5, phosphatidylinositol 3-kinase (PI3K) and Mapk pathways, targets of which have been implicated to lead to increased β-cell proliferation, survival and size. For example, transgenic overexpression of Akt1, upregulated in the islet during pregnancy, leads to dramatic increase in β-cell size (57). (B) Known transcription factors (listed with their target genes) that regulate the increase in β-cell mass during pregnancy. A red arrow indicates an increase in expression of genes in the islet during pregnancy day 14.5 as assessed by our gene expression analysis. (C) Possible prolactin receptor independent mechanisms leading to β-cell gain mechanisms. For example, increased Hgf levels in the islet endothelium correlates with increased β-cell proliferation in pregnant rats (58). Much is still to be discovered, as evidenced by the recent finding that upregulation of the developmental transcription factor MafB in β-cells during pregnancy (59).

Note: Portions of this addendum have been published in TRENDS in Endocrinology and Metabolism as a Review article entitled,

Expansion of β-cell mass in response to pregnancy.

Rieck S, Kaestner KH. *Trends Endocrinol Metab*. 2010 Mar; 21(3):151-8.

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Chapter III:

Overexpression of Hepatocyte Nuclear Factor- 4α initiates cell cycle entry, but is not sufficient to promote β-cell expansion in human islets

Abstract

One approach toward generating new β-cells for the treatment of type 2 diabetes or for cell replacement therapy for type 1 diabetes is to use instructive cues to induce proliferation of terminally differentiated β-cells. The transcription factor HNF4α (Hepatocyte nuclear factor- 4α) is required for the increase in β-cell proliferation during metabolic stress. We hypothesized that HNF4α might be able to induce proliferation of human β-cells. To address this question, we employed adenoviral-mediated overexpression of a pancreas-specific isoform of HNF4α (HNF4α8) alone, or in combination with CDK6 and CYCLIN D3, in primary human islets. HNF4α8 alone stimulated β-cells to enter the cell cycle, and led to a greater than 300-fold increase in the number of β-cells that entered S-phase, without detectable loss of glucose stimulated insulin secretion. However, $HNF4\alpha^{High}$ $BrdU^{+}$ β-cells showed signs of cell cycle arrest, such as the expression of cell cycle checkpoint markers, and lacked expression of both CYCLIN A and KI67. We observed activation of the DNA damage response associated with the dysregulated DNA synthesis, ultimately resulting in a senescence-like phenotype independent of caspase-dependent apoptosis. When we overexpressed HNF4α8 together with CDK6 and CYCLIN D3, we further increased β-cell cycle entry. However, we observed a substantial proportion of β-cells stimulated to enter the cell cycle by CDK6 and CYCLIN D3 to also exhibit a DNA damage response. In sum, HNF4α8 alone is a mitogenic signal in the human β-cell but is not sufficient for completion of the cell cycle. In addition we find that the DNA damage response is a barrier to efficient β-cell

proliferation *in vitro*, and suggest its evaluation in all attempts to stimulate β-cell replication.

Introduction

Since the first successful transplantation of cadaveric islets into brittle type 1 diabetic patients using a glucocorticoid-free immunosuppressive regiment resulted in temporary insulin independence (1), there has been much interest into the basic mechanisms and factors required to obtain β-cells *in vitro*, because the supply of islets from organ donors is far exceeded by the demand for transplants. Approaches toward this therapeutic goal include human embryonic stem cell (hESC) directed differentiation (2), transdifferentiation of related cell types such as hepatocytes or acinar cells to β-cells by the forced expression of transcription factors (3-6), and the replication of pre-existing β-cells (7). However, while progress has been made towards the differentiation of human ES cells to pancreatic endoderm (2), a well-defined protocol to drive efficient non-oncogenic proliferation of human β-cells *in vitro* remains elusive.

Because the vast majority of human β-cells reside in G_0-phase, especially after the first 30 years of life (8), the promotion of β-cell proliferation needs to accomplish (a) entry into the cell cycle and licensing of DNA at replication origins (G_1- phase), (b) initiation of DNA replication at licensed origins resulting in duplication of the genome (S-phase), and (c) progression through the remaining cell cycle phases (G_2- and M-phase). Thus far, only overexpression of the winged helix transcription factor FOXM1 (9), or combinations of a G_1/S-phase specific CDK with its D-type cyclin partner have shown evidence for induction of multiple phases of the cell cycle in adult human β-cells, with CDK6-overexpression translating to continued β-cell replication in the face of hyperglycemia *in vivo* (10). In addition, even if a factor is sufficient to induce progression

through one complete cell cycle, as shown by overexpression of E2F1- alone (11), protection of the newly formed β-cells from their vulnerability to apoptosis must also be achieved (12).

One potential sufficiency factor is hepatocyte nuclear factor (HNF) 4α (MODY1), as it was shown previously by its conditional gene ablation to be required for the physiological increase of β-cell replication during pregnancy (13). Both its membership in the nuclear hormone receptor family and the presence of isoforms uniquely expressed in the islet suggest HNF4α as being a potential drug target (14, 15). In the current study, we overexpress a pancreas-specific isoform of HNF4α8 specifically in human β-cells and demonstrate that HNF4α-overexpression alone and in combination with other mitogenic factors is sufficient for entry into the cell cycle. We further uncover that analysis of the DNA damage response is critical for the evaluation of mitogenic signals in human β-cells.

Results

Adenoviral overexpression of HNF4α8 in human β-cells

To examine the effect of HNF4α on β-cell replication in primary human islets, we overexpressed HNF4α8, an islet-specific isoform (15, 16), using an adenovirus containing the rat insulin promoter (RIP). Bright nuclear immunofluorescence staining for HNF4α8 (designated as HNF4α^{High}) was observed predominantly in β-cells (Figure 3.1A-D). Approximately 35% of either PDX1^{+} or insulin^{+} cells overexpressed HNF4α 72 hours after transduction (Figure 3.1E). HNF4α^{High} expression was only rarely seen in α-cells (glucagon^{+}) or δ-cells (somatostatin^{+}) cells (Figure 3.1C-D). A replication-competent adenovirus, encoding *E1A*, can lead to misleading results when assessing β-cell proliferation (17). Importantly, no *E1A* mRNA was found in either AdCMV-eGFP or AdRIP-hHNF4α8 treated islets 72 hours after transduction (Figure 3.1F). These results demonstrate successful and sustained overexpression of HNF4α8 in human β-cells using AdRIP-hHNF4α8.

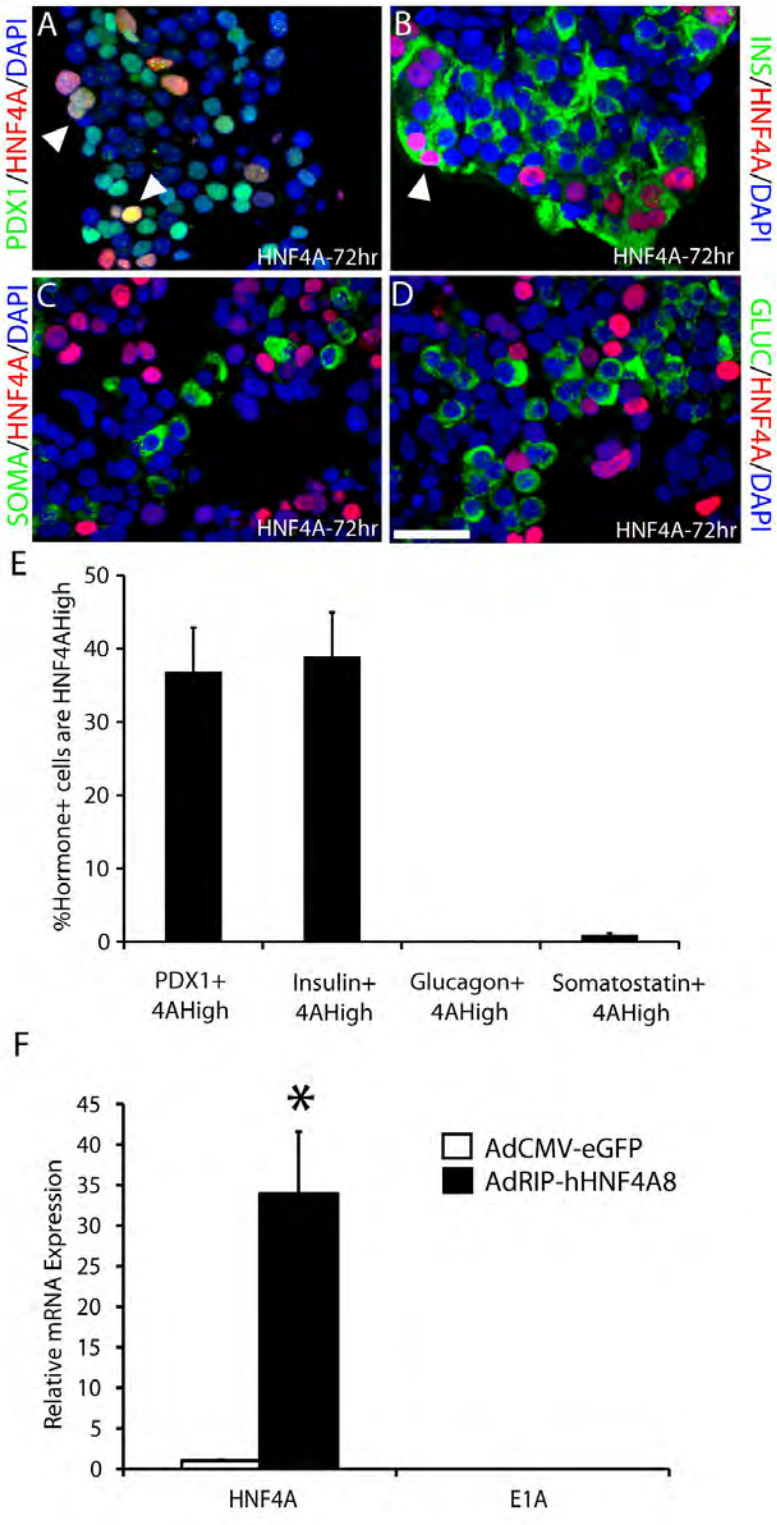

Figure 3.1: Adenoviral overexpression of HNF4α8 in human β-cells.

Figure 3.1: Adenoviral overexpression of HNF4α8 in human β-cells. Human islets were transduced with Ad-RIP-hHNF4α8 and incubated for 72 hours before embedding and processing for immunofluorescence staining. Dual immunofluorescence staining of (A-D) HNF4α (red) and DAPI (blue) with (A) PDX1 (green), (B) Insulin (green), (C) Somatostatin (green) and (D) Glucagon (green). White arrows show examples of $PDX1^{+}$ and $Insulin^{+}$ $HNF4\alpha^{High}$ cells and the scale bar in (D) indicates 25 μm. (E) Quantification of the percentage of hormone-expressing cells that are $HNF4\alpha^{High}$ (n=3). (F) Gene expression of *HNF4α* and *E1A* in AdCMV-eGFP and AdRIP-hHNF4α8 treated islets (n=5-6; *, $P<0.001$ versus CMV-eGFP condition).

HNF4α^{High} β-cells incorporate BrdU in a punctate, not diffuse, manner

To answer if HNF4α8 overexpression leads to increased β-cell proliferation and to detect all *de novo* proliferation events, we added BrdU continuously for 72 hours after adenoviral transduction. Of note, human islets were exposed to BrdU label continuously for the entire post-transduction period unless otherwise stated. No appreciable BrdU incorporation occurred into the PDX1^{+} population in both untransduced and AdCMV-eGFP transduced islets (Figure 3.2A-B), consistent with the very low rate of β-cell replication measured upon receipt of donation (only 0.05%±0.04 of PDX1^{+} cells were KI67^{+}) (Supplemental Figure 3.3A-C). However, overexpression of HNF4α8 caused a dramatic increase in BrdU incorporation, with 4.71%±1.36 of PDX1^{+} cells colocalizing with BrdU 72 hours after transduction (Figure 3.2C, M). The majority (81.5%±7.3) of BrdU incorporation colocalized with HNF4α^{High} staining (Figure 3.2D-F). Surprisingly, BrdU incorporation occurred in distinct punctate domains overlapping regions with reduced DAPI signal (Figure 3.2C, F). This effect occurred specifically in insulin^{+} cells, but not glucagon^{+}, or somatostatin^{+} cells (Figure 3.2J-L, O). In contrast, diffuse BrdU incorporation throughout the nucleus was present in non-endocrine cells (Figure 3.2E insert, G-I), as described previously (10). Of the entire HNF4α^{High} population 12.0%±2.99 incorporated BrdU, and this was similar in islets isolated from type 2 diabetic donors (9.29%±2.9) (Figure 3.2N). These data indicate that a significant fraction of β-cells overexpressing HNF4α8 enter S-phase. Interestingly, this fraction was similar

to that reported for other genes known to be sufficient for β-cell proliferation, such as CYCLIN D1, CYCLIN D2, and CDK6 (10, 18).

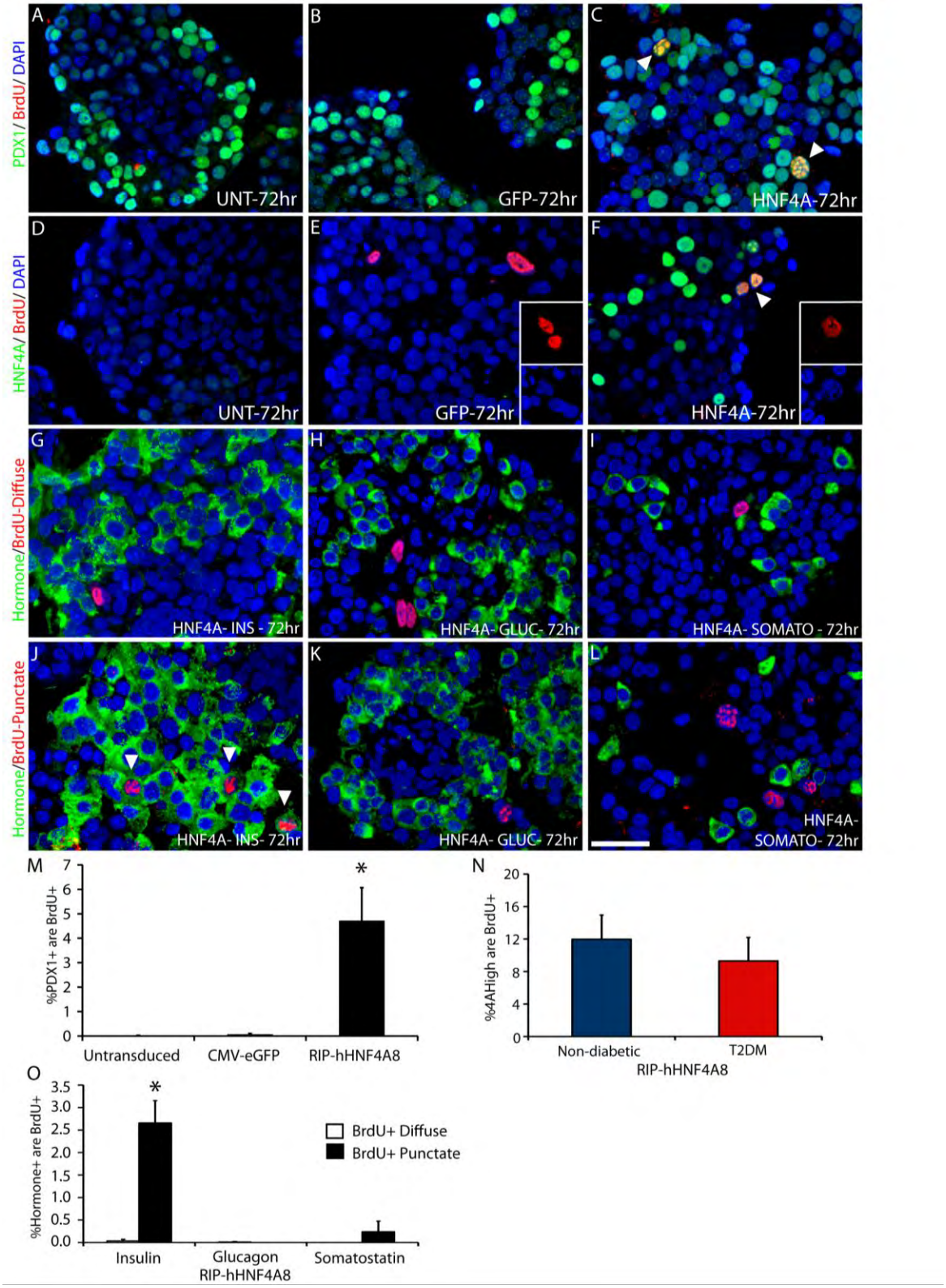

Figure 3.2: HNF4α^{High} β-cells incorporate BrdU in a punctate, not diffuse, manner.

Figure 3.2: HNF4α^{High} β-cells incorporate BrdU in a punctate, not diffuse, manner. Immunofluorescence staining of PDX1 (green), BrdU (red), and DAPI (blue) in primary human islets (A) not transduced with adenovirus, (B) transduced with AdCMV-eGFP, and (C) transduced with AdRIP-hHNF4α8. Similarly, detection of HNF4α (green), BrdU (red), and DAPI (blue) in primary human islets (D) not transduced with adenovirus, (E) transduced with AdCMV-eGFP, and (F) transduced with AdRIP-hHNF4α8. The inserts in (E) show diffuse BrdU incorporation overlapping with DAPI, and in (F) punctate BrdU incorporation overlapping with DAPI-diminished regions in the nucleus. Dual immunofluorescence staining of (G-L) BrdU incorporation (red) and (G-L) DAPI (blue) with (G, J) Insulin (green), (H, K) Glucagon (green), and (I,L) Somatostatin (green) cells in primary human islets overexpressing HNF4α8. Quantification of the (M) percentage of PDX1^{+} cells that are BrdU^{+} in all three conditions at 72 hours after transduction (n=3-4; *, P<0.02; versus both untransduced and CMV-eGFP conditions). Quantification of the (N) percentage of HNF4α^{High} cells that are BrdU^{+} at 72 hours after transduction both in non-diabetic and type 2 diabetic donors (n=3-4). Quantification of the (O) percentage of Insulin^{+}, Glucagon^{+}, and Somatostatin^{+} cells that are BrdU$^{+,Diffuse}$ or BrdU$^{+,Punctate}$ (n=3-4; *, P<0.01 versus all conditions). All primary human islets were harvested 72 hours after transduction. The scale bar in (L) indicates 25 μm.

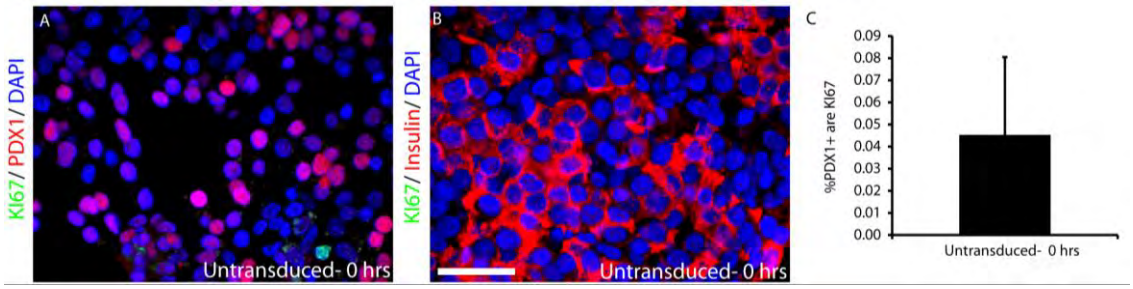

Figure 3.3: The rate of proliferation of PDX1^{+} cells upon receipt of human islet donations is extremely low.

Figure 3.3: The rate of proliferation of PDX1$^+$ cells upon receipt of human islet donations is extremely low. Immunolocalization of KI67 (green), DAPI (blue), (A) PDX1 (red) and (B) Insulin (red) of untransduced primary human islets before transduction. (C) Quantification of the percentage of PDX1$^+$ that are KI67$^+$ (n=3). The scale bar in (B) indicates 25 μm.

HNF4α^{High} β-cells do not progress through the cell cycle

To investigate if HNF4α8 overexpression is sufficient for cell cycle progression in β-cells, we next turned to additional cell cycle markers such as, KI67, a marker of all phases of the cell cycle. Surprisingly 72 hours post-transduction, HNF4α^{High} PDX1^{+} cells were not KI67^{+} (0.06% ± 0.03 of PDX1^{+} cells were KI67^{+}) similar to untransduced or AdCMV-eGFP treated controls (Figure 3.7A-C).

Next, we considered the possibility that HNF4α8-induced proliferation was completed before the 72 hour time point analyzed. We collected HNF4α8 overexpressing islets exposed to continuous BrdU at various time-points after transduction (24, 30, 36, 42, 48 hours). The BrdU incorporation rate into HNF4Aα^{High} β-cells was negligible until 36 hours post-transduction, and increased until 48 hours, with no significant difference compared to 72 hours, suggesting that entry into S-phase occurred between 36 and before 48 hours (Figure 3.4A-D). The majority of cells that incorporated BrdU diffusely expressed KI67 at 24, 30, 36 and 42 hours, but not at 48 and 72 hours, indicating exit from the cell cycle. Strikingly, cells that incorporate BrdU in a punctate manner never expressed KI67 (Figure 3.4E-H). The rare cells exhibiting both BrdU$^{+,punctate}$ and KI67 expression do not show large BrdU domains but small BrdU points, and their pattern of BrdU incorporation reflects incomplete S-phase progression at the time of islet harvest (19). Also, HNF4Aα^{High} β-cells do not express KI67 at any time-point (Figure 3.4I-L). Furthermore, HNF4α8 overexpression does not induce the expression of CYCLIN A, a marker of S-phase, G_2-phase and early M-phase (20), while BrdU$^{+,diffuse}$ cells are CYCLIN A positive (Figure 3.4N-P). Using a highly proliferative tissue as a positive control, we show CYCLIN A antibody specificity in that CYCLIN A^{+} cells comprise a

subset of $KI67^{+}$ cells (Figure 3.4M). Together, these data suggest that $BrdU^{+,punctate}$ cells do not progress through the cell cycle after having entered S-phase.

To confirm whether $HNF4A\alpha^{High}$ β-cells enter the cell cycle, and to investigate if they activate a cell cycle checkpoint, we utilized MCM7, and phospho-CHK2(Thr68) and phospho-P53(Ser15) staining, respectively. MCM7 functions as a DNA replication licensing factor whose expression is activated in early G_1-phase, when KI67 not yet expressed (21). Thus, in human tonsil MCM7 protein is not restricted to $KI67^{+}$ cells, and, conversely, $KI67^{+}$ cells do not always express MCM7 (Figure 3.4Q). Interestingly, an appreciable number (52.0% ± 6.3) of $BrdU^{+,punctate}$ singlets expressed MCM7 at 42 hours after transduction (Figure 3.4R-T), confirming that $HNF4A\alpha^{High}$ β-cells had indeed entered the cell cycle. Expression of both checkpoint markers phospho-CHK2(Thr68) and phospho-P53(Ser15) was seen predominantly in $BrdU^{+,punctate}$ cells (73.4% ± 2.2 and 78.7% ± 7.5, respectively), but not in $BrdU^{+,diffuse}$ cells 48 hours after transduction (Figure 3.4U-Y). These data suggest that while HNF4α8 is sufficient to stimulate β-cells to enter the cell cycle, it also activates the checkpoint response, indicating cell cycle arrest.

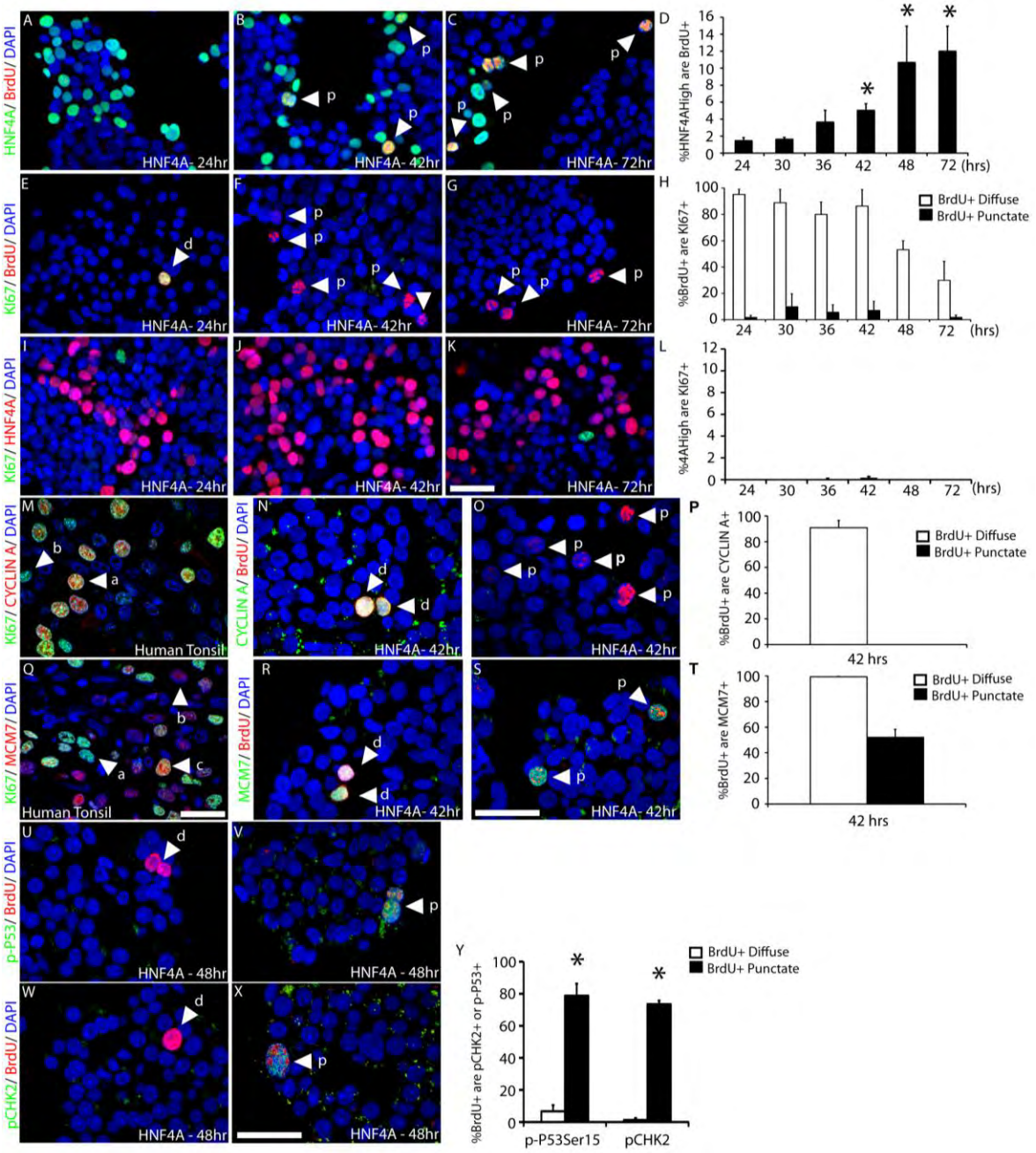

Figure 3.4: HNF4α^{High} β-cells arrest in the cell cycle.

Figure 3.4: HNF4α^{High} β-cells arrest in the cell cycle. Immunolocalization of HNF4α (green), BrdU (red) and DAPI (blue) in primary human islets (A) 24 hours, (B) 42 hours, and (C) 72 hours after transduction with HNF4α8 adenovirus. (D) Quantification of the percentage of HNF4α^{High} cells that are BrdU^{+} (n=3-5; *, P<0.03; versus 24hr). Analysis of KI67 (green), BrdU (red), and DAPI (blue) cells in primary human islets at (E) 24 hours, (F) 42 hours, and (G) 72 hours after transduction with HNF4α8. (H) Quantification of the percentage of BrdU$^{+,Diffuse}$ or BrdU$^{+,Punctate}$ that are KI67^{+} (n=3-5). Assessment of KI67 (green), HNF4α (red), and DAPI (blue) colocalization in primary human islets (I) 24 hours, (J) 42 hours, and (K) 72 hours after transduction with HNF4α8. (L) Quantification of percentage of HNF4A^{High} cells that are KI67^{+} (n=3-4). Immunostaining of (M) CYCLIN A (red), KI67 (green) in human tonsil as a positive control, and (N-O) CYCLIN A (green) with BrdU (red) in human islets 42 hours after transduction with HNF4α8. Quantification (P) of the percentage of BrdU$^{+,Diffuse}$ or BrdU$^{+,Punctate}$ that are CYCLIN A^{+} (n=3). Immunostaining of (Q) MCM7 (red), KI67 (green) in human tonsil as a positive control, and (R-S) MCM7 (green) with BrdU (red) in human islets 42 hours after transduction with HNF4α8. Quantification (T) of the percentage of BrdU$^{+,Diffuse}$ or BrdU$^{+,Punctate}$ that are MCM7^{+} (n=3). Immunostaining of (U-V) phospho-P53(ser15) (green) and BrdU (red), and (W-X) phospho-CHK2 (green) and BrdU (red) in human islets 48 hours after transduction with HNF4α8. Quantification (Y) of the percentage of BrdU$^{+,Diffuse}$ or BrdU$^{+,Punctate}$ that are either phospho-P53(Ser15)$^{+}$ or phospho-CHK2^{+} (n=3; *, P<0.001 versus BrdU$^{+,Diffuse}$ condition). White arrows indicate either diffuse (d) or punctate (p) BrdU incorporation. The scale bars in (K, Q, S, and X) indicate 25 μm.

Overexpression of HNF4α8 leads to activation of the DNA damage response associated with replication stress

Phosphorylation of CHK2 on threonine 68 and p53 on serine 15 are downstream substrates of ATR and ATM, key mediators of the DNA damage checkpoint pathway (22). Furthermore, the punctate BrdU incorporation pattern is reminiscent of DNA replication foci during S-phase (19). We hypothesized that overexpression of HNF4α8 caused repeated licensing of DNA replication from single origins (or re-replication), resulting in enlarged punctate foci, DNA breaks, and induction of the DNA damage response. To test this possibility, we first assessed the effects of HNF4α8 overexpression on phosphorylation of histone H2AX (γH2AX), an indicator of DNA double-stranded breaks (23). Indeed, overexpression of HNF4α8 caused a dramatic increase in $\gamma H2AX^{+}$ $PDX1^{+}$ cells, with 4.4%±1.4 of $PDX1^{+}$ cells colocalizing with γH2AX 72 hours after transduction, while less than 0.5% of $PDX1^{+}$ cells stained for γH2AX in untransduced or CMV-eGFP treated controls (Figure 3.5A-D). In AdCMV-eGFP transduced islets, γH2AX staining colocalized with cells positive for GFP reflecting rare toxicity of this adenovirus to islet cells (Figure 3.5B insert). The accumulation of $\gamma H2AX^{+}$ in $PDX1^{+}$ cells exactly mirrors the accrual of $BrdU^{+,punctate}$ in $PDX1^{+}$ cells, demonstrating that the DNA damage response is restricted to this cell population (Figure 3.5D). γH2AX expression was present in 9.50%±1.97 $HNF4\alpha^{High}$ cells and 88.1%±5.6 of $BrdU^{+,punctate}$ cells at 72 hours after transduction, and was sustained at the same level up to 120 hours (Figure 3.5E-J). Many $HNF4\alpha^{High}$ $BrdU^{+,punctate}$ cells show enlarged, dysmorphic nuclei which are not present in control islets (Figure 3.5G inlet), suggesting a model wherein

HNF4α8-overexpression in β-cells stimulates DNA damage that is not repaired over time, leading to a sustained DNA damage checkpoint response.

To assess if DNA damage is associated with re-replication, we pulse-labeled HNF4α8 transduced islets between 36 and 48 hours, the established time of thymidine incorporation (Figure 3.4A-D), sequentially with BrdU and with EdU. We confirmed no cross-reactivity between the two thymidine analogs by labeling HNF4α8 transduced islets with either BrdU-only or with EdU-only continuously between 36 and 48 hours, and using detection methods for both thymidine analogs simultaneously (Figure 3.5K-L). To demonstrate occurrence of re-replication throughout the 12 hour time period, we varied the non-labeling times between pulses (1 hour, 3 hours, and 6 hours). Re-replication is defined here as the incorporation of both BrdU and EdU into the same locus in the nucleus. Several possible patterns of thymidine analogue incorporation can occur. In HNF4α8 transduced islets with a 1 hour non-labeling interval, thymidine^{+} cells incorporated a single analog in a diffuse manner (possibility #1), undergoing S-phase during the first or second labeling period (Figure 3.5M). Thymidine^{+} cells also incorporated both analogs in mutually exclusive areas of the same nucleus in a diffuse manner (possibility #2), representing a cell undergoing S-phase during both labeling periods (Figure 3.5N). The percentages of cells with diffuse thymidine analogue incorporation integrating only one analog increased with increased non-labeling interval, as expected (data not shown). Strikingly, in the thymidine$^{+,punctate}$ domains, we saw overlapping incorporation of both EdU and BrdU in distinct foci, regardless of the length of the non-labeling interval (possibility #3), demonstrating that re-replication occurred in HNF4α^{High} cells (Figure 3.5O-Q). Specifically, 84.1% ± 7.6, 78.2% ± 9.6, and 78.3% ±

8.5 of all thymidine$^{+,punctate}$ cells showed the re-replication phenotype with a 1 hour, 3 hour, and 6 hour pulse, respectively (Figure 3.5R). We saw only a low percentage of thymidine$^{+,punctate}$ cells with either non-overlapping or single incorporation of EdU and BrdU in distinct foci in all non-labeling intervals (possibility #4). These data suggest that the DNA damage and checkpoint response exhibited in HNF4α^{High} BrdU$^{+,punctate}$ β-cells occurs as a consequence of replication stress.

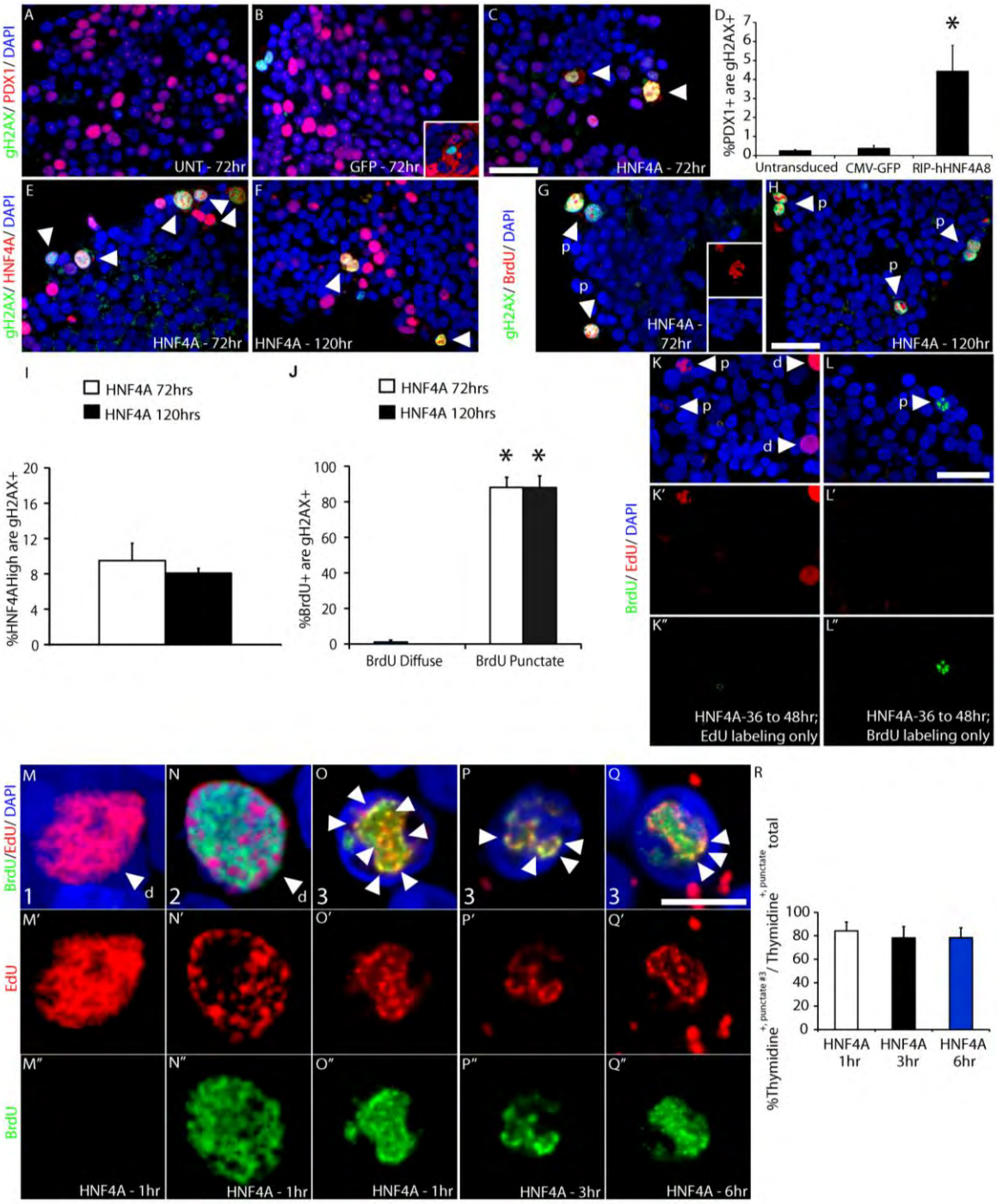

Figure 3.5: Overexpression of HNF4α8 leads to activation of the DNA damage response associated with replication stress.

Figure 3.5: Overexpression of HNF4α8 leads to activation of the DNA damage response associated with replication stress. Immunodetection of γH2AX (green), PDX1 (red), and DAPI (blue) in (A) untransduced, (B) GFP-, and (C) HNF4α8-overexpressing primary human islets at 72 hours after transduction. The insert in (B) shows GFP (red), γH2AX (green), and DAPI (blue) colocalization. (D) Quantification of the percentage of $PDX1^{+}$ cells that are $\gamma H2AX^{+}$ (n=3; $P<0.03$ versus both untransduced and CMV-eGFP conditions). Immunolocalization of γH2AX (green), and both (E-F) HNF4α (red) and (G-H) BrdU in HNF4α8-tranduced primary human islets at (E,G) 72 hours and (F, H) 120 hours after transduction. Quantification of the percentage of (I) $HNF4\alpha^{High}$ and (J) $BrdU^{+}$ cells that are $\gamma H2AX^{+}$ at 72 hours and 120 hours (n=3-4; *, $p<0.001$ versus $BrdU^{+,Diffuse}$ condition). Immunodetection of BrdU (green), EdU (red), and DAPI (blue) in (K) HNF4α8-transduced primary human islets exposed to (K) EdU-only, and BrdU-only. Individual red channel (K‘-L‘), and green channel (K‘‘-L‘‘) is shown. Simultaneous immunodetection of EdU (red) and BrdU (green) in HNF4α8 transduced islets of (M) single thymidine-analogue labeled diffuse nucleus with 1 hour non-labeling time, (N) dual labeled non-colocalizing diffuse nucleus with 1 hour labeling time, (O) dual-labeled colocalizing punctate nucleus with (O) one hour, (P) three hour, and (Q) six hour non-labeling times. Individual red channels (M‘-Q‘), and green channels (M‘‘-Q‘‘) are shown. Quantification (R) of the percentage of $thymidine^{+,punctate\ \#3}$ (#3 indicates a nucleus exhibiting overlapping incorporation of both EdU and BrdU in distinct foci within its thymidine punctate domain) over total $thymidine^{+,punctate}$ in HNF4α8 transduced islets dual labeled with one hour, three hour, and six hour non-

labeling times (n=3). The scale bars in (C, H, and L) indicate 25 μm and in (Q) indicate 5 μm.

A senescence-like phenotype, independent of caspase-mediated cell death, is the predominant fate of β-cells overexpressing HNF4α8

Sustained mitogenic signaling in the β-cell has been reported to cause cell death and loss of function (24-26). We used the TUNEL assay to determine if $HNF4\alpha^{High}$ $BrdU^{+,punctate}$ β-cells undergo apoptosis. $PDX1^{+}$ cells rarely exhibit TUNEL staining in untransduced, eGFP-, and even HNF4α8-overexpressing islets 72 hours after transduction (Figure 3.6A-D). The percentage of $HNF4\alpha^{High}$ cells that are $TUNEL^{+}$ was only 1.2%±0.4 at 72 hours, and 1.1%±0.1 at 120 hours after transduction (Figure 3.6E-F, I). While there was a very low level of $BrdU^{+,diffuse}$ cells that are $Tunel^{+}$, the percentage of $BrdU^{+,punctate}$ cells that were $TUNEL^{+}$ was only 9.8%±5.0, and 12.7%±9.9 at 72 and 120 hours after transduction, respectively (Figure 3.6G-H, J), demonstrating that caspase-dependent apoptosis is not the predominant fate of $HNF4\alpha^{High}$ $BrdU^{+,punctate}$ β-cells.

The inability of the β-cell to repair DNA damage over time can lead to cellular senescence (27). In response to a mitogenic insult, multiple senescence-promoting signals, such as DNA damage, replicative stress, reactive oxygen species, and heterochromatin formation converge on the tumor suppressor P53 (28). Indeed, P53 was activated in $HNF4\alpha^{High}$ and $BrdU^{+,punctate}$ cells at both 48 and 72 hours after transduction, with 78.2%±6.6 and 73.3%±5.1 staining positive, respectively (Figure 3.6L-N). The detection of nuclear expression of P53 was confirmed in human colon adenocarcinoma samples (Figure 3.6K). Together with cell cycle arrest, checkpoint activation, a sustained DNA damage response, and lack of caspase-dependent apoptosis, these data suggest that

HNF4α^{High} BrdU$^{+,punctate}$ β-cells exhibit a senescence-like phenotype, impeding further cell cycle progression (28).

To assess the effect of HNF4α- overexpression on β-cell function, we performed static glucose stimulated insulin assays (GSIS). The absolute amount of insulin released upon glucose stimulus by AdRIP-HNF4α8 treated islets was not statistically different from untransduced, AdCMV-eGFP treated islets, and untransduced islets at receipt of donation (Figure 3.6O). Also, there were no statistical differences in insulin content in any of the aforementioned groups 72 hours after transduction (data not shown), suggesting no loss of β-cell function or dedifferentiation in β-cells overexpressing HNF4α8 (25, 26). However, it was not possible to assess the function of the small HNF4α^{High} BrdU$^{+,punctate}$ β-cell population separate from the remainder of the islet; thus, loss of function in these cells remains a possibility.

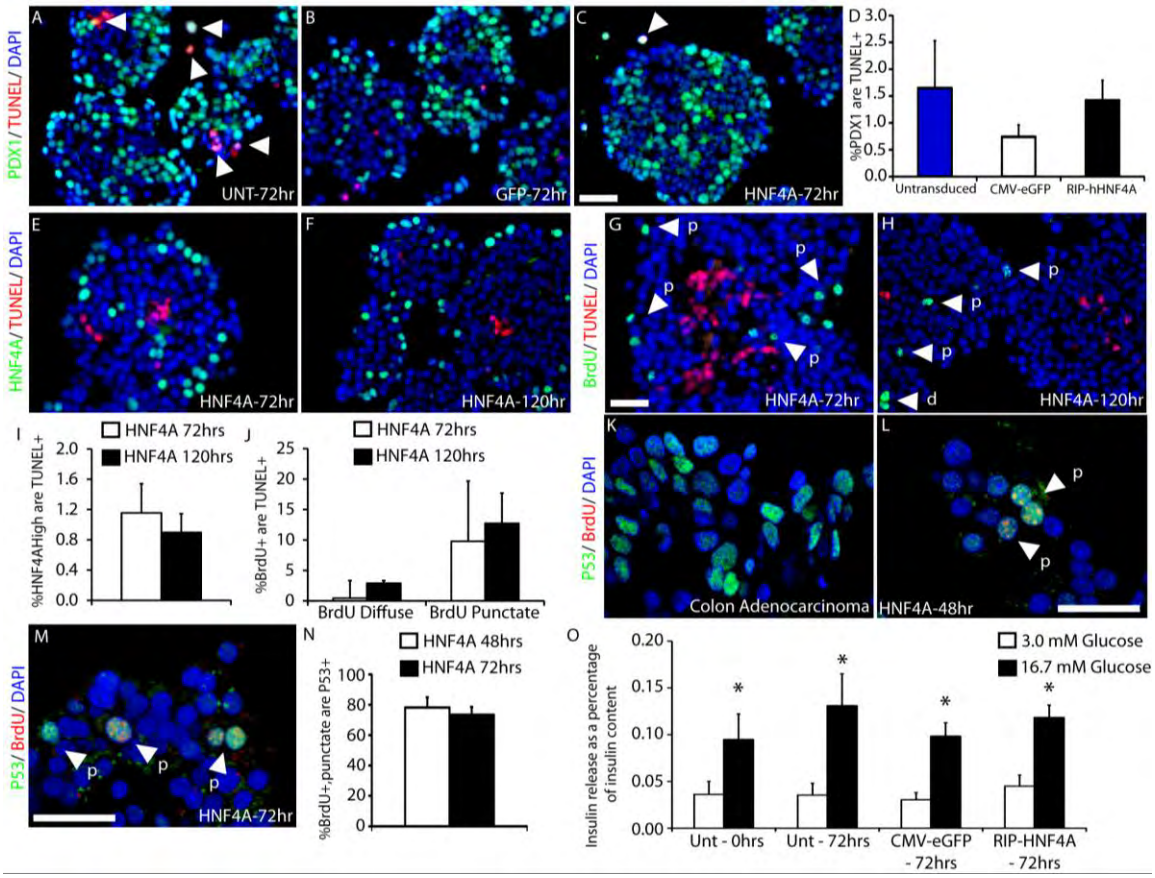

Figure 3.6: A senescence-like phenotype is the predominant fate of β-cells overexpressing HNF4α8.

Figure 3.6: A senescence-like phenotype is the predominant fate of β-cells overexpressing HNF4α8. Immunofluorescence detection of PDX1 (green), TUNEL (red) and DAPI (blue) in (A) untransduced, (B) eGFP-, and (C) HNF4α8-transduced primary human islets at 72 hours with (D) quantification of the percentage of $PDX1^{+}$ cells that are $TUNEL^{+}$ (n=3-4). Analysis of TUNEL (red) positive cells that are (E-F) $HNF4\alpha^{High}$ (green) and (G-H) $BrdU^{+}$ (green) at (E,G) 72 hours and (F,H) 120 hours. Quantification of the percentage of (I) $HNF4\alpha^{High}$ and (J) $BrdU^{+}$ cells that are $TUNEL^{+}$ at 72 hours and 120 hours (n=3-5). Immunostaining of P53 (green), BrdU (red) and DAPI (blue) in (K) human colon carcinoma, and HNF4α8 transduced islets at (H) 48 hours and (M) 72 hours after transduction. Quantification of the (N) percentage of $BrdU^{+,punctate}$ cells that are $P53^{+}$ (n=3). (O) Static glucose-stimulated insulin secretion assay (GSIS) of untransduced, eGFP-, and HNF4α8- overexpressing primary human islets 72 hours after transduction treated with 3mM and 16.7mM glucose. GSIS was also performed on untransduced primary human islets upon receipt (0 hours) (n=3-6; *, $p<0.05$ versus 3mM glucose condition). The scale bars in (C, G, L and M) indicate 25 μm.

HNF4α8 synergizes with known factors sufficient for promoting cell cycle entry in the human β-cell

The combination of the G_1/ S-phase cell cycle regulators CDK6 and a D-cyclin partner, for example CYCLIN D3, has been shown to dramatically accelerate the rate of β-cell proliferation in human β-cells *in vitro* (10, 18). While HNF4α8 is not sufficient to promote full β-cell replication alone, we hypothesized that overexpression of HNF4α8 might augment human β-cell proliferation stimulated by CDK6 and CYCLIN D3 (10). While $KI67^+$ $PDX1^+$ cells were very rare in untransduced, CMV-eGFP, and RIP-hHNF4α8- treated human islets, the overexpression of CDK6 and CYCLIN D3 caused a significant increase in the number of $PDX1^+$ cells that also expressed KI67 (3.14%±0.75). Strikingly, when we applied HNF4α8, CDK6, and CYCLIN D3 adenoviruses together, we saw a further increase in the number of $PDX1^+$ cells that were in the cell cycle 72 hours after transduction (7.97%±2.2) (Figure 3.7A-F). This finding suggests that HNF4α8 overexpression not only alone, but in combination with known cell cycle regulators, is a mitogenic signal sufficient for initiation of the cell cycle in human β-cells.

To confirm this conclusion and assess the fate of the additional $KI67^+$ $PDX1^+$ cells, we analyzed the $HNF4\alpha^{High}$ β-cell population when overexpressing HNF4α8, CDK6, and CYCLIN D3 with aforementioned markers for cell cycle progression, DNA damage, and caspase-dependent apoptosis. Indeed, 22.6%±6.0 and 3.17%±0.82 of HNF4α8 overexpressing cells were $KI67^+$ and CYCLIN A^+, respectively, 72 hours after transduction (Figure 3.7G-J). CYCLIN A^+ cells not colocalizing with $HNF4\alpha^{High}$ cells could reflect cells transduced with either CDK6 and/or CYCLIN D3 only, as their

overexpression is based on a CMV-promoter (Figure 3.7I). However, while this was an improvement relative to the lack of cell cycle progression seen when overexpressing HNF4α8 alone, at this time point, the HNF4α^{High} cell population was positive for both γH2AX (37.6%±6.4) and TUNEL (44.6%±3.4) at similar levels (Figure 3.7K-N). Additionally, these cells morphologically exhibited nuclear blebbing surrounding a central region of reduced DAPI staining (Figure 3.7M insert). The slightly higher percentage of the HNF4α^{High} cells exhibiting γH2AX and TUNEL compared to KI67 expression suggests that DNA damage induced caspase-dependent apoptosis is the predominant fate of the KI67^{+} PDX1^{+} cell population over time.

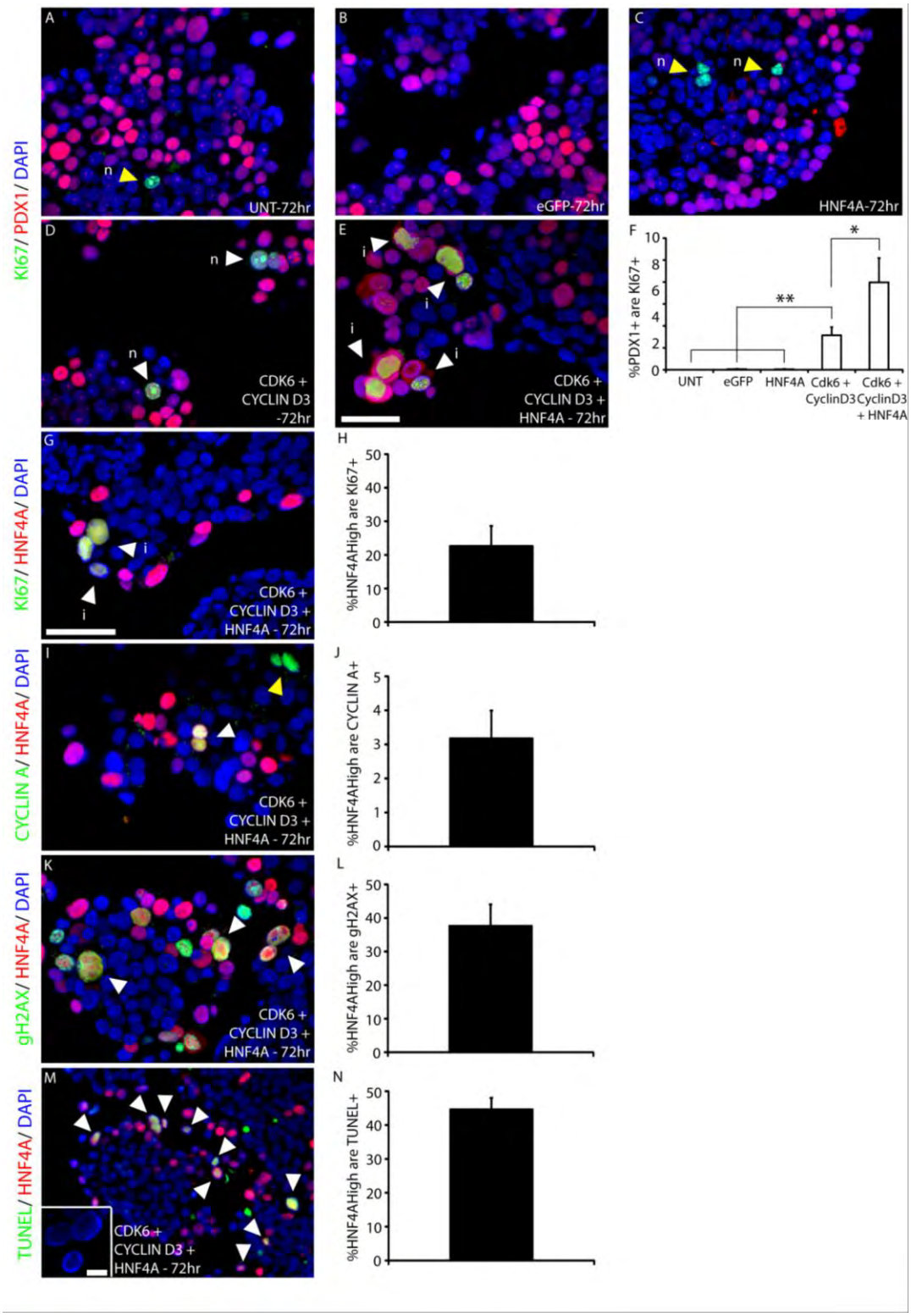

Figure 3.7: HNF4α8 synergizes with factors known to be sufficient for promoting cell cycle entry in human β-cells.

Figure 3.7: HNF4α8 synergizes with factors known to be sufficient for promoting cell cycle entry in human β-cells. Immunofluorescence detection of KI67 (green), PDX1 (red) and DAPI (blue) in (A) untransduced, (B) eGFP-, (C) HNF4α8-, (D) CYCLIN D3 and CDK6-, (E) HNF4α, CYCLIN D3, CDK6- transduced primary human islets at 72 hours with (F) quantification of the percentage of $PDX1^{+}$ cells that are $KI67^{+}$ (n=4-6; *, $p<0.04$; **, $p<0.01$). Immunofluorescent detection of HNF4α (red), DAPI (blue) and (G) KI67, (I) CYCLIN A, (K) γH2AX, and (M) TUNEL in HNF4α8, CYCLIN D3, CDK6-transduced primary human islets at 72 hours with quantification of the percentage of $HNF4\alpha^{High}$ cells that are (H) $KI67^{+}$, (J) CYCLIN A^{+}, (L) $\gamma H2AX^{+}$, and (N) $TUNEL^{+}$ (n=3). The insert in (M) shows representative DAPI staining of $HNF4A^{High}$ $TUNEL^{+}$ cells. The scale bars in (E, G) indicate 25 μm and in insert of M indicates 5 μm. Abbreviation n = normal, and i = irregular.

Overexpression of CYCLIN D3 and CDK6 also activates the DNA damage response in human β-cells

We also investigated whether β-cells induced to proliferate by overexpression of CDK6 and CYCLIN D3 alone exhibit expression of γH2AX. Strikingly, 9.86%±2.3 and 9.10%±1.8 of $PDX1^+$ and $insulin^+$ cells, respectively, were positive for γH2AX 72 hours after transduction (Figure 3.8A-C), suggesting that over time a substantial proportion of $PDX1^+$ cells stimulated to progress through the cell cycle by CDK6 and CYCLIN D3 accumulate DNA damage. To assess if γH2AX accumulation is specific to cells having engaged the cell cycle sometime during the 72 hour time period, we evaluated the percentage of $\gamma H2AX^+$ cells that incorporated BrdU, applied continuously to culture after transduction. While not all $BrdU^+$ cells were $\gamma H2AX^+$ (Figure 3.8D insert), a significant percentage of cells having progressed through S-phase expressed γH2AX (60.6%±11.5 of $BrdU^{+,total}$ are $\gamma H2AX^+$) (Figure 3.8D, F). Interestingly, while the $BrdU^+$ $\gamma H2AX^+$ cells exhibited punctate-like BrdU incorporation (82.3%±7.5 of $\gamma H2AX^+$ are $BrdU^{+,punctate}$) similar to what was seen in β-cells when overexpressing HNF4α8 alone, $BrdU^+$ $\gamma H2AX^-$ cells exhibited diffuse BrdU incorporation (5.23%±2.2 of $\gamma H2AX^+$ are $BrdU^{+,diffuse}$) (Figure 3.8D, G). In addition, the irregular manner of KI67 staining observed in $PDX1^+$ cells transduced with CDK6, CYCLIN D3, and HNF4α8 was unlike the diffuse KI67 staining observed in cycling non-endocrine cells (Figure 3.7E). As with punctate-like BrdU incorporation, the irregular KI67 staining was also observed in human islets transduced with CDK6 and CYCLIN D3, and almost always colocalized with γH2AX expression (91.2%±2.1 $KI67^{irregular}$ cells were $\gamma H2AX^+$) 72 hours after transduction

(Figure 3.8E-F). Furthermore, the percentage of $\gamma H2AX^{+}$ cells that incorporated BrdU in a punctate-like manner was much higher than the percentage which expressed $KI67^{irregular}$ (13.9%±1.0 $\gamma H2AX^{+}$ were $KI67^{irregular}$) at 72 hours (Figure 3.8E, G). Based on cell cycle arrest seen in β-cells overexpressing HNF4α8 alone and the fate of $HNF4\alpha^{High}$ cells in the triple-transduced condition this suggests, a delay of the cell cycle in the proportion of β-cells accumulating double-stranded DNA damage, ultimately resulting in caspase-dependent apoptosis. However, as with diffuse BrdU incorporation, the majority of $KI67^{normal}$ cells do not exhibit γH2AX (10.9%±2.1 of $KI67^{normal}$ cells were $\gamma H2AX^{+}$) (Figure 3.8E, F), suggesting that, in line with the overall ability of Cdk6 in combination with a D-cyclin partner to improve human islet function *in vivo*, a fraction of functional β-cells do arise by CDK6 and CYCLIN D3 stimulated proliferation (10, 29). Indeed, only cells incorporating BrdU in a punctate-like manner were positive for the checkpoint marker, phospho-P53 on serine 15 (99.0%±0.7 of $BrdU^{+,punctate}$ were phospho-$P53(Ser15)^{+}$), while of cells incorporating BrdU in a diffuse manner only 5.48%±1.38 of $BrdU^{+}$ were phospho-$P53(Ser15)^{+}$ (Figure 3.8H-I). Also, $\gamma H2AX^{+}$, $BrdU^{+,punctate}$ cells morphologically exhibit nuclear blebbing, indicating caspase-dependent apoptosis (Figure 3.8E insert). Together, these findings demonstrate that while human β-cells can be stimulated to enter in and progress through the cell cycle by overexpression of CDK6, CYCLIN D3, and further with the addition of HNF4α8, the activation of the DNA damage response in both cases highlights the importance of using multiple experimental criteria to adequately assess human β-cell expansion.

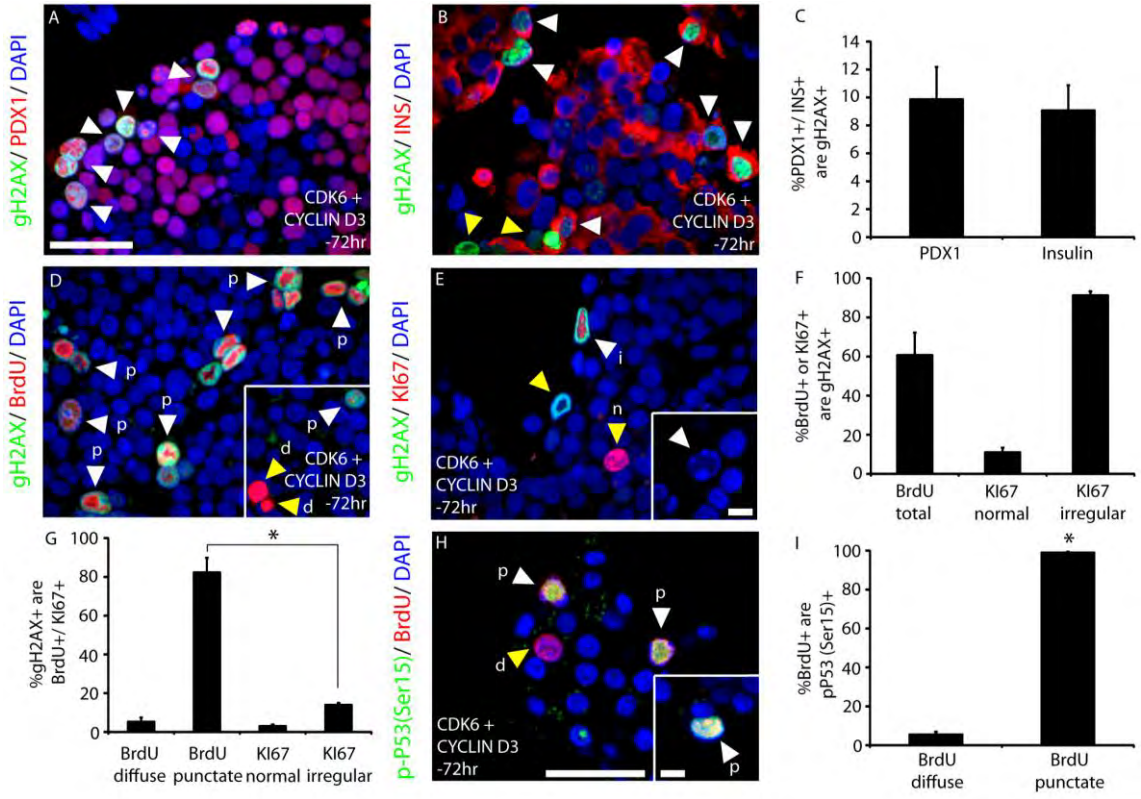

Figure 3.8: Overexpression of CYCLIN D3 and CDK6 also activates the DNA damage response in human β-cells.

Figure 3.8: Overexpression of CYCLIN D3 and CDK6 also activates the DNA damage response in human β-cells. Dual immunostaining for γH2AX (green), DAPI (blue) and (A) PDX1 (red), (B) Insulin (red), (D) BrdU (red) and (E) KI67 (red) in CYCLIN D3 and CDK6 transduced human islets. Quantification of (C) the percentage of either $PDX1^{+}$, or $Insulin^{+}$ cells that are $\gamma H2AX^{+}$ in primary human islets transduced with both CDK6 and CYCLIN D3 (n=3-4). Quantification of (F) the percentage of either $BrdU^{+,total}$, $KI67^{+,normal}$ or $KI67^{+,\ irregular}$ cells that are $\gamma H2AX^{+}$ in primary human islets transduced with both CDK6 and CYCLIN D3 (n=3-4). Quantification of (G) the percentage of $\gamma H2AX^{+}$ cells that are either $BrdU^{+,diffuse}$, $BrdU^{+,punctate}$, $KI67^{+,normal}$ or $KI67^{+,\ irregular}$ (n=3-4; *, $p<0.001$ $BrdU^{+,punctate}$ versus $KI67^{+,irregular}$ conditions). Dual immunostaining for (H) phospho-P53 on serine 15 (Green), DAPI (blue) and BrdU (red) in CYCLIN D3 and CDK6 transduced human islets. The quantification of (I) the percentage of either $BrdU^{+,diffuse}$ or $BrdU^{+,punctate}$ that are phospho-P53 $(serine\ 15)^{+}$ (n=4; *, $p<0.001$ versus $BrdU^{+,diffuse}$ condition). The white arrows indicate colocalization and yellow arrows indicate non-colocalization between two markers. All human islets were incubated for 72 hours post-transduction. The scale bars in (A, H) indicate 25 μm and in the insert of E, H indicates 5 μm. Abbreviation p = punctate, d = diffuse, n = normal, and i = irregular.

Discussion

It is well appreciated that human β-cell turnover occurs very slowly after early childhood (30, 31). Evaluating whether β-cells proliferate in states of physiological insulin resistance is difficult, because of differences in populations studied and the limited number of cases considered (32). However, in spite of this, evidence for β-cell regeneration in humans has arisen in the fetal pancreas during the perinatal period (33, 34), and the context of obesity (35). Furthermore, the presence of a significant number of paired apoptotic β-cells in pancreas sections of type 2 diabetic patients, interpreted as post-mitotic apoptosis, suggests that diabetes onset is a result of a failure of β-cell expansion rather than a decrease in existing β-cell mass only (36). The occurrence of proliferating β-cells even in the resected pancreas from an 89-year old type 1 diabetic suggests that β-cell replication can be stimulated *in vitro* (37). In this study we investigated if a pancreatic specific isoform of the MODY1 transcription factor HNF4α is sufficient to promote proliferation in primary human β-cells, based on its requirement for β-cell expansion in response to physiological insulin resistance during pregnancy (13).

The data represented here demonstrate that overexpression of HNF4α8 is sufficient to initiate cell cycle entry, license DNA at replication origins, and attempt S-phase in human β-cells, both on its own and in combination with known factors sufficient for β-cell proliferation. The punctate manner of BrdU incorporation into HNF4α^{High} β-cells is reminiscent of the patterns of DNA replication seen during S-phase in mammalian nuclei (19). To ensure that chromosomal DNA is precisely duplicated during S-phase, the cell must be able to distinguish between replicated and unreplicated DNA. The licensing

of replication origins by the binding to DNA of mini-chromosome maintenance 2-7 proteins (MCM2-7) and their subsequent removal after initiation of DNA replication ensures regulated initiation of DNA replication only once per locus (38). While HNF4α8 overexpressing cells are able to license their DNA for replication, as demonstrated by increased MCM7 expression at the time of BrdU incorporation (Figure 3.4D, S), the appearance of large punctate domains points to misregulation of the licensing system, allowing cells to inappropriately re-license their already replicated DNA. Consistent with this model, we detected re-replication as distinct overlapping foci within thymidine^{+} punctate domains by utilizing two thymidine analogs during the time when the majority of HNF4α^{High} β-cells were progressing through S-phase (Figure 3.5M-R). Although the thymidine^{+} cells are not synchronized, the high percentage of thymidine^{+} cells that exhibit the re-replication phenotype even with a 6 hour non-labeling time suggests an elongation of S-phase (39). Similarly, overexpression of Cdt1, a rate-limiting licensing factor responsible for loading of the replicative Mcm-helicase onto DNA, in the G_2-phase of the cell cycle induced re-replication of DNA, activated checkpoint pathways, and blocked further cell cycle progression in *Xenopus* egg extracts (40). Intriguingly, not only was the checkpoint activation a direct result of multiple rounds of DNA re-replication, but it coincided with the appearance of significant double stranded DNA fragments, consistent with a model of head-to-tail replication fork collision (41). Also, increased expression of CDT1 and subsequent re-replication occurs in human cancer-derived cell lines upon accumulation of constitutively active mutant of CYCLIN D1 (42). In line with this, both activation of the DNA damage checkpoint markers p-CHK2 (Thr68) and p-P53 (Ser15), and expression of γH2AX, a marker for double stranded DNA breaks, occurred

in HNF4α^{High} BrdU$^{+,punctate}$ β-cells (Figure 3.4V, X; 3.5A-J). Activation of the DNA damage response did not cease over time, suggesting that the DNA damage is too great to repair.

Surprisingly, activation of the DNA damage response is not a phenotype specific to HNF4α8 overexpression. Strikingly, a significant proportion of β-cells stimulated to progress through the cell cycle by overexpression of CDK6 and CYCLIN D3 alone also exhibited activation of γH2AX expression, and a cell cycle checkpoint (see Figure 3.8). We conclude that human β-cells are sensitive to accumulating DNA damage in response to forced cell cycle entry. In support of this, a mouse model deficient in nonhomologous end-joining (NHEJ) and expressing a hypomorphic mutant of p53, defective in apoptosis but not in cell cycle arrest, develops diabetes. In these mice, β-cell mass is progressively depleted due to accumulated DNA damage (sustained γH2AX expression), promoting a decrease in β-cell proliferation through p53-dependent cell cycle arrest (27). Strikingly, HNF4α^{High} BrdU$^{+,punctate}$ β-cells exhibited activation of P53 expression, but did not undergo caspase-dependent apoptosis (Figure 3.5I-M). Rather, HNF4α8-overexpressing cells attempting cell cycle progression display hallmarks of a senescence phenotype, such as DNA damage, tumor suppressor activation, and cell cycle arrest (28). It is clear that spontaneous replication in untransduced primary human islets is ultimately halted over time by cellular senescence, which is characterized by loss of differentiated function (43).

These phenotypes in the β-cell are consistent with the ability of oncogenes such as Ras, Mos, Cyclin E, E2F1, Cdc25A, Cdc6, and Myc to promote senescence by stimulating the DNA damage response through re-replication (44-46), for which pathways encompassing key downstream tumor suppressors p53 and Rb are necessary

(47). The mitogen-activated protein kinase (Mapk) pathway, dependent on HNF4α during pregnancy in the murine β-cell, can also induce cell cycle arrest and subsequent senescence via the increase of both p53 and p21 expression and blocks in hyper-phosphorylation of Rb (48). It is therefore tempting to conclude that HNF4α8-overexpression in the β-cell acts similarly to oncogenes such as Ras and Mos that also activate this signaling pathway (49). Indeed, rat (INS1, RINm5F) and mouse (MIN6, BTC3) insulinoma cell lines show not only increases in many cyclins and cdks, but also increases in many cell cycle inhibitors (p16, p18, p21, p57, p53) when compared to primary untransduced mouse and rat islets (50). We take this as evidence that substantial increases in β-cell cycle inhibitors are typical of rapidly dividing β-cells, and that increases in cell cycle inhibitors is not incompatible with increases in overall β-cell number, which clearly occur in insulinoma cell lines *in vitro* (50). Conversely, double-ablation of Rb and p130 in β-cells does not lead to a net change in β-cell mass despite dramatically increasing β-cell proliferation *in vivo* (51). Instead, increases in β-cell proliferation are associated with matched increases in β-cell death, and attempts at cell cycle arrest, as indicated by increased phosphorylation of p53 on serine 15 and increased p21 protein levels (51). Also, primary human β-cells transduced with CDK4 and CYCLIN D1 display increased P21 protein levels (52), placing into question whether overexpression of these proteins always lead to increases in β-cell number. Regardless of whether the overexpression of genes promoting proliferation of the β-cell stimulates a senescence-like fate, as with overexpression of HNF4α8-alone, or apoptotic fate, as with the combined overexpression of CDK6, CYCLIN D3, and HNF4α8, we propose that the accumulation of DNA damage resulting from replication stress is a barrier to efficient

human β-cell proliferation *in vitro* as also seen in precancerous lesions of the bladder, breast, colon and lung *in vivo* (46).

As a cautionary note, the criteria when demonstrating sufficiency of a factor to promote β-cell proliferation should be extended to include a detailed analysis of cell cycle entry, the fidelity of duplication of the genome during S-phase, progression through multiple cell cycle phases, and cell cycle exit. Furthermore, in addition to the already known undesirable fates of newly formed β-cells through proliferation, i.e. dedifferentiation, and apoptosis, activation of the DNA damage response should be assessed in attempts to faithfully mimic β-cell proliferation *in vitro*.

Note: Data presented in this chapter have been submitted to Diabetes as an original article entitled,

Overexpression of Hepatocyte Nuclear Factor- 4α initiates cell cycle entry, but is not sufficient to promote β-cell expansion in human islets.

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Materials and Methods

Adenovirus production: The original cDNA plasmid, pcDNA3.hHNF4α8 (NM_175914.3), was constructed by Dr. Jerome Eeckhoute (53) and is a kind gift of Dr. Frances M. Sladek. The islet-specific human HNF4α8 cDNA immediately downstream of the rat insulin promoter (RIP) was subcloned into pShuttle (Clontech, Mountain View, CA). The Adeno-X Expression System 1 (Clontech, Mountain View, CA) was used for construction of the AdRIP-hHNF4α8 adenovirus. The resulting adenovirus was amplified in HEK293 cells, purified, and its infectious titer determined. Additionally, quality control tests throughout this process, such as genome structure analysis using restriction endonuclease digests, replication-competent adenovirus assay, and endotoxin assay were successfully performed. The AdCMV-eGFP was received from Dr. Arbansjit Sandhu (University of Pennsylvania, Philadelphia, PA).

Culturing, transduction and harvest of human cadaveric islets: Human islets were supplied by the islet cell biology core of the DERC of the University of Pennsylvania, the NIH-supported National Disease Research Interchange (NDRI) (http://www.ndriresource.org), and the Methodist Hospital Research Institute (http://www.methodisthealth.com/default.cfm). The mean (± standard error) age of the all donors was 44.7 ± 11.2 years, the purity of the preparations 86.2% ± 5.5, the viability 90.2% ± 6.6, and the BMI 29.5 ± 8.9. Upon arrival, human islets were incubated in CMRL 1066 medium (Mediatech, Manassas, VA) containing 5.5mM D-glucose, 0.5% Human Albumin (Talecris Biotherapeutics, Research Triangle Park, NC), 10U/ml Heparin (Sagent Pharmaceuticals, Schaumberg, IL), 100 μg/ml Penicillin/Streptomycin (Mediatech, Manassas, VA), and 2mM L-glutamine (Invitrogen, Carlsbad, CA). Intact

islets were transduced with adenoviruses for 24 hours in non-tissue culture coated dishes, after which the adenovirus was washed out of culture by successive medium changes. Then the islets were cultured in the continued presence of a 1:100 dilution of the Bromodeoxyuridine (BrdU) reagent (Invitrogen, Carlsbad, CA) during the length of incubation after transduction. In all experiments, $5X10^6$ infectious particles per islet of either AdRIP-HNF4α8 or AdCMV-eGFP were used. $5X10^5$ infectious particles per islet of both AdCMV-CYCLIN D3 and AdCMV-CDK6 were used as described (10). Islets were fixed in 4% paraformaldehyde (Fisher Scientific, Pittsburgh, PA) for 1 hour at 4°C, the islet pellet suspended in 2% molten agarose, processed, and embedded in paraffin. 5μm sections were used for immunofluorescence analysis. All data are from non-diabetic donors, unless stated otherwise.

mRNA isolation and RT-PCR: Total RNA was isolated using the RNeasy Mini Kit (QIAGEN, Chatsworth, CA), and eluted in water. cDNA was synthesized and quantitative PCR reactions performed as described previously (54). PCR primers sequences used for E1A are forward, TATGCCAAACCTTGTACCGGAGGT and reverse, CCGGGGTGCTCCACATAATCT, as designed in (17); HNF4α forward, TGCCTACCTCAAAGCCATCAT and reverse, GCGGTCGTTGATGTAGTCCTC.

Immunofluorescence analysis: Paraffin-embedded sections were dewaxed, and heat-induced antigen retrieval performed by pressure cooker heating (Prestige Medical, Northridge, CA) using citrate buffer (pH 6.0). Non-specific binding was blocked for 10 minutes with CAS-Block (Invitrogen, Carlsbad, CA). Sections were then incubated with primary antibodies overnight at 4°C. Antibodies used were: guinea pig anti-Pdx1 (gift from Dr. Christopher Wright), guinea pig anti-Insulin (Linco Research, St. Charles, MO),

mouse anti- HNF4α (R&D Systems, Minneapolis, MN), rabbit anti- HNF4α (Santa Cruz, Santa Cruz, CA), rat anti-BrdU (Accurate Chemical, Westbury, NY), rabbit anti-Glucagon (Invitrogen, Carlsbad, CA), rabbit anti-Somatostatin (Invitrogen, Carlsbad, CA), rabbit anti-Ki67 (Vector Laboratories, Burlingame, CA), mouse anti-Cyclin A (Thermo Scientific, Fremont, CA), mouse anti-Mcm7 (Thermo Scientific, Fremont, CA), rabbit anti-γH2AX (Cell Signaling, Danvers, MA), mouse anti-p53 (Santa Cruz, Santa Cruz, CA), rabbit anti-phospho-Chk2 (Cell Signaling, Danvers, MA), rabbit anti-phospho-p53(Ser15) (Cell Signaling, Danvers, MA) and goat anti-GFP (Abcam, Cambridge, UK). Sections were incubated with Cy3-, Cy5-, or Cy2-conjugated donkey anti-rabbit, anti-mouse, anti-goat, anti-guinea pig, anti-rat IgGs (Jackson ImmunoResearch, West Grove, PA) for 2 hours at room temperature, and nuclei stained with 4',6-diamidino-2-phenylindole (DAPI). Quantification was performed on a Nikon E600 microscope, and a Q-Imaging Fast CCD camera (Q-Imaging Surrey, BC Canada) in conjunction with IVision software (Biovision, Exton, PA). High-resolution images were taken on a Yokagawa CSU-10 spinning disk mounted on a Nikon Ti-U inverted microscope, and a Hamamatsu Photonics „Orca" CCD Camera (Hamamatsu, Bridgewater, NJ). All images except for ones including Tunel staining were constructed to a single-image in-focus view of a stack of frames acquired at different positions along the Z-axis. Image J software (NIH, Bethesda, MD) was used to count percentages of colocalization between two antibodies.

Re-replication assay: To determine repeated licensing of origins in human islets, 36 hours after adenoviral transduction BrdU (Invitrogen, Carlsbad, CA; 1:100 dilution) was added to the islet culture for 5.5 hours, unless otherwise indicated. BrdU was removed by

washing the islets 3 times with PBS. Islets were placed back into fresh medium without thymidine analog for 1 hour to ensure depletion of BrdU. Then EdU (Invitrogen, Carlsbad, CA) at a concentration of 100μM was added for an additional 5.5 hours after which islets were harvested, fixed, and sectioned for immunofluorescence analysis. Non-labeling intervals of 3 hours and 6 hours were also used. After antigen retrieval, EdU-incorporation was detected with the Click-iT EdU Alexa Fluor 555 imaging Kit per manufacturer‟s instructions (Invitrogen, Carlsbad, CA). After the Click-iT reaction, BrdU was detected as described above.

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL): To assess apoptosis, TUNEL assays were performed using the Tunel Label Mix and Tunel TdT Enzyme (Roche Applied Science, Indianapolis, IN), and AlexaFluor 647-dUTP secondary antisera (Invitrogen, Carlsbad, CA). Paraffin-embedded sections were re-hydrated, washed in 0.2% Triton-X to permeablize the nuclear membrane, microwaved in citrate buffer (pH 6.0) for 16 minutes, followed by 2 hours of cooling. Sections were digested in 0.0025% Trypsin-EDTA for 12 minutes at 37°C, and incubated with TUNEL reaction mixture for 45 minutes at 37°C. The sections were then incubated with primary and Cy3-conjugated secondary antibodies and imaged as described above.

Glucose stimulated insulin secretion in vitro: Glucose-stimulated insulin secretion (GSIS) was performed under static conditions. Briefly, insulin release was measured from isolated human islets at time of receipt and 72 hours after transduction with adenovirus. Islets were pre-incubated in Krebs bicarbonate buffer (120mM NaCl, 1.8mM $CaCl_2$, 5mM KCl, 10mM HEPES, 1.2mM KH_2PO_4, 25mM $NaHCO_3$, 0.2% BSA) with 3mM glucose for 1 hour to achieve baseline insulin release. Three groups of 70 islets each were

sequentially exposed to 3mM glucose for 1 hour, the medium collected, and then shifted to 16.7mM glucose for 1 hour. The medium was again collected and islet protein extracted. Insulin release into the medium, and insulin content of islets was determined using a radioimmune assay specific for human insulin (University of Pennsylvania Diabetes and Endocrinology Research Center – Radioimmunoassay and Biomarkers Core).

Statistics: Statistical analysis between two groups was done using a one-tailed Student‟s *t*-test. Values are considered significant when $P < 0.05$. Variation measurements are given as standard error of the mean unless stated otherwise.

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Chapter IV:

Conclusions and Future Directions

Conclusions

Normally in healthy conditions, pancreatic β-cells have a long life span, exhibiting a low rate of replication. In response to the increased systemic demand for insulin as occurs during pregnancy, obesity and β-cell recovery after injury, the β-cell population as a whole is able to dramatically increase its rate of replication, at least in rodents. It is thought that cell replacement therapy is well suited for diabetic patients whose endogenous β-cell number is below the threshold to successfully alleviate hyperglycemia chronically. One possible treatment to correct this β-cell deficit in both type 1 and type 2 diabetic patients is through the transplantation of isolated islets from cadavers. However, a recent report indicates a disproportionately low number islet infusions are performed annually compared to the total amount of diabetic patients, exemplifying the paucity of donor tissue (1). A promising approach to improve this statistic depends on developing an unlimited *in vitro* supply of fully functional β-cells through the proliferation of pre-existing terminally differentiated adult β-cells. To this day, a well-defined protocol to drive efficient non-oncogenic proliferation of human β-cells *in vitro* remains elusive.

My research attempted to succeed against this challenge by identifying the mechanisms and molecular players responsible for β-cell proliferation during physiological expansion of β-cell mass and attempting to mimic human β-cell proliferation *in vitro* through the forced expression of known rodent β-cell mitogens. In the first part of my thesis, I used gene expression analysis to determine what mechanisms are involved in controlling islet replication during pregnancy in mice. In the second part

of my thesis I utilized an adenovirus, primary human islet donations, and immunofluorescence techniques to assess the consequence of the overexpression of a pancreas specific isoform of HNF4α, a known transcription factor required for murine β-cell proliferation during pregnancy (2), on human β-cell proliferation *in vitro*.

This dissertation makes several contributions to the field of β-cell regeneration. First, I provide the first systematic *in vivo* study investigating the expression profile of the proliferating islet during pregnancy in the mouse. I identify many differentially expressed genes that at the time of the study have not been previously reported to play a role in islet expansion. I compare three different mouse models of β-cell expansion and provide evidence that although both proliferative and survival mechanisms are required for universal islet expansion, the mechanisms to attain increased islet mass differ between pregnancy, obesity, and β-cell injury. In addition, I demonstrate that HNF4α8 is an initiator of cell cycle entry in primary human β-cells. While several groups have attempted to mimic human β-cell proliferation *in vitro*, I am the first to show that the DNA damage response resulting from replication stress is a barrier to the successful completion of the cell cycle by the overexpression of known cell cycle regulators.

Both proliferative and survival signals are required for islet expansion *in vivo*

By combining gene expression profiling data and gene ontology functional analysis I found a cluster of categories with genes involved in cellular proliferation and cell death that were overrepresented in the list of differentially expressed genes from pregnant day 14.5 islets as compared to non-pregnant age matched controls (Table 2.7). Thus, I hypothesized that the islet‟s ability to compensate during metabolic stress requires the simultaneous induction of both proliferative and survival pathways. Birc5

(Survivin), a member of the inhibitor of apoptosis (IAP) gene family, is a gene of particular interest as endocrine pancreas specific Birc5-deficient animals progressively lose β-cell mass after 2 weeks of age, and Birc5-deficient islet cells exhibit dysmorphic nuclei, consistent with defective cell division (3). I found that *Birc5* expression in the islet closely mirrored the proliferative profile, reflected by mRNA levels of *Ki67*, throughout pregnancy, obesity and β-cell injury (Figure 2.4, 2.8), implicating Birc5 as a crucial and universal factor that ensures the survival of proliferating β-cells through and after mitosis in various settings during increased metabolic loads later in adulthood (Figure 4.1). Indeed, I demonstrate that significantly reduced expression of both *Ki67* and *Birc5* in part explains for the onset of severe diabetes in 10 week old BTBR *leptin*$^{ob/ob}$ mice as compared to age matched diabetes resistant B6 *leptin*$^{ob/ob}$ mice (4). Consistent with the observations I made in comparing different models of β-cell expansion, Birc5‟s cytoprotective qualities might be of therapeutic value as demonstrated by transplantation of a suboptimal number of β-cell specific Birc5 transgenic islets into diabetic recipient mice resulting in long-term engraftment, and stable correction of hyperglycemia in part through the intrinsic inhibition of β-cell apoptosis without inducing new rounds of β-cell proliferation (5). Together these experiments indicate that the ideal strategy to derive fully functional human β-cells through proliferation whether endogenously in a patient or *ex vivo* should consider the utility of „survival‟ genes ensuring protection from the consequences of flawed cell division and/or yet unidentified deficiencies, to limit undesired fates such as apoptosis induced by genotoxic stress.

The transcriptional mechanisms to attain increased islet mass differ between pregnancy, obesity and a β-cell injury model

Using qPCR I compared the expression of selected genes most differentially expressed during pregnancy day 14.5 to two other models of β-cell expansion caused by an induction of β-cell proliferation: the 10 week old diabetes resistant B6 *leptin*$^{ob/ob}$ mouse and the PANIC-ATTAC transgenic mouse. I demonstrated dramatic differences in the induction of gene expression occurring between pregnancy, obesity-induced β-cell compensation, and recovery from β-cell ablation (Figure 2.8), and suggest that diverse mechanisms can be used by the islet to expand its mass at least on a transcriptional level. Given the divergent physiological contexts of pregnancy, obesity, and experimental β-cell ablation, it makes intuitive sense that the molecular mechanisms responsible for the compensatory increase in islet mass in each case are probably distinct. A hypothesis one can extend from this is that these divergent mechanisms converge upon the cell cycle genes of the adult β-cell. This is exciting as there might be many potential therapeutic interventions one can pursue to elicit β-cell proliferation; the challenge being which are most feasible as an effective treatment for diabetes mellitus.

Overexpression of HNF4α8 initiates cell cycle entry in human β-cells *in vitro*

The transcription factor HNF4α is required for the increase in β-cell proliferation during pregnancy, and is one therapeutic avenue for further investigation. To address the ability of HNF4α as a human β-cell mitogen, I employed adenoviral-mediated overexpression of a pancreas-specific isoform of HNF4α (HNF4α8). Through the use of immunofluorescence markers distinguishing between the distinct phases of the cell cycle, I demonstrated that HNF4α8 overexpression alone stimulated β-cells to enter the cell cycle, and led to a greater than 300-fold increase in the number of β-cells that entered S-phase without detectable loss of function (Figure 3.2, 3.6). In addition, when I

overexpressed HNF4α8 together with known factors sufficient for promoting cell cycle entry in the human β-cell (CDK6 and CYCLIN D3), I further increased the number of β-cells entering the cell cycle (Figure 3.7). This data extends the role of HNF4α in murine β-cell proliferation during pregnancy to a cell cycle regulator in human β-cells *in vitro*.

The DNA damage response resulting from replication stress limits cell cycle progression in primary human β-cells overexpressing cell cycle regulators

I further investigated β-cells stimulated to enter the cell cycle by overexpression of HNF4α8 for (i) active cell cycle progression through cell cycle phases, and (ii) proper duplication of the genome. Using immunofluorescence markers I demonstrate that this population of β-cells undergoes dysregulated DNA replication at one origin leading to activation of the DNA damage checkpoint, and cell cycle arrest ultimately leading to a senescence-like fate (Figure 3.4, 3.5, 3.6). DNA damage induced cell cycle arrest is not limited to overexpression of HNF4α8, but is also present in a substantial proportion of β-cells induced to enter the cell cycle by the combined overexpression of CYCLIN D3 and CDK6 (Figure 3.8), the previous gold standard for the induction of human β-cell proliferation *in vitro* (6). In addition, I show that this fraction of islet cells that have entered the cell cycle upon CYCLIN D3/CDK6 overexpression show morphological signs of cell death (Figure 3.8), which was not previously appreciated (6). While entry into the cell cycle is not being debated, these results put into doubt whether complete cell cycle progression and exit is achieved with previously published β-cell sufficiency factors transcriptionally modulated through overexpression. Rather it suggests uncoupling of the mechanisms that control the timing and execution of each cell cycle phase in primary human β-cells through excessive transcriptional activation.

These results also suggest that the expression levels of cell cycle regulators are important to consider when attempting to elicit β-cell proliferation *in vitro*. For example, the reintroduction of supraphysiological protein levels of HNF1α back into HNF1α- null mice is deleterious, as demonstrated by a severe reduction of β-cell proliferation, increased apoptosis, and subsequent β-cell depletion (7). Indeed, the tissue specific expression of HNF4α as determined by its dual promoters is dysregulated in certain human cancers (8). Also, it is important to realize that the DNA damage response is a barrier to tumorgenesis in early human bladder, lung, colon and breast cancers. Overexpression of already mentioned genes such as Cyclin E, E2F1 and c-Myc stimulates the DNA damage machinery through DNA hyper-replication (9-11), abrogation of which leads to transformation and tumor formation in both *in vitro* and *in vivo* models (11, 12). This sets an undesired but very much potential precedence that overexpression cell cycle regulators can generate genomic instability, leading to spontaneous mutations of key DNA damage response mediators such as p53, and consequent unchecked proliferation. Numerous P53 mutations and splice mutations of CHK2, including loss of heterozygosity of the CHK2 protein, accumulate with worsening grade of bladder cancers, indicating progressive defects in the DNA damage response pathway (10). While the manipulations performed in my experiments did not lead to a diminishing DNA damage response over time (Figure 3.5, 3.6), the wide appreciation and analysis of the DNA damage response pathway, including p53, until now did not exist in the context of the β-cell, and more importantly in a human β-cell that has been stimulated to enter the cell cycle via overexpression of a β-cell mitogen. My findings not only demonstrate that the DNA damage response is a barrier to efficient β-cell proliferation *in*

vitro, but also suggest genomic stability as an additional determinant of successful β-cell proliferation and expansion *in vivo* (Figure 4.1).

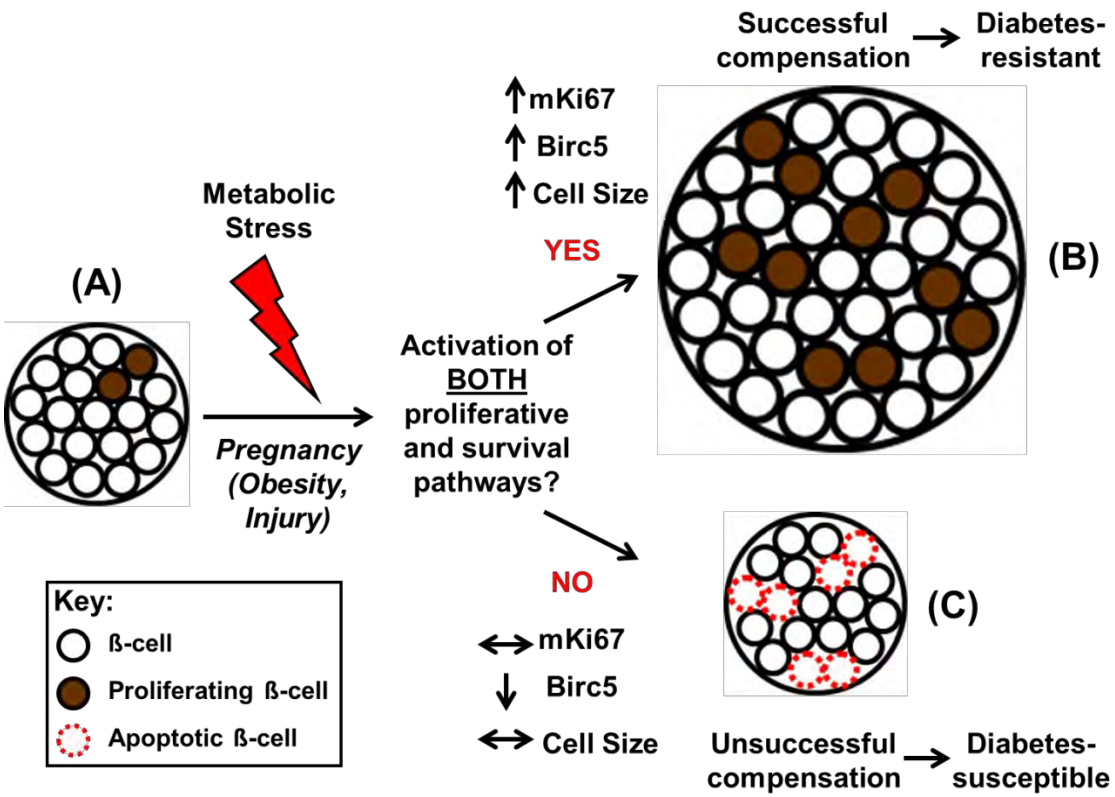

Figure 4.1: A model for β-cell expansion in response to metabolic loads.

Figure 4.1: A model for β-cell expansion in response to metabolic loads. This is a model based on the conclusions of this thesis and the literature. (A) Normally, β-cells in the mouse undergo very little turnover, exhibiting a low basal rate of replication. Pregnancy, obesity and β-cell recovery after injury are examples in which the systematic demand for insulin increases. (B) To successfully compensate for the relative insulin deficiency that occurs in each case, both proliferative and survival pathways are activated in β-cells, protecting against the onset of diabetes. An increase in β-cell size also accompanies β-cell expansion during pregnancy and obesity. (C) If the expanding β-cell mass remains predisposed to β-cell apoptosis resulting from an increased vulnerability during cell cycle progression, for example the lack of expression of „survival" factors (represented here as Birc5) and/or increased genomic instability, β-cell compensation fails. If β-cell apoptosis overcomes β-cell renewal mechanisms and persists for a prolonged amount of time, diabetes might ensue.

Note: This figure has been published in TRENDS in Endocrinology and Metabolism as a Review article entitled,

Expansion of β-cell mass in response to pregnancy.

Rieck S, Kaestner KH. *Trends Endocrinol Metab*. 2010 Mar; 21(3):151-8.

Future Directions

Investigation of the role of Cish in reversible β-cell expansion during pregnancy

It is now appreciated that a great multitude of cell cycle inhibitory proteins or molecular brakes are expressed in the β-cell postnatally, dramatically slowing the rate of β-cell proliferation during adulthood (13). Instead of looking for the therapeutic molecular accelerator that drives cell cycle progression, the converse question I can ask is which molecular brakes must be removed to allow spontaneous β-cell proliferation. The identification of differentially expressed genes associated with β-cell expansion during pregnancy (see Chapter 2) offers hints to which brakes might help generate new β-cells in a cell replacement therapy for treating diabetes mellitus.

One such gene whose expression is highly induced in the islet during pregnancy is *Cish*, a member of the suppressor of cytokine signaling (SOCS) family (Table 2.3). The SOCS family is characterized as a family of proteins capable of inhibiting Jak2/Stat signaling in various tissues, acting via a classical negative feedback loop (14). Since it is known that lactogenic hormones signal through Jak2/Stat5, and induce proliferation by upregulation of Cyclin D2 in the β-cell *in vitro* (15), a molecular regulator of this pathway is of particular interest. Specifically, Cish can utilize two mechanisms to downregulate lactogen- induced Jak2/Stat signaling. First via its SH2 domain, it can bind to phosphorylated tyrosine residues on the prolactin receptor, a tyrosine kinase receptor, thereby masking potential docking sites for downstream signaling such as Stat5. Also, via its SOCS box domain, Cish may target its bound signaling molecule to ubiquitination and proteosomal degradation by recruitment of an E3 ligase complex (14).

Thus, I propose the induction of Cish expression by lactogen signaling through Jak2/Stat5 negatively regulates growth in the β-cell during pregnancy. Indeed, I have preliminarily demonstrated that suppression of Cish induction augments phosphorylation of Stat5 by recombinant prolactin in MIN6 cells (data not shown). However, the role of Cish in reversible β-cell expansion during pregnancy *in vivo* is not known. I hypothesize that ablation of Cish will enhance β-cell proliferation during pregnancy in the mouse. To answer this question, I derived a conditional null allele for *Cish* utilizing genetic recombination and subcloning protocols (Figure 4.2A-B). I will utilize a transgenic mouse expressing Cre recombinase under the control of the mouse insulin promoter (MIP) to specifically ablate Cish in β-cells (Figure 4.2C), because the MIP-Cre/ERT transgenic mouse does not have Cre recombinase activity in the brain and tamoxifen is incompatible with pregnancy studies (16). I will use the $Cish^{LoxP/LoxP}$; MIP-Cre mouse model to assess if Cish ablation is enough to cause a normally quiescent adult β-cell to spontaneously proliferate. In addition, using the pregnancy β-cell expansion paradigm I will be able to assess if Cish ablation can augment the proliferation rate and/or survival of β-cells in response to physiological insulin resistance in the mouse. Gene expression profiles will give me mechanistic information of what potential pro-proliferative and pro-survival genes are transcriptionally upregulated upon Cish deletion, and if Cish acts as a negative regulator of the Jak2/Stat5 pathway and potentially any other signaling pathways downstream of the prolactin receptor during pregnancy such as the insulin signaling pathway (17). If there is a role for Cish in β-cell proliferation during pregnancy, combining the $Cish^{LoxP/LoxP}$; MIP-Cre mouse model with other models of β-cell expansion such as obesity (high fat diet) and β-cell recovery after ablation (PANIC-ATTAC) will

give me information about if Cish‟s role in β-cell proliferation is restricted to only the pregnancy. If these experiments employing this mouse model are successful, experiments can then be carried further into the primary human islet system to ask if the RNA interference mediated suppression of CISH expression upon lactogen stimulation has a stimulatory effect on human β-cell proliferation.

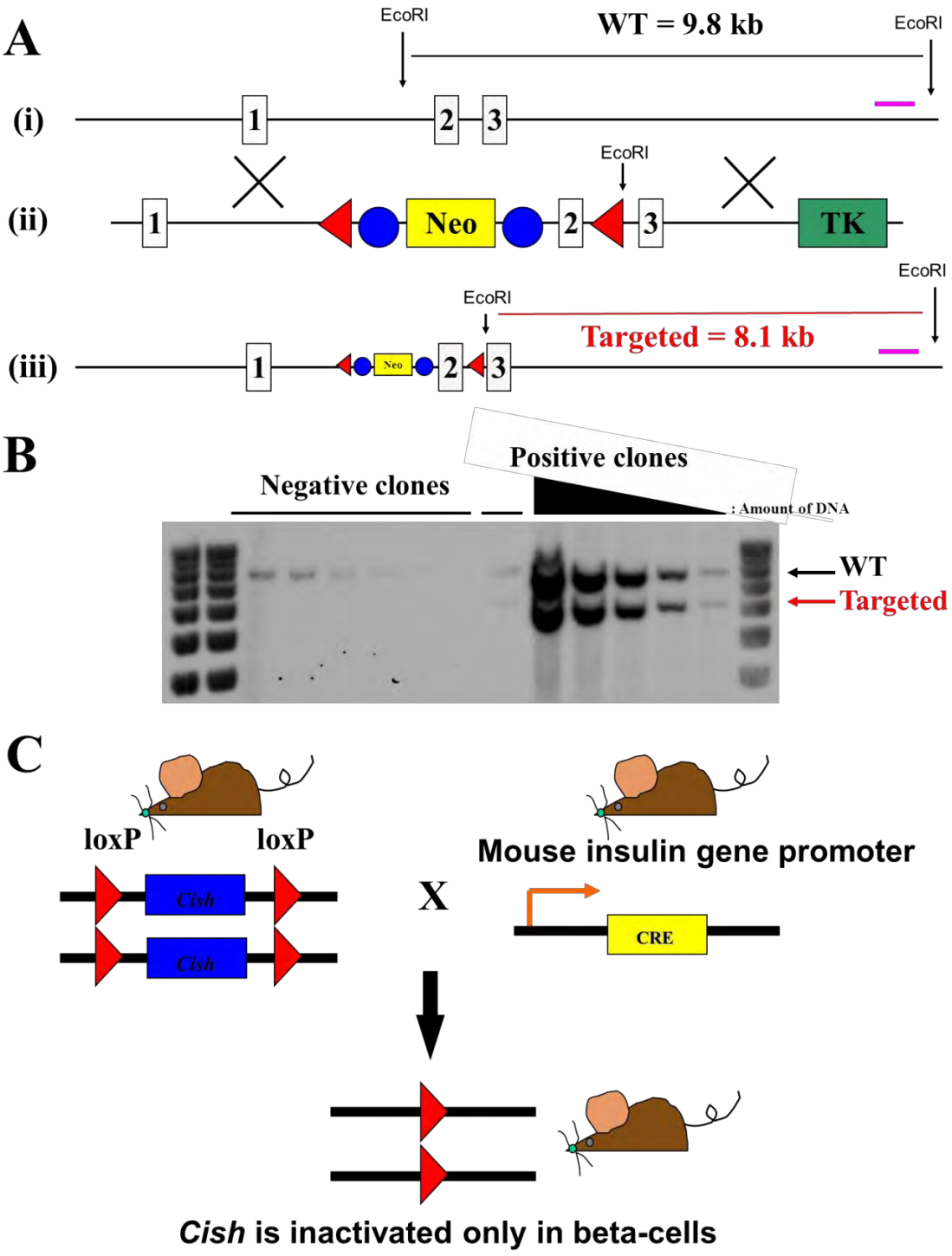

Figure 4.2: Derivation of a loxP allele for the conditional ablation of Cish in the mouse.

Figure 4.2: Derivation of a loxP allele for the conditional ablation of Cish in the mouse. (A) The homologous recombination strategy chosen to derive the $Cish^{LoxP}$ allele with (i) wild type *Cish* locus on chromosome 9, (ii) the target vector, and (iii) the resulting homologous recombination product between the target vector and wildtype *Cish* locus. Notice the additional EcoR1 site introduced by the 3‟ loxP site. (B) A southern blot showing successful recombination of the target vector into the *Cish* locus as detected by two different DNA products after EcoR1 restriction enzyme digestion. (C) A cross between a mouse homozygous for the $Cish^{LoxP}$ allele and MIP-Cre transgene mouse ablates Cish only in β-cells.

Define HNF4α dependent pathways responsible for β-cell proliferation during states of increased metabolic loads

It has been shown that HNF4α is not only required for β-cell proliferation during pregnancy (2), but its overexpression can also be utilized to stimulate primary human β-cells to enter the cell cycle (see Chapter 3). While one mechanism through which this is accomplished could be the Ras/Mapk signaling pathway (2), it is unclear as to how HNF4α regulates the cell cycle and if other known mechanisms leading to proliferation of β-cells are dependent on HNF4α. Based on my human β-cell work, I hypothesize that HNF4α transcriptionally regulates genes responsible for triggering cell cycle entry. While it was not possible to assess the small population of HNF4α8- overexpressing human β-cells that entered the cell cycle separate from the remainder of the islet by gene expression analysis, I can begin to validate this hypothesis by utilizing a transgenic mouse model that has inducible overexpression of HNF4α8 in a higher percentage of β-cells by using the RIP-RTTA (reversible tetracycline transactivator protein); TRE-HNF4α8 (tetracycline response element) system. In addition, β-cell specific deletion of HNF4α, by using the HNF4α$^{loxP/loxP}$; Ins.Cre mouse model provides an experimental platform with which to identify physiological pathways involved in β-cell proliferation dependent on HNF4α not only during pregnancy, but also other models of β-cell expansion (18). Expression profiling of pregnant control and pregnant HNF4α$^{loxP/loxP}$; Ins.Cre mice can provide other pathways transcriptionally dependent on HNF4α during pregnancy. Placing HNF4α$^{loxP/loxP}$; Ins.Cre mice on a high fat diet or crossing with leptin deficient *Ob/Ob* mice can address if β-cell proliferation is dependent on HNF4α during obesity. Together I can compare the gene expression profiles between these three

contexts (HNF4α8 overexpression, pregnancy, obesity) to assess if the differential expression of genes differs between a proliferating β-cell during two different states of physiological insulin resistance, and a β-cell stimulated to enter the cell cycle by overexpression.

Another potential mechanism for HNF4α dependent β-cell proliferation reflects a very recent but provocative idea that glucose driven glycolysis is a key mitogenic trigger for β-cells. Specifically, β-cell proliferation depends on a signaling pathway involving glucokinase and membrane depolarization (19). HNF4α directly regulates a key factor in this pathway, the potassium channel (K_{ATP}) subunit Kir6.2, through regulation of mRNA and protein expression *in vivo* and transcriptional activation of Kir6.2 *in vitro* (18). It is possible that reduced levels of Kir6.2 protein levels uncouples HNF4α- deficient β-cells from the increased glycolytic flux present during pregnancy, leading to a reduction in proliferation rate of these cells (19). Although the direct mechanism downstream of Kir6.2 to cell cycle genes is not clear, the HNF4α$^{loxP/loxP}$; Ins.Cre mouse in combination with the pregnancy β-cell expansion paradigm gives an experimental platform from which to test this hypothesis. While Kir6.2 is only a subunit of the K_{ATP} channel, mutations in it alone are able to cause impaired insulin secretion in pancreatic β-cells *in vivo* (20). Indeed, the HNF4α$^{loxP/loxP}$; Ins.Cre mouse during pregnancy is glucose intolerant as measured by glucose tolerance tests (2). In addition, if an increase in the random blood glucose and decrease in the insulin levels in the plasma of these mice during pregnancy occurs, opposite of what normally happens (21), it would suggest an uncoupling of the HNF4α- deficient β-cell to its surrounding glycolytic environment. An interesting experiment would be to attempt to rescue the β-cell proliferation defect by

molecules that modulate the activity of the K_{ATP} channel of the β-cell. Diazoxide decreases insulin secretion by opening the K_{ATP} channel and sulfonylureas like glyburide increase insulin secretion by closing the K_{ATP} channel (19). I hypothesize that hyperpolarization of the β-cell membrane by administration of diazoxide will not improve the β-cell proliferation defect. Conversely, depolarization of the β-cell membrane by administration of the glyburide would rescue the proliferation of β-cells normally reduced in the HNF4α$^{loxP/loxP}$; Ins.Cre mouse during pregnancy. This is supported by the fact that forced membrane depolarization upon injection of glyburide in the presence of increased glycolytic flux by β-ablation can further stimulate β-cell proliferation (19). An alternative rescue experiment is to reintroduce Kir6.2 protein expression back into the HNF4α-deficient β-cell by the use of a β-cell specific Kir6.2 overexpression mouse, with the expectation that this would couple the HNF4α-deficient β-cell back to an environment of increased glycolytic flux restoring β-cell proliferation. These experiments would begin to clarify whether glycolytic flux has a role in β-cell expansion during pregnancy, and if HNF4α regulates this process.

Examination of methods which potentially stimulate β-cell proliferation without activation of the DNA damage response

My findings demonstrate that overexpression of cell cycle regulators can generate replication stress in human β-cells, characterized by cell cycle arrest due to the activation of the DNA damage response (see Chapter 3). I propose that future studies attempting to understand the DNA damage response in the β-cell can help avoid undesired consequences when attempting to stimulate β-cell proliferation. While re-replication occurs in HNF4α8 overexpressing cells that enter the cell cycle and attempt S-phase, what is the nature of this DNA damage response in β-cells overexpressing other cell cycle

regulators such as the combination of CYCLIN D3 and CDK6? It is true that re-replication occurs in response to the overexpression of various oncogenes in cell lines (11), but other pathways may induce genomic instability by dysregulation of chromatin-remodelers, or by directly influencing chromatin structure such as in the telomere and centromere regions (22). In addition, is the DNA damage response generic or specific to the β-cell, and if so, why does one face this challenge only in β-cells? It is curious that out of the endocrine pancreas only the β-cell is susceptible to Birc5-deficiency (3). An extension of this line of thought is indicated by the yet unexplained observation that embryonic and adult cell division seems to be regulated independently (23, 24); the study of cell cycle control in the β-cell not only in various contexts, but also with respect to other cell types is still incomplete. There is a need for identification of the mechanisms cell cycle entry and progression employed by the β-cell prenatally. These mechanisms should be compared to β-cell expansion models already discussed such as during pregnancy, obesity, and β-cell recovery after injury. For example, is some degree of the DNA damage response physiological or always pathological? One way to examine that question would be to look at the only clearly proven type of human β-cell replication, which occurs during prenatal period of the fetus (25). If no DNA damage response is present in these proliferating β-cells, what are the proliferative and survival mechanisms employed that protect the β-cell against this during the prenatal period? The challenge becomes to identify ways in which β-cell proliferation can be stimulated while avoiding activation of the DNA damage response.

The utility of cell permeable small molecules would be an alternative to the overexpression of cell cycle regulators to stimulate β-cell proliferation. Small molecules

would allow for greater regulation of both the amount and duration of potential β-cell mitogen activation needed to drive efficient and non-oncogenic cell cycle progression. I propose that small molecules identified by a screen using a quiescent β-cell line can be confirmed by the primary human islets *in vitro* system. Known molecules stimulating β-cell proliferation include, hepatocyte growth factor (Hgf) (26), and Exendin-4 (27). Other potential molecules include ones that trigger an increase in the rate of glycolysis in the β-cell, since glucose driven glycolysis is a physiological mitogenic trigger for β-cells (19). A recent study demonstrated that a major regulator of Cyclin D2 levels is glucose acting via glycolysis and calcium channels in the β-cell (28). Indeed, a high throughput, high content screening of a growth arrested immortalized mouse β-cell line has identified agonists of calcium channels that can stimulate primary human β-cells into the cell cycle (29). It would be interesting to see if glucokinase activators, upcoming drugs aimed at improving the control of blood glucose in type 2 diabetics, could have a stimulatory effect on human β-cell proliferation (19). Other potential small molecules include ones that increase the activation of known genes regulating proliferation and potentially in combination with known genes regulating survival in the β-cell. For example, identification of small molecules that modulate the activity of pancreas specific HNF4α could be more beneficial than its overexpression with respect to its role as a „proliferation" gene. Also, small molecules activating the FoxM1 transcription factor with „survival" gene products such as Birc5 could ensure unflawed cell cycle progression. Eventual assessment of small molecules on β-cell mass in diabetic patients is needed to validate this therapeutic avenue.

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